METHODS IN ENZYMOLOGY

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Methods in Enzymology volumes provide an indispensable tool for the researcher. Each volume is carefully written and edited by experts to contain state-of-the-art reviews and step-by-step protocols.

In this volume we have brought together a number of core protocols concentrating on DNA, complimenting the traditional content which is found in past, present and future Methods in Enzymology volumes.

PREFACE

These volumes of *Methods in Enzymology* contain the protocols that made up the on-line Methods Navigator. Our philosophy when we selected the protocols to include in the Navigator was that they should be for techniques useful in any biomedical laboratory, regardless of the system the lab studies. Each protocol was written by researchers who use the technique routinely, and in many cases by the people who actually developed the procedure in the first place. The protocols are very detailed and contain recipes for the necessary buffers and reagents, as well as flow-charts outlining the steps involved. Many of the chapters have accompanying videos demonstrating key parts of the procedures. In a few cases, detailed protocols for certain important approaches could not be generated either because they are instrument-specific (e.g., next-generation sequencing) or because they are proprietary (e.g., column-based nucleic acid purifications). In these cases we have included "explanatory chapters" that outline the theoretical basis for each technique without giving a detailed protocol. The volumes are broken into distinct areas: DNA methods; Cell-based methods; lipid, carbohydrate and miscellaneous methods; RNA methods; protein methods. Our goal is that these protocols will be useful for everyone in the lab, from undergraduates and rotation students to seasoned post-doctoral fellows. We hope that these volumes will become dog-eared and well-worn in your laboratory, either physically or electronically.

> PROFESSOR JON LORSCH Johns Hopkins University School of Medicine

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CHAPTER ONE

Explanatory Chapter: PCR Primer Design

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Abstract

This chapter is intended as a guide on polymerase chain reaction (PCR) primer design (for information on PCR, see General PCR and Explanatory Chapter: Troubleshooting PCR). In the next section, general guidelines will be provided, followed by a discussion on primer design for specific applications. A list of recommended software tools is shown at the end.

1

1. THEORY

In the spring of 1983, Kary Mullis had the idea to use a pair of synthetic oligonucleotides (primers) to potentially copy *ad infinitum* a target DNA sequence using DNA polymerase; an idea worthy of a Nobel Prize. However, thermal cycling was the essence of the process, and posed a critical drawback: the double-stranded DNA was denatured by heat at the beginning of each cycle, destroying the polymerase in the process, and it had to be replaced in each round. In 1986, Mullis solved the problem by using a thermo-resistant DNA polymerase from *Thermophilus aquaticus* (Taq). With the drawback gone, the PCR became dramatically more affordable and was subject to automation.

Now, PCR is a core technique in molecular biology and an extremely large number of applications have been developed. These methods directly depend on the efficiency of the reaction, which relies on the fine-tuning of its components: DNA template, dNTPs, reaction buffer, and a set of primers that flank the target sequence and are extended by the DNA polymerase.

This chapter focuses on primer design, a critical factor to both the efficiency and the specificity of the PCR.

1.1. Thermodynamics

The changes in two thermodynamic properties of a system (ΔS – change of disorder, and ΔH – change of heat) can be used to calculate ΔG , the Gibb's free energy change. This value shows whether a process is spontaneous, and allows one to determine the equilibrium constant K, with which the concentrations of all the species that are involved in the equilibrium can be worked out. Therefore, the thermodynamic changes that happen in going from random coil to duplex can be known and used to determine the primer quality. A full thermodynamic explanation is given in SantaLucia (2007); Mann et al. (2009).

To quickly sum up, the ΔG is directly related to the equilibrium constant (*K*) of the primer/template hybridization at a given temperature (*T*).

$$\Delta G = \Delta H - T\Delta S$$

K = [primer plus template duplex]/([primer][template])
$$\Delta G = -^{**}RT \times \ln(K)$$

A key parameter that affects the efficiency of a PCR is the amount of primer bound to the template. If the concentration of duplex (primer bound to template) is different for each of the primers, the efficiency will be reduced. From the formulae above, it can be deduced that designing primers with the same ΔG (at a fixed annealing temperature) will render more efficient primers pairs, and also explains why matching $T_{\rm m}$'s is a less accurate approach than matching ΔG 's.

The more negative the ΔG the higher the K, and then the more stable the duplex. Primer software sets thresholds that are used to discard bad primers, as secondary and tertiary structures (primer-dimers, hairpins, etc.) with very low ΔG (very stable) are undesirable.

1.2. General guidelines for primer design

Primer design has two goals: specificity and efficiency of amplification. Specificity is the frequency of proper priming events – mispriming leads to amplifications off-target, though mindfully used it can serve to hunt for related sequences (e.g., degenerate primers, see below). Efficiency is the increase of product in each PCR cycle; the theoretical optimum is a two-fold increase. The application determines the balance between these parameters. For instance in diagnostics, efficiency is sacrificed for a higher specificity, minimizing the false positives at a cost of less PCR product. These two parameters are controlled by factors such as temperature, extension time, template concentration and thermodynamic properties, length of the amplicon, divalent cations (Mg^{2+} , Mn^{2+}), and detergents.

The main parameters affecting primer design and therefore the PCR reaction are discussed below.

1.3. Melting temperature (T_m) and GC content

 $T_{\rm m}$ is the temperature at which half the primer strands are bound to the target. It depends on base composition and can be roughly estimated as

$$T_{\rm m} = 4(G+C) + 2(A+T)$$

However, this equation neglects the dependence of $T_{\rm m}$ on strand concentration, salt concentration, and base sequence. Typical errors for this method compared with determining the $T_{\rm m}$ experimentally can be >15 °C, thus this equation is not recommended (SantaLucia, 2007).

The best current approach to calculate $T_{\rm m}$ is the nearest-neighbor (NN) method (SantaLucia, 1998). The previous 1986 NN model is unreliable, but still present in most common primer design tools such as **Primer3**, **OLIGO**,

and **Vector NTI**[®]. When possible, then, the 1998 model should be selected (SantaLucia, 2007).

 $T_{\rm m}$ depends on base content. Two primers with a different length can have the same $T_{\rm m}$ then, but the longer one will anneal less efficiently than its partner, thereby affecting the efficiency of the PCR. A different $T_{\rm m}$ between the pair will affect specificity (at lower annealing temperatures, the primer with higher $T_{\rm m}$ will be more prone to misprime) and efficiency (at higher temperatures, the primer with lower $T_{\rm m}$ will anneal less frequently or not at all). ΔH° of binding is different for each primer, resulting in different slopes of binding at the $T_{\rm m}$, and then in different amplification efficiencies and artifacts. Therefore, the key point is not to match $T_{\rm m}$, but equal amounts of primers bound to the target: to do so, design primers with matching ΔG° at the desired annealing temperature (SantaLucia, 2007).

Typically, the annealing temperature to start with is 4–10 °C below the $T_{\rm m}$. However, the optimal annealing temperature can be more accurately calculated as

$$T_{\rm a} = 0.3 T_{\rm m, primer} + 0.7 T_{\rm m, product} - 14.9$$

with $T_{m,primer}$ being the T_m of the less stable primer-template pair (Rychlik, 1990. Note that T_m calculations are not made with the latest NN model – SantaLucia, 1998).

In summary, for regular PCR, a match of ΔG° (otherwise no more than 2 °C of difference in $T_{\rm m}$) and GC content (40–60%) is advised. However, the GC content can be higher and work fine, so the more general rule is that it should be not be lower than that of the amplicon (Rychlik, 1993).

1.4. Primer length

A major factor controlling specificity is the annealing temperature, which is determined by the length and base composition of the primers.

The shorter the primer, the quicker the annealing with the template and the lower the specificity, therefore, primers <18 nucleotides in length are likely to perform badly with complex templates (e.g., genomic DNA). Primers 16–18 nucleotides in length will perform well only for <500 bp amplicons; for longer amplicons, longer primers are recommended, for example, 24 nt primers will do for 5 kb products (Rychlik, 1993).

The longer the primer, the lower the efficiency (a small inefficiency will propagate with the number of cycles). However, a longer primer leads to a higher specificity because of the primer length (4 times per added base) and its melting temperature. Thus, 28–35 nt primers can be used to amplify templates expected to have a high degree of heterogeneity, or for cloning closely related genes. It is important to note here that longer primers are less pure due to inefficiencies in chemical synthesis, so purification better than standard desalting might be required for certain applications (see Purification of DNA Oligos by Denaturing Polyacrylamide Gel Electrophoresis (PAGE)).

In summary, the utilization of primers of a minimal length that ensures $T_{\rm m}$ > 54 °C is recommended to balance specificity and efficiency in general conditions (Dieffenbach et al., 1993).

1.5. Primer sequence and secondary structures

The primer's sequence determines its specificity for the target but can also facilitate interaction with itself and other primers, affecting its performance.

Taq polymerase extends only the 3'-end of the primer, so its sequence is critical. Perfect base pairing for at least the last 4 bases is optimal, and minimal mismatch should exist within the last 5–6 ones (lowering the annealing temperature does not compensate well for mismatches near the 3'-end of the template, but rather it promotes mispriming). Conversely, the 5'-end sequence is not that critical, so 5' tails (e.g., restriction sites, fluorophores, etc.) can be added to the primers without significantly affecting annealing efficiency.

Primers with low 3'-end stability (AT-rich, $\Delta G > -9$ kcal mol⁻¹) but a stable 5'-end perform best in sequencing and PCR, since this structure reduces mispriming (Rychlik, 1993). Therefore, structures with high 3'-end stability such as GC clamps (more than 3 G or C bases within the last five 3'-end bases) should be avoided because the stronger bonding raises the likelihood of partial priming.

Avoid also mononucleotide *runs* longer than 4 nucleotides (e.g., GGGG) and *repeats* of more than 4 dinucleotides (e.g., ATATATAT), because they can cause mispriming and polymerase slippage (see Viguera et al., 2001 for more information on slippage).

In general, discard primers that form intramolecular or primer/primer duplexes with negative ΔG , especially if these structures are formed at the 3'-end. Although $T_{m,duplexes} < T_{m,primer/template}$ dimerization will occur to some extent, lowering the PCR efficiency by sequestering the polymerase and the primers themselves. For instance, *hairpins* can cause internal primer extension if they form at the 3'-end, eliminating that primer from the reaction (though on the 5'-end do not significantly affect the PCR). Primerdimers are extended by the polymerase and can be detected on an agarose gel as a low molecular weight band, or on a melting curve (usually performed in qPCR experiments) as extra peaks. 'Fake' primer-dimers can also appear if the primers bind off-target close to one another, which explains why sometimes the band is longer than expected, and why sequencing shows a few extra unexpected bases between the actual primers.

Prevention of dimerization is crucial in qPCR experiments and multiplex PCR. Patches like the hot start method can mitigate it but, if possible, it is preferable to avoid it from the beginning.

An approximation that meets these problems is the proprietary **ThermoBLASTTM**, by DNA software, which can be implemented into their **Visual OMPTM** primer design package. Although a BLAST search will give information on mispriming, it just considers base pairing and not thermodynamic parameters like duplex stability, so its help is limited. ThermoBLASTTM retains the computational efficiency of BLAST but uses thermodynamic scoring for base pairs, dangling ends, single mismatches, bulges, tandem mismatches, and other motifs, thereby rendering more balanced primer pairs (SantaLucia, 2007). **Pythia** is another software approach in which state-of-the art DNA binding affinity computations are directly integrated into the primer design process. It seems it is comparable to other available software, although it outperforms them on the design for primers on difficult regions (e.g., repeats).

In summary, select those primers less likely to form secondary structures and with low 3'-end stability.

1.6. PCR product length and placement within the target sequence

The product length affects the efficiency of the PCR. Generally, 150-1000 bp products are amplified for target detection, and some techniques like qPCR (see below, *real-time PCR*) have narrower windows for the length of the amplicon. For detection of gene expression, it is preferable to design the primers on contiguous exons (see below, *RT-PCR*).

If possible, avoid regions with long runs, repeats, or secondary structures, because they will make good primer design and matching more complicated. Those structures can be predicted with software such as **UNAFold** (also called Mfold⁺⁺). Some primer design software, such as **Phytia**, takes into consideration binding and folding energies, outperforming other software for designing primers for repeat regions. Proprietary software like **Visual OMPTM** also allows for prediction of secondary structures and can solve the multistate coupled equilibrium of these cases (SantaLucia, 2007).

1.7. Step-by-step guide for primer design

These are some guidelines for RT-PCR primer design, but can mostly be applied to design primers for other purposes.

Conventions:

- Sequences are always written from 5' to 3', no matter whether they are forward or reverse primers (therefore, *do not ever* write a primer sequence reversed because it will lead to confusion).
- Polymerase extends from the 3'-end.

Procedure:

1. Get the cDNA sequence. If possible, use RefSeqs.

Refseq, or **Ref**erence **Seq**uence database from NCBI, is a collection of annotated DNA, RNA, and protein sequences. Each RefSeq is reviewed and represents a single molecule from one organism (http:// www.ncbi.nlm.nih.gov/RefSeq/). Sequences other than RefSeqs do not have that assured quality and there is a chance they are truncated, untrimmed, or incorrect. Make sure you are using the latest version of a RefSeq entry (i.e., the accession with the highest version number), or simply just use the accession without the version number, as you will automatically get the latest version.

2. Primers can be designed from the NCBI itself by clicking on 'pick primers,' next to the sequence. The tool combines Primer3 and BLAST and is called **Primer-BLAST**.

Primer-BLAST allows one to design primers bridging an exon–exon junction or spanning an intron (see below for explanation, on *RT-PCR*), thereby avoiding false positives due to DNA amplification. The BLAST addition allows for the selection of organism-specific primers and selective amplification of splice variants.

- 3. Select the parameters that suit your specificity goals (see guidelines above) and the software will provide the best primer pairs (Note: Primer-BLAST uses the 1998 model for $T_{\rm m}$ calculation by default).
- 4. Primer quality can be double-checked using software such as **NetPrimer**.
- 5. Alternatively, design the primers using software like Primer3, Primer3Plus, Pythia, Visual OMPTM, OLIGO, or Vector NTI[®] (just a few examples in no particular order. The first 3 are freely available and the last 3 are proprietary).
- 6. Finally, for RT-PCR, it is recommended that primers be chosen close to the mRNA 3'-end. The reverse transcription reaction starts at this end, and premature termination of the reaction can produce shorter

molecules that lack the 5'-end. Specially if poly-(T) are used, designing the primers close to the 3'-end is suggested to avoid the underestimation of the target concentration.

1.8. Troubleshooting and general rule

If no specific product is achieved in the first attempt, alternative approaches can be used which are, however, beyond the scope of this chapter (see Explanatory Chapter: Troubleshooting PCR). A gradient PCR is very useful to determine empirically the best annealing temperature for the primer set. A touchdown PCR will increase specificity at a cost of lower efficiency. A nested PCR will enrich the sample in specific product. The use of a different polymerase can dramatically affect the results as well (e.g., switching from regular *Taq* to a highfidelity polymerase). Amplification of some DNA templates works better at extension temperatures lower than 72 °C (especially if they are <500 bp or highly AT-rich, and therefore less stable at high temperatures). The use of two-step PCRs (denaturation plus annealing-extension, by designing primers with a $T_{\rm m}$ of 68–70 °C) or tweaking the PCR mix (changing the Mg²⁺ concentration, addition of DMSO, etc.) can also help. See Mülhardt (2007) for further information on PCR troubleshooting.

However, the general rule can be: begin with well-designed primers, and if a few troubleshooting attempts fail, consider designing a new set of primers instead of trying to tackle all of the modifications possible.

1.9. Specific applications

Ultimately, primer design strategy is determined by the goal of the PCR method. Several designs are discussed below; it is important to note that PCR depends not only on primer design, but also on primer quality and purity. Chemical synthesis of primers ends up with a salty mixture of oligo-nucleotides of different lengths, or a mixture of labeled and unlabeled ones. They have to be purified, and the purification method should be chosen in terms of the final application. Therefore, a faulty PCR can happen after a perfect primer design, simply because in the reaction we are adding interfering faulty oligonucleotides. See Purification of DNA Oligos by Denaturing Polyacrylamide Gel Electrophoresis (PAGE) for further explanation.

1.10. Target cloning

There are several approaches for cloning (see Molecular Cloning and Restrictionless cloning). Primers can be designed at the ends of a target and the PCR product cloned into an A/T vector (even directionally cloned by doing some modifications, see Zhou and Gomez-Sanchez, 2000). This can be done in **Primer3Plus** by choosing the option 'cloning.'

Alternatively, restriction sites can be designed as 5'-tails in the primers, where they do not substantially affect annealing (if necessary, lower the annealing temperature for the first few cycles and then raise it to the annealing temperature considering the whole primer including the tail). Restriction enzymes will not cut efficiently if just the target restriction site is added; adding 4–6 bases at the 5'-end are usually required for best performance. New England Biolabs offers some information on this subject:

http://www.neb.com/nebecomm/tech_reference/restriction_ enzymes/cleavage_linearized_vector.asp.

http://www.neb.com/nebecomm/tech_reference/restriction_

enzymes/cleavage_olignucleotides.asp (bear in mind that these examples are oligonucleotides. Therefore, only a few bases are needed at the 5'-end of the restriction site since the 3'-end is linked to the primer).

Check that the target sequence does not contain the desired restriction sites, design the primers, and add the 5'-tails containing the restriction sites. If the sequence contains the restriction site(s), you can still use different restriction enzymes for insert and vector that generate compatible ends (e.g., *Sal* I and *Xho* I; see a complete list at http://www.neb.com/nebecomm/tech_reference/restric tion_enzymes/compatible_cohesive_overhangs.asp).

For further reference on restriction enzymes, check the NEB website at http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/ default.asp?

1.11. Reverse-transcription PCR (RT-PCR)

RT-PCR (see Reverse-transcription PCR (RT-PCR)) is sensitive enough to enable detection and quantitation of RNA from even a single cell. In contrast, two other commonly used techniques for quantifying mRNA levels – Northern blot analysis (see Northern blotting) and nuclease protection assays (see Explanatory Chapter: Nuclease Protection Assays) – require larger of amounts of RNA.

A potential problem with RT-PCR is contamination of the RNA with genomic DNA, which can result in false positives. Consequently, primers should be designed to span introns or bridge an exon–exon junction (Fig. 1.1). When they span one or more introns, the amplified product from genomic DNA will be bigger than expected, or there will be no product at all if the distance between the primers exceeds the DNA polymerase

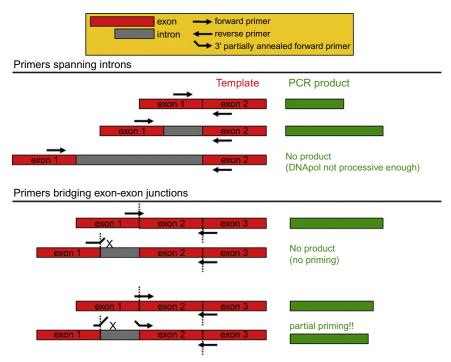


Figure 1.1 *Considerations on RT-PCR primer design.* DNA contamination in RNA can result in false positives. Consequently, primers should be designed to span introns or bridge an exon–exon junction, so the cDNA-derived PCR product is distinguishable from the genomic DNA-derived one. When bridging exon–exon junctions, take care not to design a primer with too long a 3'-end or GC clamps which allow for extension, since you will not be able to distinguish between cDNA- and genomic DNA-derived amplifications (example at the bottom).

processivity. When they bridge an exon–exon junction, they will not amplify genomic DNA template because the intron will hinder the annealing. Make sure you avoid GC clamps and that only a few nucleotides from the 3'-portion of the primer can base pair with the 3'-exon, because a substantial pairing can still prime the polymerase. See above *Step-by-step guide for primer design*, where a RT-PCR-oriented designing protocol is provided.

1.12. Real-time PCR (quantitative PCR, qPCR)

(Adapted from http://www.eurogentec.com). Guidelines for primers:

- Length: 18–30 nucleotides
- %GC: 30-80% (ideally, 40-60%)

- $T_{\rm m}$: 63–67 °C (ideally, 64 °C), so the annealing temperature would be 58–60 °C (Note: as discussed above, matching ΔG is more accurate than matching $T_{\rm m}$. Otherwise, avoid $T_{\rm m}$ differences larger than 4 °C).
- Avoid mismatches, runs, repeats, and self- and primer-primer complementarity.
- Avoid a T residue at the 3'-end (allows mismatching).
- Make sure the primers are specific and target a single amplicon by BLAST.
- The considerations given in the section on RT-PCR should also be observed if a RT-qPCR is to be performed.
- For qPCR, it is preferable to design primers bridging exon-exon junctions (see Fig. 1.1), since these will amplify specifically the mRNA. If there is genomic DNA contamination, primers spanning introns will amplify the contaminant template and the resulting product will interfere with the accurate quantification of the target. It is then recommended that a separate control be set up to detect genomic DNA contamination.

Guidelines for probes:

- Length: 18–30 nucleotides (optimal length is 20 nt. If >30 nt are required, use an internal quencher on dT around the 20th nucleotide rather than at the 3'-end).
- %GC: 30-80%.
- $T_{\rm m}$: 8–10 °C higher than the $T_{\rm m}$ of the primers (8 °C for genotyping, 10 °C for expression profiling).
- Select the strand that gives the probe more Cs than Gs.
- Place the probe the close to the primers without overlapping them.
- Avoid mismatches, runs, repeats, and self- and probe-primer complementarity.
- Avoid a G residue at the 5'-end because it quenches the fluorophore.
- Make sure the probe is specific and targets a single amplicon (the same as the primers) by BLAST.
- If genotyping by multiplexing, position the polymorphism in the center of the probe and match the probe's ΔG if possible, otherwise match the $T_{\rm m}$. Guidelines for amplicons:
- Length for SYBR[®] Green I assays: 80–150 nucleotides (shorter amplicons will increase the PCR efficiency, and longer ones will give a higher Δ Rn as more dye will be incorporated).
- Length for 5' exonuclease probe assays: 80–120 nucleotides (shorter amplicons will increase the efficiencies of the PCR and 5' nuclease reactions).

- %GC: 30-80% (ideally, 40-60%).
- Avoid secondary structures in the amplicon (they can be checked with UNAFold).

It is *critical* that the primers are well designed. No secondary structures and very close ΔG 's, no annealing to parts of the template that can result in unbalanced binding, so that the efficiency of amplification is close to 100% and no off-target amplification occurs.

See Explanatory Chapter: Quantitative PCR and Wong and Medrano (2005) for further information regarding real-time PCR.

1.13. Multiplex PCR

The goal of multiplex PCR is to amplify several targets in the same tube. Since first described by Chamberlain et al. (1988), it has had many applications and is typically used for genotyping, where simultaneous analysis of multiple markers is required, for detection of pathogens or genetically modified organisms, or for microsatellite analyses. Multiplex assays are difficult to establish, so they are worthwhile only for analyses that will be repeated many times, since at the end, they save space, money, and time.

Two or more primer pairs are used at a time. They could be designed and work separately, but when put all together, primers from one pair can interact with primers from another one. This makes the optimization of multiplex PCR quite difficult, as each primer pair could have different requirements. To match the $T_{\rm m}$ and ΔG is just not enough because of the potential interactions, and an effort made in primer design is worthwhile.

Sets of primer pairs can be checked for cross-dimerization using tools like **AutoDimer**.

If starting from scratch, there is software for multiplex PCR primer design: **MIPS** is specially made for designing multiplex PCR experiments for SNP genotyping, **PrimerStation** is specific for the human genome, and the commercially available **Visual OMPTM** can deal with multistate equilibrium reactions and multiplex design as well (SantaLucia, 2007).

1.14. Sequencing

As stated above, sequences are written from 5' to 3' and the polymerase extends from the 3'-end. Sequencing is done on just one strand, so you have to make sure you design the primer for the right one.

Additionally consider that whilst PCR is exponential, sequencing is not. Therefore, bad primers that work for PCR after tweaking the reaction might not be suited for automated sequencing: mismatched primers will create templates that are perfect matches in a PCR, allowing amplification, but that will not happen with sequencing since only one strand is replicated.

Desired conditions:

- Use accurate sequence data, as mismatching will reduce the specificity and efficiency of the sequencing reaction, leading to bad readings or overlapped sequences. Verify the site-specificity of the primer. The last 8–10 bases at the 3'-end should be unique. If you repeatedly get overlapped sequences, primers might be partially annealing to the vector or on a different place on the insert, and you should redesign them.
- Design the primers no <50 bases from the sequence you are interested in (because the first reads are usually inaccurate), but not farther than 300 bases from it (usable sequence reads reach up to 700–1000 bases). Sequence data is often most accurate about 80–150 nucleotides away from the primer.
- Optimal primer length is 20–25 nucleotides (acceptable range is 18–30 nt).
- %GC content: 40–60%.
- $T_{\rm m}$ within the range of 55–65 °C.
- Discard candidate primers that self-anneal
- Try to choose primers with <3 Cs or Gs at the last 5 bases at the 3'-end, and that do not end with C or G. This will reduce the likelihood of partial unspecific priming.

Some software, such as **Primer3Plus**, has a specific option for designing sequencing primers.

Additionally, software has been developed specifically for directed sequencing, such as **JCVI Primer Designer**. This particular software is designed for pipelining high-throughput PCR primer design, and high-quality directed sequencing results have been obtained using it (as a note, this software is based on Primer3 but does not use the latest NN $T_{\rm m}$ calculation method of SantaLucia (1998)).

Anyway, always check with your sequencing facility as they might have specific requirements for primer design, template concentration, etc.

1.15. Degenerate primers

A PCR primer sequence is called 'degenerate' if some of its positions have several possible bases. The 'degeneracy' of the primer is the number of unique sequence combinations it contains. Hence, these primers are supplied as mixtures of similar, but not identical, oligonucleotides. They have various applications such as amplification or cloning of related genes from the same or different organisms, cloning of genes based on protein sequence, or genome walking with techniques such as TAIL-PCR (thermal asymmetric interlaced PCR, Liu and Whittier, 1995).

Sequence comparison of multiple members of protein families has revealed protein motifs and domains that play important roles in protein function. Closely related sequences can be cloned using degenerate primers (a pool of primers containing most, or all, possible nucleotide sequences encoding a conserved amino acid motif) or consensus primers (a single primer containing the most common nucleotide at each codon position within the motif).

The goal is then to design primers that match as many sequences of homologous genes as possible. A naïve solution would be to align the sequences without gaps, count the number of different nucleotides in each position along the alignment, and seek a primer-length window (20–30 bp) where the product of the count is low. Such a solution is insufficient because of gaps, the inappropriate objective function of the alignment, and most notably, the exceedingly high degeneracy: when degeneracy is too high, unrelated sequences may be amplified as well, losing specificity (Linhart and Shamir, 2002; 2005). The degeneracy has to be high to maximize the coverage, but has to be bound to decrease the probability of amplifying unrelated sequences. This problem and the approaches to solve it are addressed in Najafabadi et al. (2008).

Below, some software approaches are suggested for the design of degenerate primers:

PAMPS (Pairwise Alignment for Multiple Primer Selection). In contrast to previous algorithms, this one does not restrict the output to the exact primer length that was given, which allows selecting an appropriate primer in terms of annealing temperature. PAMPS can be used to design degenerate primers for amplifying genes with uncertain sequences, such as new members of gene families or libraries of antibody variable fragments. PAMPS has been shown to outperform previous algorithms (HYDEN and MIPS) in this task. This software is freely available.

Other tactics have been developed based on COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOPs). A **CODEHOP** is a hybrid primer consisting of a 15–20 nt consensus 5'-*clamp* and a 9–12 nt degenerate 3'-*core* region. The *core* gives a broader specificity for distantly related target gene templates, while the *clamp* allows for a robust amplification during the later cycles of the PCR. CODEHOP works well for small sets of proteins, taking into account the codon usage of the target genome and the desired annealing temperature. However, it is inappropriate for constructing primers with very high degeneracy on large sets of long genomic sequences, and software like PAMPS should be used for that task. An interactive version of the software, iCODEHOP, is freely available.

MAD-DPD (Minimum Accumulative Degeneracy Degenerate Primer Design). MAD-DPD can be considered as a first approach in primer design when a high degeneracy is desired and the number of degenerate primers to be constructed is known or has to be kept low. A drawback of this software is that it does not consider parameters like melting temperature, self-annealing, or secondary structures. This software is freely available.

MIPS (Multiple, Iterative Primer Selector). The software MIPS outperforms HYDEN on the task of designing multiplex PCR experiments for SNP genotyping. This software is freely available.

HYDEN (HighlY DEgeNerate primers) is a software useful for constructing primers and primer pairs with high degeneracy and yet high specificity. This software is freely available.

Use of degenerate primers can greatly reduce the specificity of the PCR amplification, a problem that can be partly solved by using touchdown PCR.

Code	Amino acid	Stands for
A	А	Adenine
С	С	Cytosine
G	G	Guanine
Т	Т	Thymine
U	U	Uracil
R	A or G	pu R ine
Y	C or T (U)	p Y rimidine
М	A or C	a M ino
К	G or T (U)	Keto
s	C or G	S trong (triple hydrogen bonds)
W	A or T (U)	W eak (double hydrogen bonds)
В	C or G or T (U)	not A
-		Continued

Genetic	code l	(IUPAC)

Continued

D	A or G or T (U)	not C	
н	A or C or T (U)	not G	
V	A or C or G	not T (U)	
Ν	A or C or G or T (U)	a N y nucleotide	

1.16. Modified primers

Modifications can be positioned at the 3'- or 5'-end of the oligonucleotide, or internally within the sugar-phosphate backbone or at the nucleobases. These modified primers are used in applications such as *in situ* hybridization, sequencing, fragment analysis, real-time PCR, fluorescent assays, chemiluminescent assays, microscopy, antisense experiments, and gene cloning and discovery (e.g., degenerate primers). The modifications can also allow for the oligonucleotides to be immobilized onto a surface, enabling spotdetection (e.g., in microarrays, chemiluminiscent, and fluorescent assays).

These modifications include

- functionalization with amino, phosphate, or thiol groups
- fluorescent dyes
- hapten or enzyme coupling
- unnatural bases, for example, Br-dU, I-dU
- phosphothioates
- multiply-labeled oligonucleotides, for example, doubly labeled probes
- Locked Nucleic Acid (LNA[™])

The applications of each modification fall beyond the scope of this chapter, but they can be found at major oligonucleotide supplier's Web sites (e.g., Invitrogen, Sigma-Aldrich, IDT DNA, GeneLink, Exiqon, Thermo Electron, etc., in no particular order).

1.17. Recombineering

Recombineering stands for *in vivo* homologous **recombi**nation-mediated genetic engi**neering**. This technique relies on bacteriophage-based recombination systems such as λ Red, or RedET, which mediate recombination between the host's genome and a construct. Restriction-ligation techniques

^{*} LNA nucleosides are a class of nucleic acid analogues in which the ribose ring is 'locked' by a methylene bridge connecting the 2'-O atom and the 4'-C atom. This modification enhances the base pairing, increasing the $T_{\rm m}$ and then the specificity, making them very useful in a variety of applications such as Northern blotting, *in situ* hybridization, and real-time PCR, microarrays (see http://www. exiqon.com/ for further information).

are not used, thereby allowing for manipulation of longer constructs and more versatility, including marker-free approaches (Karsten Tischer et al., 2006). Recombineering with ssDNA oligonucleotides has been shown to be very efficient (Costantino and Court, 2003); however, it can also be performed with dsDNAs.

Primer design is key for efficient recombineering (Sawitzke et al., 2007. See Recombineering: Highly Efficient *in vivo* genetic engineering using single-strand oligos):

- The primer should correspond in sequence to the DNA strand that is replicated discontinuously (the lagging-strand). Twenty-fold reductions in efficiency are expected if the leading strand is chosen.
- It should be 70 bases long, and the base changes no closer than 10 bases from an end. The longer the oligo, the more likely it is to have errors introduced during synthesis. However, further purification is not typically helpful.
- It should be designed to avoid the DNA-mismatch repair (MMR) system (otherwise, the recombineering should be performed in a MMR-deficient strain), following these guidelines:
 - it should create a C/C mispair at the target base or 6 bases away from it (Costantino and Court, 2003),
 - or change 5 bases in a row,
 - or change 4–5 wobble positions in a row, in addition to the designed change.

For troubleshooting and the most up-to-date information on recombineering, see http://redrecombineering.ncifcrf.gov/.

1.18. Primers for homologous recombination

Gene replacement can be performed in yeast by homologous recombination using PCR products. Primer design is critical for this purpose, and some guidelines are given below and those should be taken into account apart from those at the beginning of this chapter (see the full protocol in Yeast – Gene Replacement Using PCR Products).

- Homology to the gene to be replaced or interrupted: 50 nucleotides are recommended and up to 100 nucleotide-long homology regions could be needed. In some cases, as little as 40 nucleotides might work. If the gene is to be replaced, the homology regions have to be upstream and downstream of the coding sequence.
- Homology to the selectable cassette: 20 nucleotides are recommended.

 Since the primers are >70 nucleotides long, additional purification rather than simply desalting might be needed (see Purification of DNA Oligos by Denaturing Polyacrylamide Gel Electrophoresis (PAGE)).

1.19. Software tools list

Please note that no commercial interests have influenced the software choice below, where an attempt has been made to balance the use of freely available and commercial software. This review is not intended as an in-depth comparison and marking of software tools, hence although these ones will perform well for your primer design, better ones might be available in the market for more specific applications.

AutoDimer (Vallone and Butler, 2004). http://www.cstl.nist.gov/ strbase/NIJ/AutoDimer.htm

CODEHOP (Rose et al., 2003; Boyce et al., 2009). https://icodehop. cphi.washington.edu/i-codehop-context/Welcome

HYDEN (Linhart and Shamir, 2002; 2005). http://acgt.cs.tau.ac.il/ hyden/HYDEN.htm

JCVI Primer Designer (Li et al., 2008). http://sourceforge.net/projects/ primerdesigner/

MAD-DPD (Najafabadi et al., 2008). http://bioinf.cs.ipm.ir/down load/MAD_DPD08172007.zip

MIPS (Souvenir et al., 2003; 2007). http://www.cs.wustl.edu/% 7Ezhang/projects/mips.zip

NetPrimer. http://www.premierbiosoft.com/netprimer/index.html OLIGO. http://www.oligo.net/

PAMPS (Najafabadi et al., 2008). http://www.biomedcentral.com/ content/supplementary/1471-2105-9-55-S1.zip

Primer3 (Rozen and Skaletsky, 2000). http://frodo.wi.mit.edu/primer3/ Primer3Plus (Untergasser et al., 2007). http://www.bioinformatics.nl/ cgi-bin/primer3plus/primer3plus.cgi

PrimerStation (Yamada et al., 2006) http://ps.cb.k.u-tokyo.ac.jp Pythia (Mann at al., 2009). http://frodo.wi.mit.edu/primer3/

ThermoBLASTTM. http://dnasoftware.com/tabid/110/Default.aspx

UNAFold (Mfold⁺⁺, Markham and Zuker, 2008). http://dinamelt. bioinfo.rpi.edu/download.php http://mfold.bioinfo.rpi.edu/

Vector NTI[®]. http://www.invitrogen.com/site/us/en/home/LINNEA-Online-Guides/LINNEA-Communities/Vector-NTI-Community/Vector-NTI.html

Visual OMPTM. http://dnasoftware.com/tabid/108/Default.aspx

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Referenced Protocols in Methods Navigator

General PCR Explanatory Chapter: Troubleshooting PCR Purification of DNA Oligos by Denaturing Polyacrylamide Gel Electrophoresis (PAGE) Molecular Cloning Restrictionless cloning Reverse-transcription PCR (RT-PCR) Northern blotting Explanatory Chapter: Nuclease Protection Assays Explanatory Chapter: Quantitative PCR Recombineering: Highly Efficient *in vivo* genetic engineering using single-strand oligos Yeast - Gene Replacement Using PCR Products

CHAPTER TWO

Explanatory Chapter: How Plasmid Preparation Kits Work

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Abstract

To isolate plasmid DNA from bacteria using a commercial plasmid miniprep kit (if interested, compare this protocol with Isolation of plasmid DNA from bacteria).

1. THEORY

Modern laboratories depend upon high-quality, contaminant-free plasmid DNA preps in order to carry out many standard applications, subcloning, sequencing, transfections, and microinjections being a few examples. Many miniprep kits are commercially available, all of which can be used to quickly and cleanly purify plasmid DNA. These kits all work on the same principles, which are described in detail.

2. EQUIPMENT

Micropipettors Micropipettor tips 1.5-ml microcentrifuge tubes

3. MATERIALS

Plasmid isolation kit (e.g., miniprep or maxiprep kit, from any commercial supplier).

4. PROTOCOL

4.1. Preparation

Grow 3-5 ml overnight cultures of bacteria containing plasmid of interest at 37 °C, with shaking.

Add RNase A to the Resuspension Buffer and ethanol to the Wash Buffer according to the manufacturer's instructions.

4.2. Duration

Preparation	Overnight
Protocol	About 1 h

See Fig. 2.1 for the flowchart of the complete protocol.

5. STEP 1 PELLET BACTERIA AND RESUSPEND IN RESUSPENSION BUFFER

To begin, pellet your bacterial culture by centrifugation, remove the supernatant, and resuspend the cells in resuspension buffer.

Resuspension buffer contains Tris–HCl, pH 8.0, EDTA, and RNase A. Tris–HCl, pH 8.0, acts as a buffering agent. EDTA is a chelator, meaning that it soaks up free divalent cations (such as Mg^{2+} and Ca^{2+}) from the surrounding solution. There are two main effects of removing divalent cations

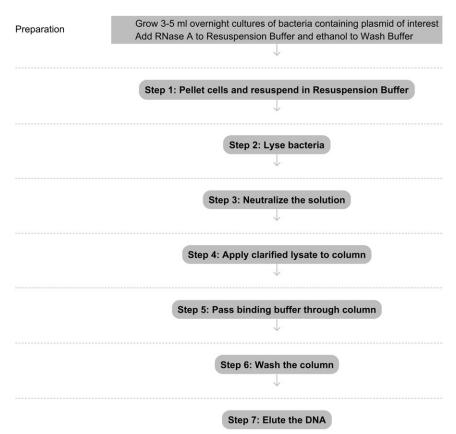


Figure 2.1 Flowchart of the complete protocol, including preparation.

with EDTA: (1) destabilization of bacterial lipid membranes and (2) inhibition of DNases, which often require divalent cations as cofactors. Therefore, EDTA prepares cells for lysis and prevents the degradation of your plasmid DNA of interest. The RNase A in the resuspension buffer enzymatically degrades all RNAs since later purification steps cannot separate RNA from plasmid DNA.

6. STEP 2 LYSE BACTERIA

Lysis buffer is then added to the resuspended cells in order to break them open and release cellular contents, including your plasmid of interest. (If interested, compare this protocol with a milder one used to extract proteins.) Lysis buffer contains SDS and NaOH, which serve to break bacterial cells open. SDS, a detergent, breaks down the lipid membrane, while NaOH helps break down the bacterial cell wall. The action of these two chemicals releases both plasmid DNA and long genomic DNA into solution. Once the DNAs are in solution, NaOH disrupts the hydrogen bonding between DNA bases, separating the double-stranded DNA into singlestranded DNA.

7. STEP 3 NEUTRALIZE THE SOLUTION

To neutralize the pH and precipitate some contaminants, neutralization buffer is then added to the tube.

This solution contains a high concentration of potassium acetate, pH 5.5, which is in equilibrium with acetic acid and K^+ . Neutralization of the alkaline conditions created by the lysis buffer establishes an environment in which hydrogen bonds can reform, allowing the small, single strands of your plasmid DNA to reanneal. Your double-stranded plasmid DNA will now easily dissolve in solution while the long stretches of bacterial genomic DNA will be unable to reanneal and dissolve. The potassium ion from the potassium acetate creates a high-salt environment, which precipitates the cellular debris, the single-stranded genomic DNA, and the SDS (as KDS, the white precipitate that forms in your tube). Your plasmid DNA will be left in the supernatant after centrifugation.

8. STEP 4 APPLY CLARIFIED LYSATE TO COLUMN

After spinning down the lysates, the clear supernatant containing your plasmid is poured over the silica membrane column and centrifuged.

Your plasmid DNA will reversibly bind to the silica in the column, with the help of guanidine HCl, a chaotropic salt, provided in the neutralization buffer from Step 3. Chaotropic salts are large monovalent ions with a low charge density. They weakly interact with H₂O and disrupt the network of hydrogen bonding in the surrounding H₂O. Disruption of this network effectively dehydrates macromolecules, such as your DNA, that are saturated with hydrogen bonded H₂O molecules. Disruption of intra- and intermolecular hydrogen bonding allows your DNA to open up, increasing its surface area and allowing it to bind reversibly to the silica membrane through an unknown mechanism.

9. STEP 5 PASS BINDING BUFFER THROUGH COLUMN

Before beginning the wash step, you have the option of passing a binding buffer through the column.

This buffer contains isopropanol and some guanidine HCl, which again helps to secure your DNA to the silica. The isopropanol dissolves salts and other contaminants. With your plasmid of interest safely bound to the silica in the column, you are now able to wash it to remove contaminants.

10. STEP 6 WASH THE COLUMN

Wash the column with the provided wash buffers.

Wash buffers are a buffered solution containing 80% EtOH. Washing with EtOH removes the chaotropic salts but does not disrupt the bonds between your DNA and the silica membrane.

11. STEP 7 ELUTE THE DNA

Elute your DNA.

At this point, your DNA should be the only thing bound to your column. Because your DNA is dehydrated, application of an aqueous buffer will rehydrate it and allow your DNA to be eluted. Depending upon your intended downstream use, you will be able to elute your DNA in your choice of several different aqueous buffers: ddH_2O , TE, or Tris–HCl, pH 8.5.

11.1. Tip

Which elution buffer to choose?

 ddH_2O : Water is the best choice if you are going to use your DNA for saltsensitive applications such as PCR or sequencing.

TE: TE contains both Tris–HCl, (pH 8.0) and EDTA. DNA will be stable in TE over a longer period of time than in water, making this a good choice for long-term plasmid storage.

Tris–HCl, pH 8.5: Typically the elution buffer provided with kits is Tris–HCl, pH 8.5. This slightly basic buffer makes dissolving DNA easier than with ddH_2O . This buffer also does not contain EDTA, which could inhibit downstream reactions.

11.2. Glossary of Buffers (note x M denotes unknown concentration)

Resuspension Buffer – 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and $100 \ \mu g \ \mu l^{-1}$ RNase.

Lysis Buffer – 200 mM NaOH, 1% SDS.

Neutralization Buffer (Proprietary) - x M potassium acetate, pH 5.5, and x M guanidine HCl. (While the concentration of guanidine HCl in the neu-

tralization buffer is not known, it is listed on the MSDS as an ingredient.) Binding Buffer (Proprietary) – Isopropanol and x M guanidine HCl.

Wash Buffer - 10 mM Tris-HCl, pH 7.5, and 80% EtOH.

Elution Buffer Choices (Miniprep) – ddH_2O ; Tris–HCl, pH 8.5; and TE (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA).

REFERENCES

Referenced Protocols in Methods Navigator Isolation of plasmid DNA from bacteria.

CHAPTER THREE

Explanatory Chapter: Introducing Exogenous DNA into Cells

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Abstract

The ability to efficiently introduce DNA into cells is essential for many experiments in biology. This is an explanatory chapter providing an overview of the various methods for introducing DNA into bacteria, yeast, and mammalian cells.

1. THEORY

Transformation, transfection, transduction: each of these words describes a process by which exogenous genetic material is introduced into cells. Care should be taken when using these terms as they have specific meanings and thus are not interchangeable.

The term transformation originated in 1928 when Frederick Griffith noted that innocuous *Streptococcus pneumoniae* bacteria became virulent after being exposed to heat-killed virulent strains. He reasoned that they must have taken up some sort of 'transforming' factor that gave them that new virulence. Although he did not know it at the time, these bacteria must have been able to incorporate the DNA encoding virulence genes from the dead strains. Since then, the term *transformation* has come to mean a process by which cells incorporate and express exogenous genetic material, specifically bacteria taking up nonviral, typically plasmid, DNA. Transfection is a portmanteau of 'trans' and 'infection'; however, this is misleading, as transfected cells are not actually *infected* with anything. The term *transfection* has since evolved to describe the process by which a eukary-otic cell incorporates and expresses any nonviral genetic material (plasmid DNA, siRNA, or dsRNA).

Transduction is the process by which genetic material is transferred to a cell through a viral vector.

2. PROTOCOL

2.1. Transforming bacteria

Bacteria are only able to take up foreign genetic material if they are 'competent' to do so. Typically, bacteria are not naturally competent, but competency can be induced by two different transformation techniques: (1) calcium chloride transformation or (2) electroporation.

Calcium chloride transformation (sometimes referred to as heat shock) is the cheapest, simplest, and most commonly used method for transforming bacteria with plasmid DNA. Cells are chilled on ice in a solution containing calcium chloride (CaCl₂). The presence of divalent cations (Ca²⁺) prepares the bacterial cell membrane for permeabilization. Bacteria are then briefly heat shocked (typically either 37 °C for 5 min or 42 °C for 30–60 s) and then returned to ice. During the heat shock, the cell membrane becomes permeable to nucleic acids and allows the plasmid DNA to enter the cells. If the plasmid has an origin of replication, it will be retained in the bacteria during divisions and can be selected for based on a selectable marker.

Note: Competent cells can be kept at $-80 \degree C$ and only need to be thawed directly before transformation.

2. Electroporation of bacteria is typically employed when the plasmid of interest is too large to be taken up through calcium chloride transformation or when high transformation efficiency is required. During electroporation, cells are briefly exposed to an electric field (typically 10–20 kV cm⁻¹), which creates holes in the membrane that plasmids are able to move through. At the termination of the electric current, the holes in the cell membrane are repaired by bacterial machinery. The plasmid can then be selected for by standard means.

Note: Electroporation competent cells can be kept at -80 °C in a 10% glycerol solution.

2.2. Transforming yeast

There have been many attempts to induce competency in yeast; however, none has been successful. Therefore, cultures of yeast must be grown up prior to transformation each and every time. Typically, log-phase growth yeast cultures are best for transformation; however, the best OD for transformation varies by strain of yeast and by method of transformation. The two most common methods for transformation will be discussed here: (1) lithium acetate transformation and (2) electroporation.

- 1. Lithium acetate transformation is the most common method of transforming yeast. To carry out this protocol, yeast are cultured to log growth and then resuspended in a solution containing lithium acetate. PEG (polyethylene glycol) and DNA to be transformed (+ carrier DNA if needed) are then added. This mixture of yeast and DNA is then heat-shocked for ~15 min at 42 °C. The presence of the lithium cations in the solution destabilizes the membrane and prepares it for permeabilization. It is not entirely understood why PEG and heat shock are required; however, they have both been shown to be necessary for the uptake of DNA. If the concentration of DNA to be transformed is low, it is beneficial to add carrier DNA from salmon testes which stimulates recombination between the yeast genome and the transformed exogenous DNA of interest.
- 2. If the amount of DNA to be transformed is limiting, *electroporation of yeast* is an easy and fast way to transform small amounts of DNA efficiently. The rationale behind electroporation of yeast cells is identical to that discussed above for bacterial cultures. To electroporate yeast, log growth phase cells are concentrated and resuspended in a solution of 1 M sorbitol. A small amount of DNA is added, without carrier DNA, and the mixture subjected to an electric field. Addition of sorbitol to the solution maintains the osmotic stability of the yeast so that they do not lyse under hypotonic conditions. In the case of electroporation, a carrier DNA is not used because it has been shown to decrease the efficiency of transformation.

2.3. Introducing genetic material into eukaryotic cells

There are several different methods for transfecting genetic material into eukaryotic cell lines depending upon downstream applications. In addition to various methods of transfection, genetic material can either be transfected transiently or stably. During transient transfection, only a percentage of plated cultured cells will express the transfected construct(s). This percentage, the transfection efficiency, varies with the cell line and transfection method being used. For many applications such as co-immunoprecipitations and Western blotting, transient transfection is sufficient. If a homogenous population of cells expressing a gene is required, the cells must be stably transfected instead. To do this, cells are transfected normally with a plasmid containing the gene of interest and an antibiotic resistance gene. The cells are then passaged under antibiotic selection until a population of cells all expressing the gene(s) of interest is obtained. Since it is relatively time consuming to generate a stable cell line, it is only done for experiments in which it is absolutely necessary.

The following sections will give overviews of several different methods for introducing DNA into higher eukaryotes: (1) chemical-based methods; (2) electroporation; (3) viral transduction; (4) particle-based approaches; and (5) microinjection. Exact methods will not be addressed here since they vary widely by purpose, cell type, transfection reagent, and lab.

- 1. Chemical-based methods for transfection have the advantage that they are relatively easy, can transfect large amounts of DNA, are fast, and are cheap to use. For those reasons, they are the most commonly used for routine, every day transfections. While there are many types of kits which employ different methods of chemical delivery, they all work based on the same basic principle: DNA is bundled together with something that will interact with the plasma membrane of cells, that bundle is then deposited onto a layer of adherent cells and a proportion of them will take up the exogenous DNA via endocytosis. The oldest of these methods is calcium phosphate-mediated transfection. Calcium phosphate is mixed with the DNA to be transfected and a fine precipitate is formed. This is added to the cell media and, after it settles on the cells, it is taken up. Most commercially available kits use lipids to mediate these transfections. The DNA of interest is incubated with synthetic cationic lipids, which encase it in a liposome. These liposomes are then bathed over the cells to be transfected. The positively charged liposomes interact with the negatively charged lipids of the cellular plasma membrane and are eventually endocytosed, releasing the DNA into the cell.
- 2. *Electroporation of eukaryotic cells* follows the same principles as discussed above for bacteria. Electroporation is typically employed when the plasmid to be transfected is quite large; for example, electroporation is sometimes used to transfect mouse embryonic stem cells with the targeting vectors needed for generation of a knockout mouse.

- **3.** Viral transduction is used when cell lines, such as primary cell cultures, are difficult to transfect through other mechanisms, when it is necessary to stably introduce a gene into the cell's genome; or when it is necessary to express a protein at high levels. There are several different types of viruses used, the most common of which will be discussed here. For mammalian tissues, it is often useful to use adenovirus transduction. Adenovirus has the advantage that it infects a wide variety of cell types and allows for high expression of transduced genetic material. However, the viral DNA does not integrate into the host cell chromosomes and is not replicated, thus, it is most useful for experiments where efficient, high-level, short-term expression is needed. Retrovirus vectors have long been used for long-term stable expression of genes in mammalian cells; however, they have the drawback of infecting and integrating only in dividing cells. Another mammalian cell specific vector is the lentivirus family of retroviruses. Lentiviruses are similar to retroviruses in that they integrate into the host genome but they have the advantage that they can infect nondividing cells as well as dividing cells. Baculoviruses are able to infect many types of invertebrate cells so they are often used for expressing proteins in insect cells, including Sf9 cells, derived from pupal ovarian tissue of the fall armyworm, Spodoptera frugiperda, and S2 cells, derived from late embryonic stages of Drosophila.
- 4. *Particle-based approaches* are often employed when other transfection techniques fail. These approaches have the benefit that they are extremely targeted: the nuclei of single cells are bombarded with DNA attached to a particle (e.g., gold beads, nanoparticles). The particles are shot into a cell using a gene gun or other bolistic delivery system. Nucleic acids associated with magnetic nanoparticles can be introduced into cells by applying a magnetic force. However, this technique has several serious drawbacks. It is expensive, time consuming, and has a low efficiency of transfection.
- 5. *Microinjection* is frequently used to create genetically engineered or transgenic animals. This technique requires quite a bit of precision and expertise, therefore it is typically only employed in specialized circumstances. To microinject DNA into a cell, the DNA of interest is loaded into a microinjection needle, $\sim 0.5-5 \ \mu m$ in diameter. The needle is then carefully and slowly pushed through both the plasma membrane and nuclear envelope of the cell to be injected. At that time, a small amount of the

DNA is injected into the nucleus, and the needle carefully removed. Injected cells are then allowed to recover and later screened for insertion of the DNA of interest.

SOURCE REFERENCE

Griffith, F. (1928). The significance of pneumococcal types. *Epidemiology and Infection*, 27(2), 113–159. Cambridge Univ Press. ISSN=0950-2688.

CHAPTER FOUR

Agarose Gel Electrophoresis

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Abstract

Agarose gel electrophoresis is used to separate DNA or RNA molecules based upon their size.

1. THEORY

The separation of nucleic acids based upon their size is required for many common laboratory practices (e.g., subcloning, genotype diagnostics, RT-PCR). Separation of nucleic acids by agarose gel electrophoresis works by harnessing the negative charge of the phosphate backbone of nucleic acids. DNA and RNA molecules have a net negative charge spread evenly over their entire length so they will move through an agarose matrix in an electric field toward the positive pole. Shorter nucleic acids will be able to migrate through the matrix faster than larger ones during a given period of time. Depending upon the percentage of agarose used to make the gel, the range of linear separation will vary.

2. EQUIPMENT

pH Meter and calibration standards Magnetic stir plate Microwave oven Agarose gel casting tray Agarose gel comb Agarose gel electrophoresis tank Power supply Transilluminator or other UV light source Micropipettors Micropipettor tips Magnetic stir bars Beaker, 1 1 Erlenmeyer flask, 250 or 500 ml

3. MATERIALS

Agarose Tris base EDTA, disodium salt Glacial acetic acid Boric acid (H₃BO₃) Sodium hydroxide (NaOH) Lithium hydroxide (LiOH) Ethidium bromide or other fluorescent DNA intercalator (e.g., SYBR-Safe, Invitrogen) Ficoll Bromophenol blue Xylene cyanol FF DNA ladder

3.1. Solutions & buffers

There are several different buffers that you can choose from for your electrophoresis. The recipes for all of these buffers are included here. Briefly the advantages and disadvantages of each buffer are:

TAE – The most common of all buffers, TAE has a low buffering capacity that prohibits gels from being run at extremely high voltages. However, it is best at resolving large DNA or RNA fragments.

TBE – TBE has been shown to be better than TAE at resolving fragments smaller than 3 kb. Gel purification of DNA bands may not work as well in TBE as in TAE. Refer to the manual of your specific kit for instructions and limitations.

SB – Sodium borate is a Tris-free buffer. Sodium borate has low conductivity and therefore gels can be run at higher voltages. SB produces sharp bands and nucleic acids can be purified for all downstream applications. However, SB is not as efficient as Trisbased buffers at resolving bands larger than 5 kb.

LB – Lithium borate buffer is similar to SB but has an even lower conductivity. LB can resolve very small size differences if used in conjunction with high-percentage agarose gels. However, lithium borate is somewhat expensive and thus not necessary for most common electrophoretic needs.

5	0	X	I	A	F	

Component	Final concentration	Stock	Amount
Tris base	2 M	N/A	242 g
EDTA, pH 8.0	$1 \times$	0.5 M	100 ml
Glacial acetic acid	N/A	N/A	57.1 ml

Dissolve Tris in \sim 750 ml of deionized water. Add EDTA and acetic acid. Adjust the final volume to 1000 ml with water. There is no need to adjust the pH of this solution.

$1 \times TAE$

Add 20 ml of $50 \times$ TAE stock to 980 ml of deionized water

10 imes	TBE
----------	-----

Component	Final concentration	Stock	Amount
Tris base	890 mM	N/A	108 g
EDTA, pH 8.0	20 mM	0.5 M	40 ml
Boric acid	890 mM	N/A	55 g

Dissolve Tris and boric acid in approximately 750 ml of deionized water. Add EDTA. Adjust final volume to 1000 ml with water. There is no need to adjust the pH of this solution.

$1 \times \text{TBE}$

20× SBComponentFinal concentrationStockAmountBoric AcidN/AN/A45 gNaOHN/AN/A8 g

Dissolve boric acid and NaOH in 950 ml of deionized water. Adjust the volume to 1000 ml with water. There is no need to adjust the pH of this solution.

$1 \times SB$

Add 50 ml of $20 \times$ SB stock to 950 ml of deionized water

$20 \times LB$

Component	Final concentration	Stock	Amount
Boric acid	N/A	N/A	36 g
Lithium hydroxide	N/A	N/A	8.392 g

Dissolve boric acid and lithium hydroxide in 950 ml of deionized water. Adjust the volume to 1000 ml with water. There is no need to adjust the pH of this solution.

$1 \times LB$

Add 50 ml of $20 \times$ LB stock to 950 ml of deionized water

1% Agarose Gel Mix

Component	Final concentration	Stock	Amount
Agarose	1% (w/v)	N/A	1 g
Buffer of choice	$1 \times$	$1 \times$	100 ml
Ethidium bromide	$0.5 \ \mu g \ ml^{-1}$	10 mg ml^{-1}	5 µl

Mix agarose and buffer in a flask and microwave until dissolved. Add ethidium bromide (or other DNA intercalating dye) **after** removing from the microwave oven, so as not to vaporize the ethidium bromide. NOTE: This is just a template recipe. The concentration of agarose gel that you will need depends upon the size(s) of the nucleic acid(s) you wish to resolve.

Agarose Concentration	Linear Resolution of Gel
0.5%	1000–30 000 bp
0.7%	800–12 00 bp
1.0%	500–10 000 bp
1.2%	400–7000 bp
1.5%	200–3000 bp
2.0%	50–2000 bp

$6\times$ DNA loading dye

Component	Final concentration	Stock	Amount
Ficoll	15% (w/v)	N/A	1.5 ml
Bromophenol blue	0.25% (w/v)	N/A	25 mg
Xylene cyanol FF	0.25% (w/v)	N/A	25 mg

Add water to 10 ml

4. PROTOCOL

4.1. Preparation

Prepare the running buffer you wish to use.

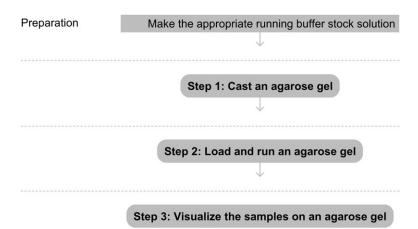


Figure 4.1 Flowchart of the complete protocol, including preparation.

4.2. Duration

Preparation	About 2 h
Protocol	About 1 h

4.3. Caution

Ethidium bromide is a known mutagen and carcinogen. It, and all other nucleic acid intercalators, should be handled with care. Wear gloves. Do not microwave any buffer containing ethidium bromide or any other dye. The dyes can vaporize and be inhaled. Instead, wait until the buffer has cooled slightly and then add the dye. Wear gloves when handling the gel and solutions containing ethidium bromide. Dispose of gel and buffer in accordance with local regulations.

See Fig. 4.1 for the flowchart of the complete protocol.

5. STEP 1 CASTING AN AGAROSE GEL

5.1. Overview

Cast an agarose gel for ~ 30 min before you are ready to run your samples.

5.2. Duration

30 min

1.1 First, refer to the table in (Materials section) and determine what percentage gel you need to resolve all of your samples. For example, if you are digesting DNA from plasmid minipreps to check for the insertion of a 1.5 kb fragment in a vector of 7 kb, you will need a 1% gel in order to linearly resolve all bands.

Once you have determined what percentage gel you need, add the appropriate amount of agarose to a 250 or 500 ml flask. Add 100 ml of $1 \times$ buffer.

- 1.2 Microwave on high for about 2 min, or until all agarose is dissolved.
- **1.3** Remove from microwave and allow the agarose to cool on the benchtop for a few minutes.
- **1.4** Once the agarose solution has cooled slightly, add your DNA dye of choice. Mix and pour into your assembled casting tray. Take care to pop any bubbles with a pipette tip. Insert the desired type of comb.
- **1.5** Allow gel to cool and solidify. This will take \sim 30 min.
- **1.6** Remove comb (and tape, if used) and place gel in electrophoresis tank filled with 1× running buffer.

5.3. Tip

Do not make your gel with $H_2O!$ Water does not have the ionic strength to conduct electricity through the gel and your samples will not migrate correctly.

5.4. Tip

You must run your gel in the same buffer that you use to make the gel. Otherwise it will not properly conduct electricity and your gel will not run correctly.

5.5. Tip

Assemble the gel-casting tray while microwaving or cooling your agarose solution. Take care not to move the gaskets or else your gel will leak. If you do not have a setup with a mold, secure lab tape over the ends of the gel-casting tray.

5.6. Tip

Wide combs are typically used for purification, while thin combs are used for diagnostics (Fig. 4.2(a) and 4.2(b))

5.7. Tip

If you are in a hurry, you can cast your gel at $4 \degree C$ and it will solidify faster. See Fig. 4.3 for the flowchart of Step 1.

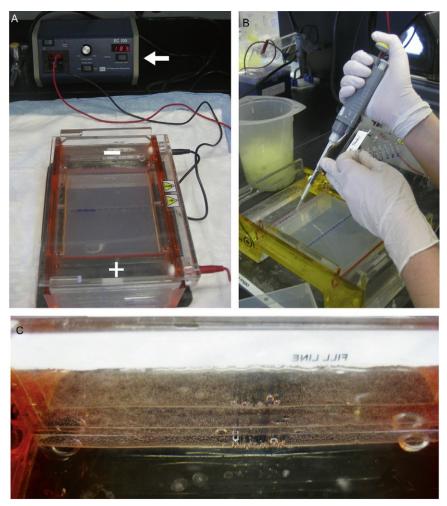


Figure 4.2 Loading and running a gel. (a) A typical gel electrophoresis apparatus consists of a gel box full of running buffer connected to a power source (arrow). Here the positive end is at the bottom of the box. Note the wide wells that the DNA is loaded in – these wells are ideal for purification because they can hold a large volume. (b) In contrast, thin wells are ideal for running a large number of diagnostic samples, such as in the case of genotyping experimental animals. (c) In order to ensure that the power source is connected correctly, look for the presence of bubbles emanating from the wire at the negative side of the gel box.

6. STEP 2 LOADING AND RUNNING AN AGAROSE GEL

6.1. Overview

Here you will load your samples on the gel and separate them in an electric current.

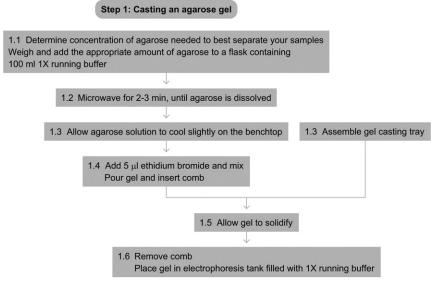


Figure 4.3 Flowchart of Step 1.

6.2. Duration

About 1 h

- 2.1 Determine the amount of sample you would like to run on the gel and add the appropriate amount of $6 \times$ DNA loading buffer. For example, if you are running a genotyping PCR, add 2 μ l $6 \times$ DNA loading buffer to 10 μ l of sample. If you are purifying a fragment for subcloning, you will want to load more.
- **2.2** Load an appropriate DNA ladder in one well, either 100 bp or 1 kb. Carefully pipette your samples into the wells. Be careful not to damage the wells or accidentally get your sample in adjacent wells.
- **2.3** Connect your gel tank to the power source. Make sure that you have connected the positive wire from your tank to the positive electrode on the power source, and likewise connected the negative to the negative. Turn your power source on (Fig. 4.2(a)).
- **2.4** When you are ready to visualize your gel, turn off the power supply and disconnect it from the gel tank. Carefully remove your gel. Do not put your hands in the tank while the gel is still running.

6.3. Tip

If you need to load a larger volume of sample than your well can hold, you can centrifuge it in a Speed-vac to concentrate it. Additionally, using the long thin gel loading pipet tips will allow you to load more sample than regular tips.

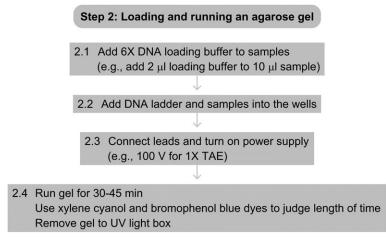


Figure 4.4 Flowchart of Step 2.

6.4. Tip

You can run your gel at a variety of voltages depending upon the buffer that you choose to use. Keep track of how far your sample has migrated by looking at the dye front on the gel.

6.5. Tip

To ensure that everything is connected correctly and an electric current is being supplied to the tank, check for the presence of bubbles at the positive pole of the tank (Fig. 4.2(c)).

See Fig. 4.4 for the flowchart of Step 2.

>

7. STEP 3 VISUALIZATION OF SAMPLES ON AN AGAROSE GEL

7.1. Overview

Since your sample is stained with a fluorescent dye, you will be able to visualize it using a UV light source.

7.2. Duration

5 min

3.1 Carefully place your gel on the glass top of a UV light box. Turn the light on. You should now be able to see your DNA light up (Fig. 4.5(a) and 4.5(b).

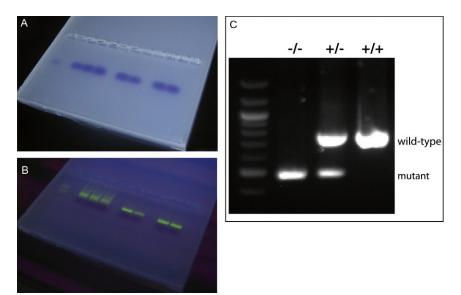


Figure 4.5 Visualization of DNA. (a) Image of an agarose gel illuminated with white light. Note the dye front. (b) Image of the same agarose gel illuminated with UV light. DNA is in green (stained with SYBR-Safe). (c) Gel electrophoresis can be used to genotype experimental animals or diseases. Here wild-type and mutant alleles can be distinguished based on a size difference.

3.2 Take a picture of your gel with a camera mounted to your UV box (Fig. 4.5(c)). If necessary, excise your DNA bands with a razor blade and transfer them to 1.5-ml microcentrifuge tubes for later gel purification and cloning.

7.3. Tip

Do not look directly at UV light. Always wear UV goggles when using UV light. Take care not to expose your skin to UV light too much as well.

7.4. Tip

Note that different DNA stains illuminate different colors – ethidium bromide glows orange and SYBR-Safe glows green, for example.

7.5. Tip

Minimize the amount of time that your sample is exposed to UV light. UV light induces crosslinks in DNA by creating pyrimidine dimers between adjacent cytosine and thymine residues. This is particularly crucial and therefore should be kept in mind if DNA is to be cut from your gel for purification and used for downstream sub-cloning.



Analysis of DNA by Southern Blotting

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Abstract

The purpose of this protocol is to detect specific DNA sequences in a complex sample by hybridization to a labeled probe.

1. THEORY

Southern blotting is routinely used to analyze DNA samples for a number of different applications. It was first described by Edwin Southern (Southern, 1975), hence the name, Southern blotting. In most applications, genomic DNA is digested with restriction enzymes and size-fractionated by agarose gel electrophoresis. The two DNA strands are denatured in the gel by alkaline treatment and then transferred to a nylon membrane by capillary action. The DNA fragments are immobilized on the membrane, which is now an exact replica of the size-fractionated DNA molecules in the gel. Specific DNA sequences are detected by hybridizing the membrane with a labeled probe that is complementary to the sequence of interest and then visualized according to the nature of the labeled probe. Originally probes were labeled using $[\alpha$ -³²P]-labeled deoxynucleotides; however, a number of nonradioactive labeling and detection techniques have been developed (Cate et al., 1991; Engler-Blum et al., 1993).

In its basic form, Southern blotting is used to determine the size of a particular restriction fragment in a complex mixture of digested genomic DNA. It can be used to study normal gene rearrangements, as in T-cell receptor or immunoglobulin gene rearrangements, or chromosomal rearrangements or translocations of oncogenes in tumor cells. It is relatively quantitative and can be used to determine the number of copies of a gene in the genome. By reducing the stringency of hybridization, it can be used to find related sequences in the genome or find similar sequences found in other species. It can also be used with restriction mapping using different restriction enzymes to characterize a particular region and in some cases to identify single nucleotide polymorphisms that affect a particular restriction enzyme recognition site. It is also used to verify that no gross rearrangements have occurred during the cloning process for a piece of DNA.

2. EQUIPMENT

Water baths (37, 65, 95 °C) Agarose gel rig Power supply UV cross-linker (e.g., Stratalinker) Plexiglass shields, radioactive waste disposal Hybridization oven Hybridization bottles Trays (for washing membranes) Film processor – or – Phosphorimager X-ray film – or – Phosphor-capture screen Micropipettors Micropipettor tips 1.5-ml microcentrifuge tubes Glass tray Glass plate Whatman 3MM chromatography paper Nylon membrane (e.g., Hybond) Paper towels Aluminum foil Plastic wrap 17-gauge needle Syringe 0.45-µm disposable cellulose acetate membrane Adhesive dots with phosphorescent ink Adhesive dots with radioactive ink

3. MATERIALS

Restriction enzymes 10× restriction enzyme buffer Agarose TBE buffer 6× DNA loading buffer Tris base Sodium chloride (NaCl) Sodium hydroxide (NaOH) Sodium citrate Random primed DNA labeling kit (e.g., Roche) $[\alpha^{-32}P]$ -dCTP, 3000 Ci mmol⁻¹, aqueous solution Digoxigenin-11-dUTP random primed DNA labeling kit (e.g., DIG-High Prime, Roche) DIG nucleic acid detection kit (e.g., Roche) Sodium dodecyl sulfate (SDS) Ficoll 400 Polyvinylpyrrolidone Bovine serum albumin, Fraction V Formamide Poly(A) (optional) Salmon sperm DNA, type III, sodium salt Phenol Phenol:chloroform:isoamyl alcohol

3.1. Solutions & Buffers

Step 2 Denaturation Buffer

Component	Final concentration	Stock	Amount
NaOH	0.5 N	10 N	25 ml
NaCl	1.5 M	5 M	150 ml

Add purified water to 500 ml

Neutralization Buffer

Component	Final concentration	Stock	Amount
Tris–HCl, pH 8.0	1 M	2 M	250 ml
NaCl	1.5 M	5 M	150 ml

$20 \times SSC$

Component	Final concentration	Amount
NaCl	3 M	175.3 g
Sodium citrate	0.3 M	88.2 g

Dissolve in 800 ml purified water. Adjust pH to 7.0 using 1 N HCl (a few drops). Add purified water to 1 l. Sterilize by autoclaving

Component	Final concentration	Stock	Amount
SSC	6×	$20 \times$	3 ml
Denhardt's solution	5×	50×	1 ml
SDS	0.5% (w/v)	20%	0.25 ml
Salmon sperm DNA	$100 \ \mu g \ ml^{-1}$	10 mg ml^{-1}	0.1 ml
Formamide	50% (v/v)		5 ml
Poly(A) (optional)	$1 \ \mu g \ ml^{-1}$	10 mg ml^{-1}	1 µl

Step 4 Prehybridization Solution

Add purified water to 10 ml. Mix and filter through a 0.45- μ m disposable cellulose acetate membrane

ComponentFinal concentrationAmountFicoll 4001% (w/v)1 gPolyvinylpyrrolidone1% (w/v)1 gBovine serum albumin1% (w/v)1 g

$50 \times$ Denhardt's solution

Add purified water to a final volume of 100 ml

Salmon sperm DNA, 10 mg ml⁻¹

Dissolve salmon sperm DNA in purified water at a concentration of 10 mg ml⁻¹ in a 1.5-ml microcentrifuge tube (may take 2–4 h, stir if necessary). Adjust concentration of NaCl to 0.1 M. Extract the solution once with phenol and once with phenol/chloroform/isoamyl alcohol. Recover the aqueous phase that contains the DNA. Pass the aqueous phase quickly through a 17-gauge needle 12 times to shear the DNA. Add two volumes of ice-cold ethanol and centrifuge the tube in a microcentrifuge at maximum speed for 10 min. Resuspend the pellet in purified water at a concentration of 10 mg ml⁻¹. Check the concentration by measuring the absorbance at A_{260} and adjust the concentration to 10 mg ml⁻¹. Boil the DNA solution for 10 min. Dispense into 100 µl aliquots and store at -20 °C. Just before use, heat the DNA solution in a boiling water bath for 5 min and immediately place the tube on ice

Poly(A), 10 mg ml⁻¹ (optional)

Dissolve 10 mg poly(A) RNA in 1 ml sterile purified water. Store at -20 °C in 50 µl aliquots

Component	Final concentration	Stock	Amount
SSC	$2 \times$	$20\times$	50 ml
SDS	0.1% (w/v)	20%	2.5 ml

Wash Solution #1

Add purified water to 500 ml

Wash Solution #2

Component	Final concentration	Stock	Amount
SSC	$0.1 \times$	$20\times$	250 ml
SDS	0.1% (w/v)	20%	2.5 ml

Add purified water to 500 ml

Maleic Acid Buffer

Component	Final concentration	Amount
Maleic acid	0.1 M	11.6 g
NaCl	0.15 M	8.77 g

Dissolve in 800 ml purified water (maleic acid will not go into solution). Add NaOH pellets while stirring, adjusting pH to 7.5. Add purified water to 1 l

10× Blocking Reagent

Dissolve The Blocking Reagent powder (Roche) in Maleic Acid Buffer at a concentration of 10% (w/v). Stir, shake, and heat to dissolve, the solution will remain turbid. Sterilize the solution by autoclaving

Washing Buffer

Component	Final concentration	Stock	Amount
Maleic acid	0.1 M		11.6 g
NaCl	0.15 M		8.77 g
Tween-20	0.3%	20%	15 ml

Prepare Maleic Acid Buffer as above. Add Tween-20 after adjusting the pH to 7.5 and then add purified water to 1 l

Detection Buffer

Component	Final concentration	Stock	Amount
Tris–HCl, pH 9.5	0.1 M	1 M	10 ml
NaCl	0.1 M	5 M	2 ml

Mix with 88 ml purified water

4. PROTOCOL

4.1. Duration

Preparation	About 1 day
Protocol	About 4–5 days

4.2. Preparation

Isolate genomic DNA from the appropriate source.

4.3. Caution

Consult your institute Radiation Safety Officer for proper ordering, handling, and disposal of radioactive materials.

See Fig. 5.1 for the flowchart of the complete protocol.

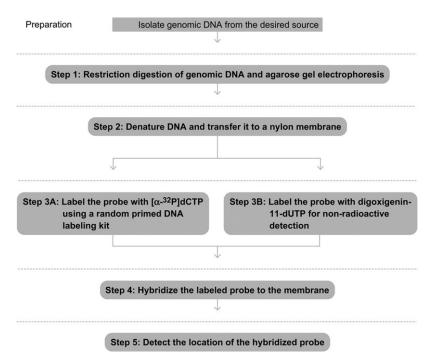


Figure 5.1 Flowchart of the complete protocol, including preparation.

5. STEP 1 RESTRICTION DIGESTION OF GENOMIC DNA AND AGAROSE GEL ELECTROPHORESIS

5.1. Overview

Genomic DNA is digested to completion using the desired restriction enzyme(s). Next, the DNA fragments are separated by agarose gel electro-phoresis according to size.

5.2. Duration

About 24 h

1.1 Digest 10 µg of genomic DNA with the appropriate restriction enzyme(s). Add to a 1.5-ml polypropylene microcentrifuge tube:

Genomic DNA (10 µg)	$X \mu l$
$10 \times$ enzyme buffer	5 µl
Restriction enzyme $(1-2 \text{ U} \mu \text{g}^{-1})$	1–5 µl
Purified water	to 50 µl

1.2 Incubate reaction at 37 °C, overnight.

- **1.3** Heat reaction at 65 °C for 20 min to denature the restriction enzyme (optional).
- **1.4** Add 10 μ l of 6× DNA sample buffer.
- **1.5** Pour an agarose gel and separate the DNA fragments by electrophoresis (see Agarose Gel Electrophoresis).

5.3. Tip

The total amount of restriction enzyme(s) added to the reaction mix should be < 10% of the final volume. If too much is added, the higher glycerol concentration may adversely affect the digestion.

See Fig. 5.2 for the flowchart of Step 1.

6. STEP 2 DENATURE DNA AND TRANSFER IT TO A NYLON MEMBRANE

6.1. Overview

The DNA will be denatured in the gel by soaking it in alkaline buffer and then transferred to a nylon membrane by capillary action.

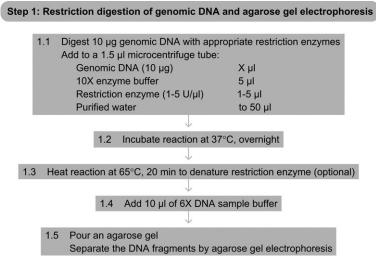


Figure 5.2 Flowchart of Step 1.

6.2. Duration

Overnight

- **2.1** Remove the gel from the electrophoresis apparatus and place it in a glass tray with 500 ml Denaturation Buffer. Let gel soak for 45 min at room temperature while rotating slowly on a platform rotator.
- **2.2** Pour off Denaturation Buffer and replace it with 500 ml Neutralization Buffer. Let gel soak for 1 h at room temperature while rotating slowly on a platform rotator.
- **2.3** Set up the transfer. Place a piece of glass across a larger glass tray so that it rests on the sides of the tray. Cut two pieces of Whatman 3MM chromatography paper so that they cover the piece of glass and hang over the edges to reach the bottom of the glass tray beneath it. Wet the paper thoroughly and fill the glass tray with $10 \times$ SSC. Roll a pipette over the paper to remove air bubbles.
- **2.4** Place the gel upside down on the wetted chromatography paper and remove air bubbles. Cover the parts of the Whatman paper not covered with the gel with aluminum foil.
- **2.5** Cut a piece of nylon membrane to the size of the gel and wet it thoroughly with $2 \times$ SSC. Place the membrane on top of the gel and remove air bubbles as before.
- **2.6** Cut two pieces of Whatman 3MM chromatography paper the same size as the membrane. Wet them thoroughly in $2 \times$ SSC and place them on top of the membrane. Remove air bubbles as before.

- 2.7 Cut a stack of paper towels about 4–5 inches high, the same size as the membrane, and place on top of the Whatman paper.
- 2.8 Place a piece of plexiglass on top of the stack of paper towels. Cover the entire setup with plastic wrap and place a weight (about 500 g) on top of the plexiglass. Allow DNA to transfer out of the gel overnight (18–20 h).
- **2.9** After the transfer is complete, disassemble the transfer setup. Invert the gel with the membrane on a dry surface.
- **2.10** Mark the positions of the wells on the membrane using a pencil. Discard the gel.
- **2.11** Immerse the membrane in $6 \times$ SSC and wash for 5 min at room temperature with gentle rotating.
- **2.12** Expose the membrane to UV light (302 nm) for 2–3 min using a UV cross-linker.

6.3. Tip

Add more $10 \times SSC$ as needed during the day before wrapping the transfer setup in plastic wrap for overnight transfer.

6.4. Tip

Remove the wet paper towels from the bottom of the stack and replace with dry ones during the day before wrapping the transfer setup in plastic wrap for overnight transfer.

6.5. Tip

In the case that nitrocellulose membranes are used instead of nylon membranes, the DNA is fixated to the membrane by heat.

See Fig. 5.3 for the flowchart of Step 2.

7. STEP 3A LABEL THE PROBE WITH [α -³²P]-DCTP USING A RANDOM PRIMED LABELING KIT

7.1. Overview

The probe will be labeled with $[\alpha-^{32}P]$ -dCTP using a random primed DNA labeling kit (e.g., Roche).

7.2. Duration

About 1 h

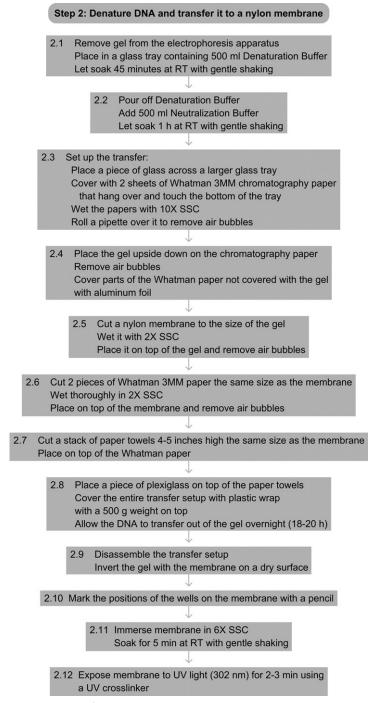


Figure 5.3 Flowchart of Step 2.

7.3. Caution

Consult your institute Radiation Safety Officer for proper ordering, handling, and disposal of radioactive materials.

- **3A.1** Thaw the $[\alpha^{-32}P]$ -dCTP behind proper shielding.
- **3A.2** Add 25–50 ng of linear probe DNA to purified water in a total volume of 9 μl.
- **3A.3** Heat the tube in a boiling water bath (>95 °C) for 10 min. Immediately place the tube on ice.
- 3A.4 Briefly spin the tube of denatured DNA and add in the following order:3 μl dNTP stock mix
 - 2 µl reaction mixture (containing random hexanucleotides)
 - 5 μ l 50 μ Ci [α -³²P]-dCTP
 - 1 µl Klenow polymerase
- **3A.5** Mix the reaction by pipetting. Centrifuge the tube briefly.
- **3A.6** Incubate the reaction at 37 °C for 30 min.
- **3A.7** Add 2 μ l of 0.2 M EDTA, pH 8.0 or heat at 65 °C for 10 min.
- **3A.8** Pass the reaction mix through a QuickSpin Sephadex G-50 column to remove unincorporated nucleotides.

See Fig. 5.4 for the flowchart of Step 3A.

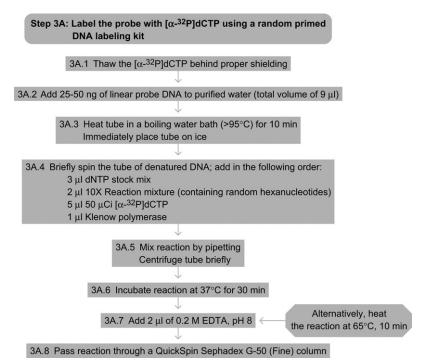


Figure 5.4 Flowchart of Step 3A.

8. STEP 3B LABEL THE PROBE WITH DIGOXIGENIN-11-DUTP FOR NONRADIOACTIVE DETECTION

8.1. Overview

The probe will be labeled with digoxigenin-11-dUTP for nonradioactive detection using a random primed DNA labeling kit (e.g., DIG-High Prime, Roche).

8.2. Duration

Overnight

- **3B.1** Add 1 μ g of linearized probe DNA to purified water in a total volume of 16 μ l in a 1.5-ml microcentrifuge tube.
- **3B.2** Heat the DNA in a boiling water bath for 10 min to completely denature the DNA. Immediately place the tube on ice.
- **3B.3** Add 4 μ l of DIG-labeling mix (5× concentration).
- **3B.4** Incubate the reaction at 37 °C, overnight.
- **3B.5** Add 2 μl of 0.2 M EDTA or heat the tube at 65 °C for 10 min to stop the reaction.

8.3. Tip

The $5 \times$ DIG-labeling mix contains a mixture of deoxynucleotides plus digoxigenin-11-dUTP, $5 \times$ reaction buffer with random hexanucleotides, and Klenow polymerase.

8.4. Tip

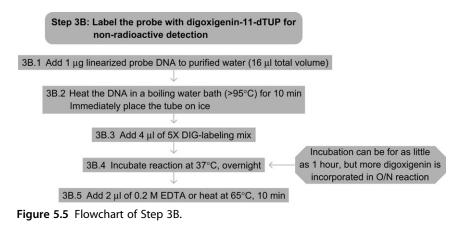
The duration of the labeling reaction should be from 1 to 20 h. The longer incubation time will increase the yield of Dig-labeled probe DNA.

See Fig. 5.5 for the flowchart of Step 3B.

9. STEP 4 HYBRIDIZE THE LABELED PROBE TO THE MEMBRANE

9.1. Overview

The labeled probe will be hybridized to the denatured DNA on the membrane. After hybridization, the membrane will be washed to reduce nonspecific background binding.



9.2. Duration

Overnight + 2 h

- **4.1** Place the membrane in a hybridization bottle containing 0.1–0.2 ml prehybridization buffer per square centimeter of membrane.
- **4.2** Incubate at 42 °C for 1 h, rotating in a hybridization oven.
- **4.3** Denature the probe at 95 °C for 10 min. Immediately place the tube on ice.
- 4.4 Add the denatured probe to the prehybridization buffer.
- 4.5 Hybridize it to the membrane at 42 °C, overnight.
- **4.6** Remove the membrane from the hybridization bottle and place it in a tray with 100 ml Wash Solution #1. Discard the hybridization solution (in radioactive waste, if appropriate, otherwise, in hazardous waste due to the formamide).
- **4.7** Incubate with shaking for 15 min at room temperature. Discard the waste (as appropriate for radioactive waste).
- 4.8 Repeat wash using Wash Solution #1.
- **4.9** Wash the membrane with 100 ml Wash Solution #2 at 65 °C for 30 min, with shaking. Discard wash solution.
- 4.10 Repeat wash using Wash Solution #2.
- **4.11** Wash the membrane with $6 \times$ SSC for 5 min at room temperature.

9.3. Tip

Do not add the denatured probe directly on top of the membrane. Instead add it to the prehybridization buffer and mix it before letting it contact the membrane.

See Fig. 5.6 for the flowchart of Step 4.

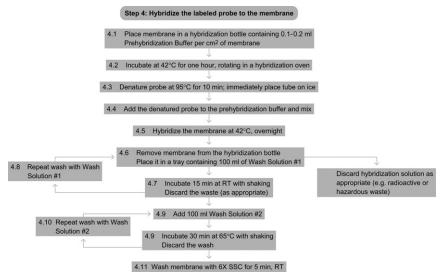


Figure 5.6 Flowchart of Step 4.

10. STEP 5 DETECT THE LOCATION OF THE HYBRIDIZED PROBE

10.1. Overview

The radiolabeled probe will be detected by autoradiography. The hybridized digoxigenin-labeled probe will be detected using an alkaline phosphatase-conjugated antibody that recognizes digoxigenin. Reactivity will be visualized by colorimetric assay using NBT/BCIP as a substrate.

10.2. Duration

- 2 h overnight, depending on the strength of the signal
- **5.1** For detecting a radiolabeled probe, wrap the membrane in plastic wrap, and expose to X-ray film or a phosphor-capture screen.
- **5.2** Develop the film or scan the phosphor-capture screen to see the bands. Fig. 5.7 shows an autoradiograph of a Southern blot probed with a different probe in each of the six panels.
- 5.3 For detecting a digoxigenin-labeled probe, rinse the membrane in Washing Buffer. Dilute the 10× Blocking Reagent 1:10 in Maleic Acid Buffer to give a 1× Blocking Solution.
- 5.4 Incubate the membrane in 100 ml 1× Blocking Solution for 30 min at room temperature with gentle shaking.

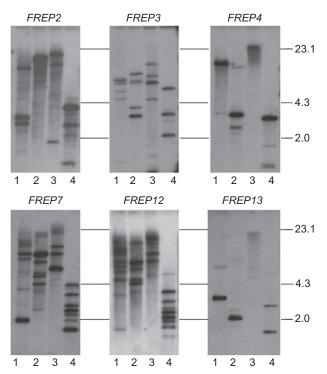


Figure 5.7 Shown is a typical autoradiograph of a Southern blot. Each panel was hybridized with a different probe. **Reprinted with permission from Zhang and Loker (2004).

- 5.5 Dilute the anti-digoxigenin-AP conjugated antibody 1:5000 in 20 ml $1 \times$ Blocking Solution (4 µl in 20 ml).
- **5.6** Incubate the membrane in the diluted antibody conjugate for 30 min at room temperature with gentle shaking.
- **5.7** Wash twice with 100 ml Washing Buffer for 15 min each wash with gentle shaking.
- 5.8 Equilibrate the membrane in 20-ml Detection Buffer for 2–5 min.
- **5.9** Add 200 μl of NBT/BCIP stock solution to 10-ml Detection Buffer in a foil-wrapped tube.
- **5.10** Incubate the membrane in Detection Buffer containing the NBT/ BCIP substrate in the dark without shaking. Check periodically to monitor color development.
- **5.11** When it reaches the desired color, place the membrane in 50 ml purified water to stop the reaction.
- 5.12 Photograph or photocopy the membrane to record the results.
- See Fig. 5.8 for the flowchart of Step 5.

Step 5: Detect the location of the hybridized probe

5.1 Wrap the membrane in plastic wrap Expose it X-ray film or a phosphor capture screen

in 50 ml purified water

Figure 5.8 Flowchart of Step 5.

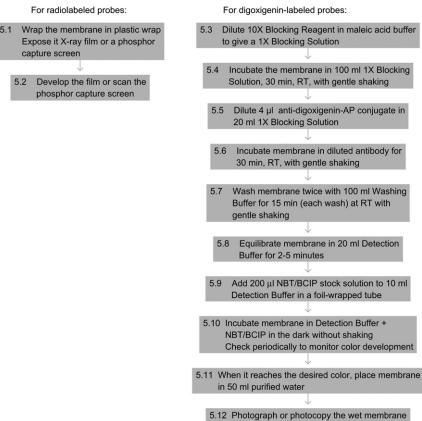
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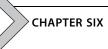
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Agarose Gel Electrophoresis.





Purification of DNA Oligos by Denaturing Polyacrylamide Gel Electrophoresis (PAGE)

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Abstract

After chemical synthesis, the oligonucleotide preparation contains the desired full-length oligonucleotide but also all of the DNA molecules that were aborted during each cycle in the synthesis, and the by-products generated during the chemical reactions. The purification of oligonucleotides is a critical step for demanding applications where the exact length or sequence of the oligonucleotide is important, or for oligonucleotides longer than 50 bases. There are several methods of increasing oligonucleotide purity, the choice of which will depend on modifications of the oligonucleotides and their intended use. Polyacrylamide gel purification (PAGE purification) is the method of choice when the highest percentage of full-length oligonucleotide is desired. This chapter describes a protocol for oligonucleotide purification using denaturing polyacrylamide gel electrophoresis, and purification from the gel slice by two different methods: by diffusion or by electroelution. This chapter also includes recommendations as well as protocol advice.

1. THEORY

Oligonucleotides are synthesized by the addition of one base at a time, extending from the 3' to the 5'-end, attached to a solid-phase support. When the chemical synthesis of the oligonucleotide is complete, the DNA chain is released from the solid support by incubation with ammonium hydroxide. Ammonium also acts as a deprotective agent, cleaving off the groups protecting the phosphates in the phosphodiester bonds. After this, hot ammonia is added to hydrolyze the protecting groups from the exocyclic amino groups of deoxyadenosine, deoxycytosine, and deoxyguanosine. The oligonucleotide could be supplied to the user in the ammonia solution as a crude preparation. In this case, before use, it may be necessary to carry out an additional step to remove the salts and by-products from the oligonucleotide preparation generated during deprotection.

After deprotection, the oligonucleotide preparation is usually desalted, quantified, evaporated to dryness in a lyophilizer, and supplied to the user in the form of a powder. The desalted oligonucleotide solution contains the full-length oligonucleotide but also contains a mixture of truncated products that were accumulated during the chemical synthesis. Truncation occurs when the specified base fails to add to the chain in the corresponding cycle (Hecker & Rill, 1998). Thus, the percentage of truncated products accumulated in the preparation is determined by the synthesis efficiency (the fraction of full-length product after synthesis) and the length of the molecule. Long oligonucleotides, which require more cycles to be synthesized, are less pure than short oligonucleotides. These failures may compete with the full-length product in some applications and may need removal before the oligonucleotide can be used. However, desalted oligonucleotide preparations are usually suitable for many applications, such as standard PCR or microarrays, especially if the oligonucleotide is <20 nucleotides in length.

Further purification for oligonucleotides longer than 50 bases is recommended because the percentage of full-length product in the preparation is typically no higher than 80%. For demanding applications where the exact length of the oligonucleotide is important, such as gel-shift assays, sitedirected mutagenesis, sequencing, production of cloning adapters, or first-strand cDNA synthesis for the generation of libraries, additional purification is recommended, even for shorter oligonucleotides (see more information on the techniques above on Standard in vitro Assays for Protein-Nucleic Acid Interactions – Gel shift Assays for RNA and DNA Binding and Site-Directed Mutagenesis).

Purification may be required after synthesis or after enzymatic modification of the oligonucleotide. There are several methods for achieving this, and the choice depends on the nature of the oligonucleotide and the application that it is intended for.

Reverse-phase HPLC purification is based on hydrophobicity. It uses a nonpolar stationary phase, and aqueous buffers and organic solvents as the mobile phase to elute the analytes; polar compounds are eluted first, while nonpolar compounds are retained. This is the best method for purifying oligonucleotides modified with a hydrophobic group, such as biotin, fluorescent dye labels, or NHS-ester conjugations. The mass recovery is higher than when using PAGE purification and it is amenable to the purification of greater amounts of oligonucleotides (mmole scale), although it does not remove incomplete products so effectively. Oligonucleotide purification cartridges, based on reverse-phase chromatography, provide a fast method of desalting and purifying oligonucleotides.

Polyacrylamide gel electrophoresis (PAGE) resolves oligonucleotides according to their length and thereby offers enough resolution to differentiate full-length products from failed molecules (Atkinson and Smith, 1984). Polyacrylamide gels are more effective for separating small fragments of DNA than agarose gels (see Analysis of RNA by analytical polyacrylamide gel electrophoresis and Agarose Gel Electrophoresis). The sole disadvantage of polyacrylamide gels is that they are more difficult to prepare and handle than agarose gels. Polyacrylamide gels are run in a vertical configuration in a constant electric field. After electrophoresis, the oligonucleotide is eluted from the gel and concentrated, using ethanol precipitation (find more on precipitation of nucleic acids on RNA purification – precipitation methods) or reverse-phase chromatography. This is the method of choice when the highest percentage of full-length oligonucleotides is desired for demanding applications. It is also strongly recommended for oligonucleotides longer than 30 bases. Also, several samples can be run simultaneously and no expensive equipment is required. The only drawback of this technique is the small amount of oligonucleotide obtained per purification. Typically, oligonucleotides are used at the scale of 1 µmol or less, and the mass recovery after PAGE purification is <50% and even lower in the case of modified oligonucleotides. Nevertheless, although the yield is low, it is satisfactory for most biochemical applications.

The oligonucleotide preparation is loaded onto a denaturing polyacrylamide gel at an amount of at least 1 mg per lane. The range of resolution of the gel depends on the concentration of polyacrylamide. For short oligonucleotides (from 15 to 35 bases), 13–15% polyacrylamide gels are recommended; for longer oligonucleotides (from 35 to 70 bases), 8–13% polyacrylamide gels are recommended. The percentage of the gel should be adapted to the running system. We usually use the Bio-Rad Mini Protean II system and run 15% gels to purify oligonucleotides of 25 bases in length. After identification of the correct band by UV visualization, the band is excised and extracted from the gel.

Two different methods of purifying oligonucleotides from polyacrylamide gels are described in this chapter. The simplest method is elution by means of diffusion. This is based on the motion of molecules from a region of higher concentration to one of a lower concentration. The result of diffusion is a gradual mixing of material. This method is time consuming but it does not require too much labor or any equipment. Basically, the polyacrylamide band containing the oligonucleotide of interest is sliced into small pieces and covered with elution buffer. After incubation, the DNA is purified from the supernatant by ethanol precipitation. The yield is limited, between 20% and 70%, depending on the length of the oligonucleotide. The greater the amount of elution buffer used, the more oligonucleotide is diffused from the gel.

The second method is electroelution into a dialysis bag (McDonell et al., 1977). The gel piece containing the oligonucleotide is excised and placed into a dialysis bag with a buffer. The application of an electric current causes the DNA to migrate out of the gel into the dialysis bag buffer. The oligonucleotide is recovered from this buffer and purified. Using this method, the oligonucleotide can be isolated with a good yield, although it is time consuming if a large number of samples are purified at the same time, because it requires the insertion of each gel band into individual dialysis bags. This method also works well for larger DNA fragments and can even be used to extract DNA from agarose gels.

2. EQUIPMENT

Chemical fume hood Microcentrifuge Speed-vac Vortex mixer Heating block Spectrophotometer Rocking shaker Power supply UV lamp or transilluminator PAGE gel system (e.g., Bio-Rad Mini Protean II system (Bio-Rad)), including glass plates, comb, and spacers Horizontal electrophoresis system Micropipettors Micropipettor tips Gloves 2.0-ml microcentrifuge tubes 1.5-ml microcentrifuge tubes 0.5-ml microcentrifuge tubes Costar[®] Spin-X[®] centrifuge tube filter (Corning) Scalpel/razor blade

Plastic wrap Syringe 20-gauge needle 21-gauge needle Dialysis tubing Dialysis tubing clips

3. MATERIALS

n-Butanol Ethidium bromide Sodium acetate (NaOAc) Ethanol GlycoBlue[™] (Ambion) Molecular weight marker (e.g., 10 bp DNA ladder, invitrogen) Tris base Boric acid (H₃BO₃) EDTA, disodium Bromophenol blue Xylene cyanol Formamide Ammonium acetate (NH₄OAc) Magnesium acetate (MgOAc) 40% acrylamide:bisacrylamide (19:1) Urea Ammonium persulfate (APS) N, N, N', N'-tetramethylethylenediamine (TEMED) Ultrapure water (e.g., purified through a Milli-Q system)

Caution	<i>N</i> -Butanol is irritating to the mucous membranes, upper respiratory tract, skin and especially the eyes. Avoid breathing the vapors. Wear appropriate gloves and safety glasses. Use in a chemical fume hood. Keep away from heat, sparks, and open flames.
	Ethidium bromide is a powerful mutagen. Consult label for specific handling and disposal procedures. Avoid breathing the dust. Wear appropriate gloves when working with solutions that contain this molecule.
	Formamide is teratogenic. The vapor is irritating to the eyes, skin, mucous membranes, and the upper respiratory tract. It may be harmful

(Continued)

by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses. Always use in a chemical fume hood when working with concentrated solutions of formamide. Keep working solutions covered as much as possible.

Unpolymerized acrylamide is a potent neurotoxin and is absorbed through the skin. Polyacrylamide is considered to be nontoxic but it should be handled with care because it might contain small quantities of unpolymerized acrylamide.

APS and TEMED are extremely destructive to tissue of the mucous membranes and upper respiratory tract, eyes, and the skin. Wear appropriate gloves, glasses, and protective clothing. Always use in a chemical fume hood.

3.1. Solutions & buffers

Component	Final concentration	Stock	Amount
Tris base	890 mM		54 g
Boric acid	890 mM		27.5 g
EDTA, pH 8.0	20 mM	0.5 M	20 ml

Add water to 11

Step 2 5×TBF

$1 \times TBE$

Add 200 ml of 5×TBE to 800 ml water

2×Formamide loading buffer

Component	Final concentration	Amount
Formamide	95%	95 ml
H ₂ O		5 ml
EDTA	5 mM	2.7 g
Bromophenol blue	0.1%	1 g
Xylene cyanol	0.1%	1 g

Ethidium bromide (10 mg ml^{-1})

Dissolve 10 mg of ethidium bromide in 1 ml water

Component	Final concentration	Amount
Ammonium acetate	0.5 M	3.85 g
Magnesium acetate	10 mM	0.14 g
H ₂ O		100 ml

Step 4.A Elution buffer

Step 4.B 0.25×TBE

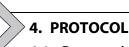
Add 50 ml of 5×TBE to 800 ml water

Step 5 3M NaOAc, pH5.2

Dissolve 12.3 g of sodium acetate in 40 ml of water. Adjust pH to 5.2. Add water to a volume of 50 ml

75% ethanol

Mix 75 ml ethanol with 25 ml water



4.1. Preparation

Order or synthesize the desired oligonucleotide.

4.2. Duration

Preparation	1–2 days (variable)
Protocol	About 8–10 h

See Fig. 6.1 for the flowchart of the complete protocol.

5. STEP 1 BUTANOL PRECIPITATION OF CRUDE OLIGONUCLEOTIDE

5.1. Overview

When the oligonucleotide is supplied as a crude oligonucleotide, it may be necessary to perform a butanol precipitation. This procedure removes ammonia and the by-products produced during deprotection (Sawadogo

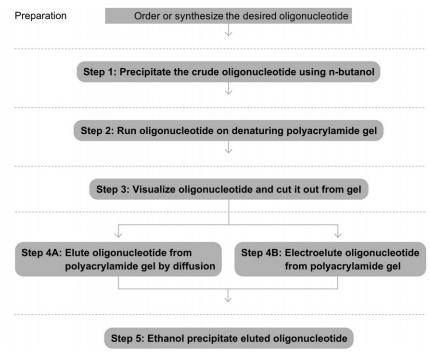


Figure 6.1 Flowchart of the complete protocol, including preparation.

and Van Dyke, 1991). When opening a tube of crude oligonucleotide for the first time, spin the sample and vent the tube in a chemical fume hood by opening it slowly to allow the ammonia gas to escape.

5.2. Duration

About 4 h

- 1.1 Dissolve the lyophilized deprotected oligonucleotide in 100 μ l of ultrapure water to give a 10 μ M solution. Mix by vortexing.
- **1.2** Centrifuge at $10\ 000 \times \text{g}$ for 5 min at room temperature and transfer the supernatant to a new 1.5-ml microcentrifuge tube.
- **1.3** Extract the oligonucleotide by adding 1 ml of *n*-butanol (not water saturated). Mix by vortexing and centrifuge at $12\ 000 \times g$ for 1 min at room temperature.
- 1.4 Remove the water-containing butanol and discard it. Redissolve the pellet in $100 \ \mu l$ ultrapure water.
- **1.5** Repeat the *n*-butanol extraction.

- **1.6** Evaporate the solution to dryness in a speed-vac. The pellet, if it is visible, should be yellowish.
- 1.7 Resuspend the pellet in ultrapure water and quantify the oligonucleotide. Dilute 2 μ l of the oligonucleotide into 200 μ l water to estimate the concentration of the oligonucleotide by measuring the absorbance at 260 nm, or use 1 μ l in a NanoDrop spectrophotometer (see Explanatory Chapter: Nucleic Acid Concentration Determination).

5.3. Tip

If the oligonucleotide is supplied as an ammonia solution, it can be evaporated to dryness in a speed-vac at room temperature. The ammonia will also be removed during butanol precipitation, so another possibility is to do the protocol without lyophilization, starting at Step 1.3.

5.4. Tip

When initially dissolving the crude oligonucleotide, the solution may turn slightly cloudy after vortexing because of the presence of insoluble benzamides.

See Fig. 6.2 for the flowchart of Step 1.

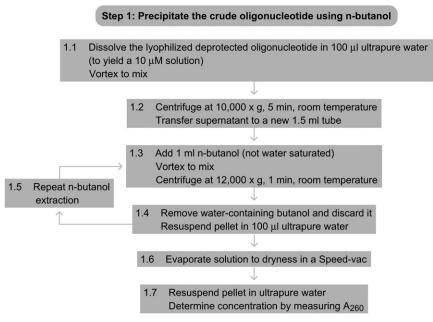


Figure 6.2 Flowchart of Step 1.

6. STEP 2 PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS

6.1. Overview

Oligonucleotides are heat denatured and loaded into a preparative denaturing polyacrylamide gel (for more information, see RNA purification by preparative polyacrylamide gel electrophoresis) to separate the full-length oligonucleotide from the truncated products produced as impurities during the chemical synthesis of the oligonucleotide.

6.2. Duration

About 2 h

2.1	Prepare a	15%	denaturing po	lyacrylamide gel:

Component	Final concentration	Stock	Amount
Urea	0.42 g ml^{-1}		2.1 g
H ₂ O			1.25 ml
TBE	$0.5 \times$	$5 \times$	500 µl
Acrylamide-bisacrylamide (19:1)	15%	40%	1.87 ml
APS	0.1%	10%	50 µl
TEMED	$0.01 \times$		5 µl

Dissolve the urea in the water by heating in a microwave oven, but do not let it boil. When the urea is completely dissolved, add the $5 \times TBE$, and acrylamide–bisacrylamide; swirl to mix. Add sterile water to a final volume of 5 ml. Just before pouring the gel, add 10% APS and TEMED, mix and pour the gel immediately into the cassette made with the two glass plates and the spacers, and then insert the comb. Allow gel to polymerize for about 30 min; 5 ml is enough to prepare one gel for the Bio-Rad Mini Protean II system.

- **2.2** Prepare the sample by adding an equal volume of 2×formamide loading buffer. Mix well by vortexing and incubate for 5 min at 65 °C to denature the DNA. Keep on ice until ready to load the gel. Load at least 1 mg of oligonucleotide per lane.
- **2.3** Place the gel into the tank. Be sure that the short plate faces toward the inner reservoir.
- **2.4** Add 1×TBE buffer to both reservoirs covering the wells, and rinse out the wells with a syringe and a 20-gauge needle.
- **2.5** Prerun the gel in $1 \times \text{TBE}$ at 100 V for 30 min. Rinse out the wells again with a syringe and a 20-gauge needle.
- 2.6 Load the samples.

- **2.7** Run the gel at 200 V until the bromophenol blue is two-thirds down the gel.
- **2.8** Pry apart the glass plates and stain the gel for 5 min in 10 μ g ml⁻¹ of ethidium bromide in 1×TBE.

6.3. Tip

Try to keep the volume of the sample loaded onto the gel down to a minimum (10 μ l or less) to obtain a better resolution. If necessary, dry down the oligonucleotide in a speed-vac and resuspend in ultrapure water. Determine the concentration of the oligonucleotide by measuring the absorbance at 260 nm.

6.4. Tip

In a 15% polyacrylamide gel, bromophenol blue runs around 15 nucleotides and xylene cyanol around 60 nucleotides. If the oligonucleotide migrates at the same rate as the tracking dyes, this will interfere with detection of the oligonucleotide by UV. The loading buffer can be prepared without the tracking dyes, and instead, load 5 μ l of formamide tracking dye mixture into an unused well. Alternatively, the tracking dyes can be substituted by 0.2% Orange G, which migrates with the buffer front and does not interfere with UV detection.

6.5. Tip

During the prerunning, the ammonium persulfate migrates from the wells and the gel will become warmed up, which helps to keep the DNA denatured during electrophoresis, resulting in better resolution.

6.6. Tip

Load a molecular weight marker into a separate lane and keep an empty lane between the marker and the oligonucleotide to avoid contamination.

See Fig. 6.3 for the flowchart of Step 2.

>>7

7. STEP 3 ISOLATION OF OLIGONUCLEOTIDES FROM POLYACRYLAMIDE GELS

7.1. Overview

After the oligonucleotide samples are run on a polyacrylamide gel, the fragment of interest is identified and cut out from the gel.

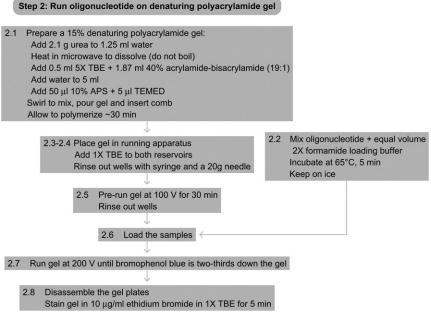


Figure 6.3 Flowchart of Step 2.

7.2. Duration

10 min

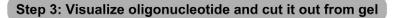
- **3.1** Place the gel on a piece of plastic wrap.
- **3.2** Examine the gel using a UV lamp. Identify the band of interest, which should be the slowest migrating band.
- **3.3** Cut out the region of the gel containing the full-length oligonucleotide. Use a clean, sharp scalpel or razor blade. Keep the size of the polyacrylamide slice as small as possible to avoid contamination.

7.3. Caution

Protect yourself from exposure to UV by wearing face and eye protection, as well as a lab coat.

7.4. Tip

Preparations of oligonucleotides that are up to 20 nucleotides in length generally contain only one band because the efficiency is more than 99%. Long oligonucleotides are less pure than short oligonucleotides, so the preparations of very long oligonucleotides may contain several bands of prematurely truncated products. The slowest migrating



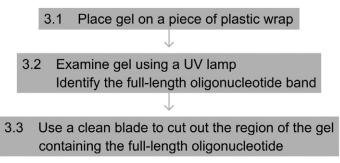


Figure 6.4 Flowchart of Step 3.

band usually corresponds to the desired product. Very rarely, however, the oligonucleotide may migrate not according to its molecular weight.

7.5. Tip

If there is not enough oligonucleotide to be detected by UV, consider radiolabeling a portion of the sample by phosphorylating it to use as a marker.

See Fig. 6.4 for the flowchart of Step 3.

8. STEP 4A ELUTION OF OLIGONUCLEOTIDE FROM POLYACRYLAMIDE GEL BY DIFFUSION

8.1. Overview

After the oligonucleotide of interest is identified and isolated from the gel, the band is sliced into small pieces and the oligonucleotide is recovered from the gel by diffusion.

8.2. Duration

3 h

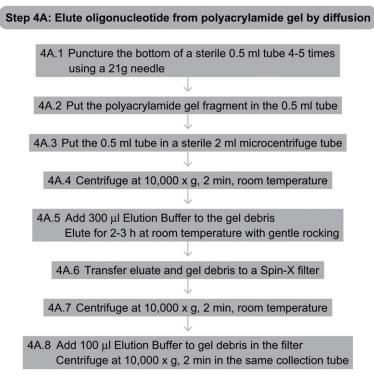
- **4A.1** Puncture the bottom of a sterile 0.5-ml microcentrifuge tube 4–5 times using a 21-gauge needle.
- **4A.2** Place the polyacrylamide gel fragment into the 0.5-ml microcentrifuge tube.
- **4A.3** Place the 0.5-ml microcentrifuge tube into a sterile 2-ml microcentrifuge tube.

- **4A.4** Centrifuge the stacked tubes at $10\ 000 \times g$ in a microcentrifuge for 2 min at room temperature to crush the gel by forcing it through the holes.
- **4A.5** Add 300 μ l of elution buffer to the gel debris and elute the DNA by rocking the tube gently at room temperature for 2–3 h.
- 4A.6 Transfer the eluate and the gel debris to a Costar Spin-X filter.
- **4A.7** Centrifuge the filters at $10\ 000 \times g$ for 2 min at room temperature.
- **4A.8** Add 100 μ l of elution buffer to the gel debris in the filter. Centrifuge at 10 000 × g for 2 min to collect the extra eluate in the same collection tube.

8.3. Tip

It is also possible to grind up the gel band in a tissue homogenizer for 10 min after addition of the elution buffer, or with a disposable pipette tip using a circular motion and pressing the fragments of the gel against the sides of the tube.

See Fig. 6.5 for the flowchart of Step 4A.





9. STEP 4B ELUTION OF OLIGONUCLEOTIDE FROM POLYACRYLAMIDE GEL BY ELECTROELUTION

9.1. Overview

After the oligonucleotide of interest is identified and isolated from the gel, the band is placed in a small dialysis tube containing TBE buffer. The tube is placed in a horizontal electrophoresis tank and the DNA is electroeluted out of the gel band.

9.2. Duration

About 2 h

- **4B.1** Seal one end of a piece of dialysis tubing with a secure knot. Fill the dialysis bag with 0.25×TBE and transfer the gel slice into it.
- **4B.2** Squeeze out most of the buffer, leaving just enough to keep the gel slice in constant contact with the buffer.
- **4B.3** Place a dialysis tubing clip above the gel slice to seal the bag. Seal above the clip with a secure knot.
- **4B.4** Immerse the bag in a shallow layer of 0.25×TBE buffer in a horizontal electrophoresis tank. Prevent the dialysis bag from floating and keep the gel fragment parallel to the electrodes.
- **4B.5** Apply an electric current through the bag at 7.5 V cm^{-1} for 45-60 min.
- **4B.6** Reverse the polarity of the electric current for 20 s to release the DNA from the wall of the dialysis bag.
- **4B.7** Recover the bag from the electrophoresis tank and gently mix the eluted DNA into the buffer.
- **4B.8** Transfer the buffer to a 1.5-ml microcentrifuge tube. Wash out the empty bag with a small volume of $0.25 \times \text{TBE}$ and transfer to the same tube.

9.3. Tip

Wear gloves when handling the dialysis tubing.

9.4. Tip

Avoid trapping air bubbles or clipping the gel slice itself.

9.5. Tip

Under these conditions, the yield of a DNA fragment 0.1-2.0 kb in length is typically about 85%. The electroelution conditions should be adapted to each situation. If the electroelution is too short, the DNA will remain in the gel slice and the yield will be low. If the electroelution is too long, the DNA will become attached to the wall of the dialysis tubing.

9.6. Tip

To confirm that the oligonucleotide has been eluted, remove the gel slice from the bag and stain it with ethidium bromide (10 µg ml⁻¹) in $0.25 \times TBE$ buffer for 20 min and visualize by UV illumination.

See Fig. 6.6 for the flowchart of Step 4B.

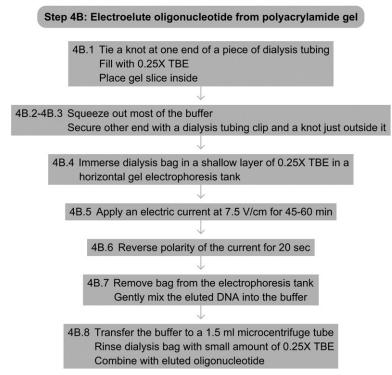


Figure 6.6 Flowchart of Step 4B.

10. STEP 5 ETHANOL PRECIPITATION

10.1. Overview

After elution of the oligonucleotide from the gel slice, it may be concentrated by ethanol precipitation. It is also possible to purify it using commercial resins.

10.2. Duration

1 h

- 5.1 Add 1/10 volume of 3 M NaOAc, pH 5.2, and 2.5 volumes 100% ethanol. Incubate at -20 °C for 30 min.
- **5.2** Centrifuge at $6000 \times \text{g}$ for 30 min at 4 °C.
- 5.3 Remove the supernatant. Wash the pellet by adding $500 \,\mu l$ of 70% ethanol.
- **5.4** Centrifuge at $10\ 000 \times g$ for 5 min at room temperature.
- 5.5 Remove the supernatant. Air dry for 5 min and resuspend in ultrapure water.

10.3. Tip

Add 3 μ l of GlycoBlueTM to increase the size and visibility of the DNA pellet. It consists of a blue dye covalently linked to glycogen, a branched chain carbohydrate.

See Fig. 6.7 for the flowchart of Step 5.

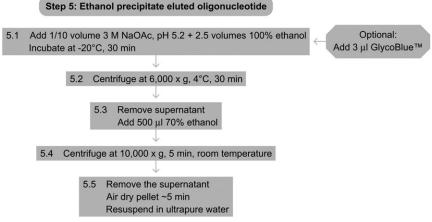


Figure 6.7 Flowchart of Step 5.

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Explanatory Chapter: Nucleic Acid Concentration Determination.

RNA purification by preparative polyacrylamide gel electrophoresis.



Molecular Cloning

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Abstract

This protocol describes the basic steps involved in conventional plasmid-based cloning. The goals are to insert a DNA fragment of interest into a receiving vector plasmid, transform the plasmid into *E. coli*, recover the plasmid DNA, and check for correct insertion events.

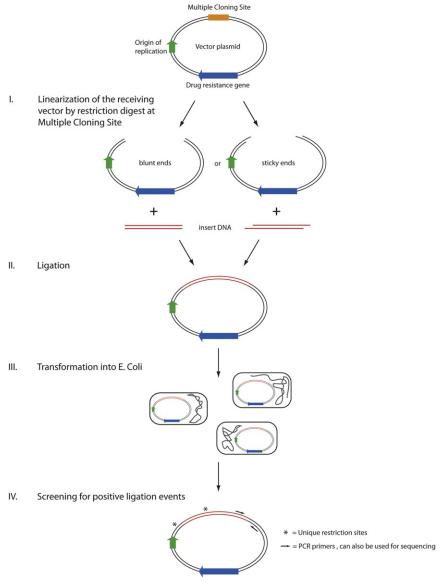
1. THEORY

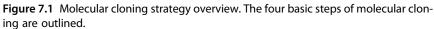
Molecular cloning is an essential technique to create DNA-based experimental tools for expression in bacterial or mammalian cells. Examples of such DNA constructs include a promoter element fused to a reporter gene or a cDNA sequence under the control of a ubiquitous promoter. Molecular cloning entails the preparation of the vector and insert DNAs, ligation of the insert into the vector, transformation of competent *E. coli*, and identification of positive clones (Fig. 7.1).

Traditionally, molecular cloning is defined as the isolation and amplification of a specific DNA fragment. Most of these fragments are created either by digesting an existing piece of DNA with restriction enzymes or by targeting it via PCR. Short inserts of ~ 100 bp can also be commercially synthesized as complementary single-stranded oligos, which are subsequently annealed to form a double-stranded fragment.

After successful isolation, the DNA of interest is ligated into a vector plasmid, a double-stranded circular piece of DNA that can be propagated in *E. coli*. Vectors used in the laboratory represent a smaller version of naturally occurring plasmids that include several basic features: a replication origin, a drug-resistance gene, and unique restriction sites to facilitate the insertion of DNA fragments. Often, several different restriction sites are clustered together in so-called 'polylinker regions' or 'multiple cloning sites,' making it easier to choose convenient and unique restriction enzyme combinations for a variety of inserts.

The choice of restriction enzymes is critical when designing a cloning strategy. While some sever the double-stranded DNA in one place, creating 'blunt' ends, others leave an overhang of a few bases at the cut site. These complementary 'sticky' ends find one another easily, increasing the efficiency of the ligation reaction and thus the chances for a successful cloning event. Thoughtful combination of restriction enzymes can also help to control the directionality of the insert, which is critical to many applications. **Molecular Cloning Strategy Overview**





2. EQUIPMENT

Microcentrifuge Shaking incubator (37 °C) Incubator (37 °C) Heating block or water bath (42 °C) Heating block (65 °C) (optional) Bunsen burner agarose gel electrophoresis equipment 1.5-ml microcentrifuge tubes 15-ml polypropylene tubes Micropipettors Micropipettor tips (autoclaved)

3. MATERIALS

Vector DNA (receiving vector, insert vector or insert PCR product) Insert DNA Restriction enzymes $10 \times$ Restriction enzyme buffers $10 \times BSA$ Agarose Tris base EDTA Glacial acetic acid Ficoll Bromophenol blue Xylene cyanol Ethidium bromide Sodium chloride (NaCl) Bacto tryptone Yeast extract Bacto/Difco agar $10 \times$ DNA T4 ligase buffer T4 DNA ligase Chemically competent E. coli Antibiotic (e.g., ampicillin, kanamycin)

DNA Gel purification kit PCR purification kit *optional:* 10× Restriction enzyme buffer #2 (NEB2) 2.5 mM dNTPs Klenow fragment 0.5 M EDTA

3.1. Solutions & Buffers

Step 1 0.5 M EDTA, pH 8.0

Dissolve 14.6 g EDTA (MW 292.24 g mol⁻¹) in 95 ml water. Adjust pH to 8.0 using NaOH and add water to 100 ml.

50 imes TAE

Component	Final concentration	Stock	Amount
Tris base	2 M	N/A	242 g
EDTA, pH 8.0	1×	0.5 M	100 ml
Glacial acetic acid	N/A	N/A	57.1 ml

Dissolve Tris in \sim 750 ml of deionized water. Add EDTA and acetic acid. Adjust the final volume to 1000 ml with water. There is no need to adjust the pH of this solution.

$6 \times$ DNA loading dye

Component	Final concentration	Stock	Amount
Ficoll	15% (w/v)	N/A	1.5 ml
Bromophenol blue	0.25% (w/v)	N/A	25 mg
Xylene cyanol FF	0.25% (w/v)	N/A	25 mg

Add water to 10 ml

Step 3 LB medium			
Component	Amount		
NaCl	10 g		
Bacto tryptone	10 g		
Yeast extract	5 g		

Add water to 1 l and autoclave. For LB plates, add 15 g Bacto/Difco agar, autoclave and cool to ${\sim}55~^\circ\text{C}$ before adding the antibiotic and pouring the plates

4. PROTOCOL 4.1. Preparation

Ensure that good-quality DNA preps are available for the receiving vector and any vectors used to retrieve the insert (see Isolation of plasmid DNA from bacteria).

Identify the restriction enzymes needed and their buffer requirements for single and double digests.

For PCR product inserts:

Add the sequence of any desired restriction site(s) to the 5' end of each primer (see Explanatory chapter: PCR –Primer design). It is a good idea to precede these sites with a few random nucleotides, such as GATC, since some restriction enzymes can cut only at sites surrounded by double-stranded DNA. Note that these regions should be excluded from annealing temperature calculations since they do not contain any homology to the target sequence (Fig. 7.2).

Once you obtain the primers, run the PCR reaction and gel-purify the product, eluting in $30 \ \mu l$ of ddH₂O.

Design one or more strategies to test for vectors that carry the correct insert (in the appropriate orientation).

Examples:

- 1. Identify restriction enzymes that cut inside (and outside) of the new insert, producing distinct bands.
- Design PCR primers, one outside and one inside the insert, to use for colony PCR.
- **3.** Identify or design primers that can be used to sequence all or part of the insert.

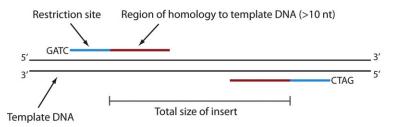


Figure 7.2 Primer design for molecular cloning. The schematic shows the elements of PCR primers to amplify a fragment of DNA to be cloned.

4.2. Duration

Preparation	variable
Protocol	4 days

5. STEP 1 RESTRICTION DIGESTS OF VECTOR AND INSERT

5.1. Overview

Linearize the receiving vector by restriction digest and cut out the insert from another vector or digest a gel-purified PCR product to create sticky ends.

If using DNA oligos, anneal the two single-stranded pieces to form a double-stranded insert with sticky ends according to the manufacturer's directions and go directly to Step 2.

5.2. Duration

2.5–3 h

1.1 Set up the restriction digests for both the vector and the insert in 1.5-ml microcentrifuge tubes:

DNA	3 μ g for vector DNA, 30 μ l for PCR product
$10 \times \mu$ Buffer	5 µl
$10 \times \mu$ BSA	5 µl
Enzyme 1	1 µl
Enzyme 2	1 μl (if required)
ddH2O	to 50 µl

1.2 Incubate at 37 $^{\circ}$ C for 1 h.

- **1.3** Pour a 0.8% agarose gel in 1× TAE buffer containing ethidium bromide (see Agarose Gel Electrophoresis).
- 1.4 Add 10 μ l of 6 × DNA gel loading dye and load onto the agarose gel. Run the gel at 100 V for 30–45 min.
- **1.5** Gel-purify the receiving vector and the insert, using a DNA Gel Purification kit. This purification step is essential as it removes any enzymatic activity that might interfere with the ligation reaction. When

running the gel, include an uncut vector control to confirm that the digests are complete. Any residual uncut plasmid will produce a high background of negative colonies.

1.6 Optional: To create blunt ends, fill in any overhangs left by restriction enzymes. Set up the following reaction in a 1.5-ml microcentrifuge tube:

DNA	2.5 µg
$10 \times \mu$ NEB Buffer 2	2.5 µl
dNTPs (2.5 mM)	1 µl
Klenow fragment (5000 U µl µ1)	0.5 µl
ddH ₂ O	to 25 µl

- **1.7** Incubate at room temperature for 30 min.
- **1.8** Stop the reaction with 1 μ l of 0.5 M EDTA.
- 1.9 Purify using a PCR purification kit or by gel-extraction.

5.3. Tip

To help prevent unwanted self-ligation of the vector, add 1 μ l of a phosphatase, for example, Calf Intestinal Phosphatase (CIP) or antarctic phosphatase, to the vector digest ONLY. This is especially important when using just one enzyme to create sticky ends or enzymes that create blunt ends.

5.4. Tip

Some restriction enzyme combinations require a sequential digest. If this is the case, add the first enzyme for 1 h, then heat-inactivate it by incubating the reaction at $65 \degree C$ for 20 min. Add the second enzyme (adjusting the buffer conditions if necessary) and incubate for another hour at $37 \degree C$.

5.5. Tip

If the insert is a purified PCR product, gel purification is not necessary at this point. Heat-inactivation of the enzyme(s) through incubation at 65 °C for 20 min is sufficient. However, using a PCR purification kit will improve the quality of the insert DNA by removing all enzymatic activity and is highly recommended.

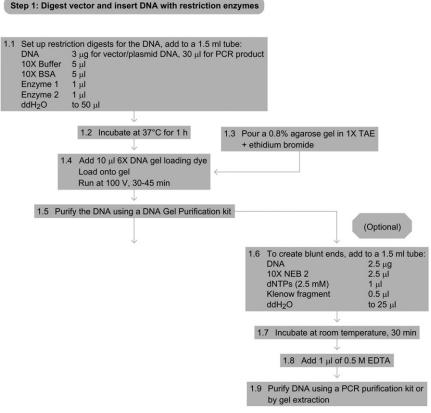


Figure 7.3 Flowchart of Step 1.

5.6. Tip

After digesting and gel purifying the DNA, run 2–5 μ l on an agarose gel to verify recovery and determine the amounts to use in the ligation.

See Fig. 7.3 for the flowchart of Step 1.

6. STEP 2 LIGATION 6.1. Overview

Ligate the insert into the linearized receiving vector.

6.2. Duration

Overnight

2.1 Set up the ligation reactions, including a negative control reaction using ddH₂O instead of the insert DNA. Add to a 1.5-ml microcentrifuge tube:

Vector DNA	100 ng	
Insert DNA	≥400 ng	
$10 \times$ T4 DNA ligase buffer	2 µl	
T4 DNA ligase	1 µl	
ddH ₂ O	to 20 µl	

2.2 Incubate overnight at room temperature.

2.3 Place the ligation at -20 °C until ready to transform competent *E. coli*.

6.3. Tip

After thawing, T4 ligase buffer often still contains small white precipitates. Prior to adding the buffer to the ligation reaction, vortex it thoroughly until the solution is completely clear. Incomplete resuspension may alter the concentration of buffer ingredients and interfere with the ligation reaction.

6.4. Tip

For sticky end ligations, a T4 ligase stock of $1 \ U \ \mu l^{-1}$ usually works well. For more complicated ligations, including blunt-end ligations, use a more concentrated T4 ligase at 400 $U \ \mu l^{-1}$.

6.5. Tip

The overnight ligation described here usually produces reliable and efficient results, especially for difficult (including blunt-end) ligations. However, manufacturerrecommended incubation times and temperatures for their ligases vary widely and most eliminate the overnight step. In general, increasing the incubation time will increase the efficiency of the reaction.

See Fig. 7.4 for the flowchart of Step 2.

7. STEP 3 TRANSFORMATION INTO CHEMICALLY COMPETENT E. COLI

7.1. Overview

The ligated plasmid is transferred into chemically competent *E. coli* via heatshock (see Transformation of Chemically Competent *E. coli*, or alternatively

	Step 2: Ligate vector and insert DNAs
	$\begin{array}{llllllllllllllllllllllllllllllllllll$
	2.2 Incubate at room temperature, 2 h-overnight \downarrow
3	Store ligation reaction at -20°C until ready to transform E. coli

Figure 7.4 Flowchart of Step 2.

2.3

see Transformation of *E. coli* via electroporation if you are using electrocompetent cells) to select against linear (nonligated) pieces of DNA. The transformed bacteria are then grown on media containing an antibiotic corresponding to the resistance gene in the receiving vector.

7.2. Duration

2 h+overnight incubation

- **3.1** Let competent cells that on ice (\sim 30 min).
- **3.2** Add 20 μ l of the ligation reaction and or negative control to about 200 μ l of cells (the ratio of the ligation reaction to *E. coli* should be at least 1:10).
- **3.3** Mix gently to avoid damage to the cells (do not pipette up and down).
- **3.4** Incubate on ice for 30 min.
- **3.5** Heat shock at $42 \degree C$ for 1 min.
- **3.6** Immediately add 1 ml of room temperature LB (or other medium, e.g., SOC).
- **3.7** Incubate at 37 °C for 1 h with shaking (100–150 rpm).
- **3.8** Centrifuge the bacteria at a low speed (\sim 7500 rpm) for 2 min.
- **3.9** Aspirate all but $100 \ \mu l$ of the LB.
- **3.10** Carefully resuspend the pellet and spread all 100 μ l evenly onto a selective plate.
- 3.11 Incubate overnight at 37 °C with the plates turned upside down.

7.3. Tip

Use autoclaved pipette tips for each step. Alternatively, sterilize tips before touching any bacteria by passing them briefly through a Bunsen burner flame.

7.4. Tip

The amounts of the competent cells and ligation mix can be reduced to 50 and 5 μ l, respectively.

7.5. Tip

If time is limited, Steps 3.4 and 3.7 may be shortened to 15 and 30 min respectively without compromising the transformation efficiency too much.

7.6. Tip

During the incubation, let selective plates warm up at 37 °C. Cold plates lead to lower transformation efficiency.

7.7. Tip

Alternately spread the transformed E. coli onto two plates, with 80% on one plate and 20% on the other, to ensure the growth of well isolated colonies.

See Fig. 7.5 for the flowchart of Step 3.

8. STEP 4 IDENTIFY SUCCESSFUL LIGATION EVENTS

8.1. Overview

Colonies appearing on the selective plates after transformation represent individual, unique ligation outcomes. The plasmid DNA is extracted from several colonies and checked for successful ligation events using one or more of the previously determined screening strategies (see Protocol Preparation).

8.2. Duration

Day 1: 5 min or 3–5 h (if colonies are screened by PCR; see Colony PCR).

Day 2: about 1 h for the mini-prep (see Isolation of plasmid DNA from bacteria), otherwise the duration depends on the screening strategy used to identify positive clones.

- **4.1** Use a sterile pipette tip to pick individual, round, medium-sized colonies from the selective plate containing transformed cells from the ligation reaction with the insert.
- **4.2** Transfer each colony into a 14-ml polypropylene round-bottom tube containing 3–5 ml LB plus the appropriate antibiotic.
- **4.3** Incubate overnight at 37 $^{\circ}$ C, shaking at ~250 rpm. For optimal growth, tilt the tubes slightly in the shaking rack.
- 4.4 The next day, isolate the plasmid DNA, using a miniprep kit.

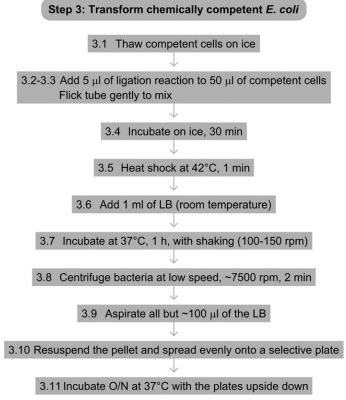


Figure 7.5 Flowchart of Step 3.

4.5 Confirm successful ligation events using one or more of the previously worked out screening strategies.

8.3. Tip

After transformation, the selective plate for the ligation should contain a lot more colonies than the negative control plate. If not, this may be an indication that the restriction digest of the receiving vector was incomplete or that the receiving vector was able to self-ligate too easily. Try increasing the time of the receiving vector digestion and add a phosphatase to limit self-ligation.

8.4. Tip

If primers are available, perform a colony PCR at this point (see Colony PCR). Draw a grid on the back of a selective plate, numbering each field. Pick one colony at a time

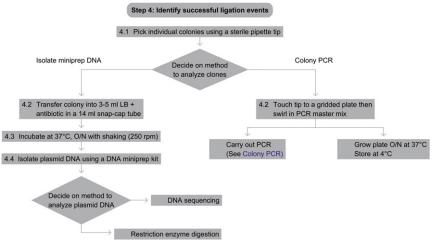


Figure 7.6 Flowchart of Step 4.

using a sterile pipette tip. Lightly touch the selective plate in one of the numbered fields, and then transfer the rest of the colony into a PCR tube with the same number, containing a PCR master mix. Repeat for several colonies. Incubate the plate upside down at 37 °C for a few hours while performing the PCR. Positive clones identified by the colony PCR can be picked directly from the corresponding numbered field on the selective plate.

See Fig. 7.6 for the flowchart of Step 4.

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Referenced Protocols in Methods Navigator

Isolation of plasmid DNA from bacteria. Explanatory chapter: PCR -Primer design. Agarose Gel Electrophoresis. Transformation of Chemically Competent *E. coli*. Transformation of *E. coli* via electroporation. Colony PCR.

CHAPTER EIGHT

Rapid Creation of Stable Mammalian Cell Lines for Regulated Expression of Proteins Using the Gateway[®] Recombination Cloning Technology and Flp-In T-REx[®] Lines

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Abstract

The biochemical analysis of cellular processes in mammalian cells is often facilitated by the creation of cell lines coexpressing or overexpressing an affinity-tagged wild-type or mutant protein of interest in an inducible or noninducible stable manner (Malik and Roeder, 2003). The affinity tag allows for standardization of purification protocols to characterize interacting proteins or nucleic acids and minimizes the need for generating protein-specific antibodies at the early stages of analysis (for more information on affinity tags, see Purification of His-tagged proteins, Affinity purification of a recombinant protein expressed as a fusion with the maltose-binding protein (MBP) tag, Purification of GST-tagged proteins, Protein Affinity Purification using Intein/Chitin Binding Protein Tags, Immunoaffinity purification of proteins or Strep-tagged protein purification). The establishment of stable cell lines with inducible expression is critical to studying proteins that reduce cell growth and/or viability upon overexpression.

Over the past several years, our laboratory has developed an expression platform for analyzing RNA-interacting proteins, including the establishment of stable mammalian cell lines expressing proteins of interest using a recombination-based cloning technology (Landthaler et al., 2008; Hafner et al., 2010). Our aim is to determine the mRNA targets of the hundreds of RNA-binding proteins encoded in the human genome by the isolation and molecular characterization of their ribonucleoprotein complexes (RNPs).

1. THEORY

✓ Invitrogen commercially introduced recombination-based Gateway[®] Cloning Technology (Walhout et al., 2000). It represents a fast and easy way to transfer a gene of interest (GOI) from one vector into another within a multitude of vectors already available. The reactions are performed in less than 1 h at room temperature without the need for sequencing the new vector.

Here we describe how the process of generating stable cell lines can be shortened to just under a month by adapting conventional cloning-based Flp-In recombination vectors, a separate Invitrogen technology, to the Gateway technology.

Following the recommendations of Malik and Roeder (2003), we use a 17-amino acid N-terminal FLAGHA-double affinity tag. Excellent antibody products are commercially available for these affinity tags. Cell lines expressing FLAGHA-affinity-tagged proteins also represent an excellent source for characterizing antibodies raised against the protein of interest.

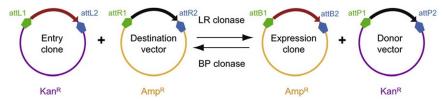


Figure 8.1 The Invitrogen Gateway[®] Recombination Cloning Technology.

In brief, a Gateway recombination reaction mimics either the excision (LR reaction) or the integration (BP reaction) of phage lambda into or from the *E. coli* genome (Fig. 8.1).

- LR reaction: The phage DNA is excised from the bacterial genome by recombination between the attL and attR sites. This excision reaction requires two vectors, the entry clone (with attL sites) and the destination vector (with attR sites). The LR reaction requires three enzymes, phage protein integrase (Int), bacterial protein integration host factor (IHF), and phage excisionase (Xis). These three enzymes are collectively referred to as 'LR Clonase.' The attL sites give rise to attB sites and the attR sites to attP sites.
- BP reaction: The phage DNA is integrated into the bacterial genome. It requires two vectors, the expression clone with attB sites and the donor vector with attP sites. The BP reaction requires Int and IHF only. These two enzymes are collectively referred to as 'BP Clonase.' Since the attB sites give rise to attL sites and the attP sites to attR sites the cycle completes itself enabling a new round of cloning.

Since both reactions are reversible, this allows for a very flexible approach in shuttling the insert DNA from one expression vector to another.

We adapted two regular Invitrogen vectors pcDNA5/FRT and pcDNA5/ FRT/TO for their use as Gateway vectors and added an N-terminal FLAGHA tag, naming the resulting vectors pFRT_DESTFLAGHA and pFRT_TO_ DESTFLAGHA (Fig. 8.2). These plasmids were deposited at www. addgene.com, together with many destination vectors our laboratory generated for stably expressing human RNA-binding proteins or components of the RNAi machinery. Plasmids pFRT_DESTFLAGHA_GOI (cDNA of gene of interest) are prepared for stable nonregulated (co-)expression of proteins, whereas the pFRT_TO_DESTFLAGHA_GOI vectors are prepared for inducible expression of otherwise toxic proteins or protein levels. Protein synthesis is induced by addition of tetracycline derivatives to the culture medium of stable GOI-containing cell lines.

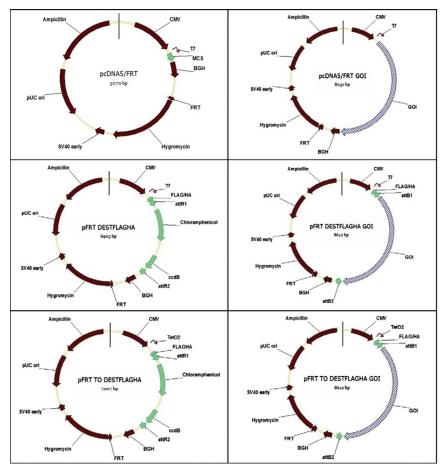


Figure 8.2 Vector maps of pcDNA5/FRT, pcDNA5/FRT_GOI, pFRT_DESTFLAGHA, pFRT_DESTFLAGHA_GOI, pFRT_TO_DESTFLAGHA and pFRT_TO_DESTFLAGHA_GOI vectors. Shown are individual vector properties including replication origins, antibiotic resistance and recombination sites.

For transfer of the GOI into the pFRT vectors, we first establish a Gateway entry vector pENTR_GOI by cloning the cDNA PCR product of the GOI in the correct reading frame into commercial pENTR4, although any of the other available entry vectors can equally be used (Fig. 8.3).

The pENTR4_GOI also allows access to a multitude of different Gateway expression vectors for recombinant protein purification. For bacterial expression (see Small-scale Expression of Proteins in *E. coli*), there is a choice between untagged (pDEST14) and tag-expressing vectors, such as

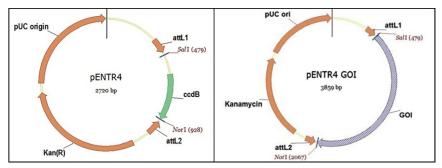


Figure 8.3 pENTR4 and pENTR4_GOI, which was cloned using Sall and Notl restriction sites.

glutathione S-transferase (GST, N-terminal, pDEST15; C-terminal, pDEST24) and 6xHis (N-terminal, pDEST17). Analogous vectors exist for baculovirus expression (see Recombinant protein expression in baculovirus-infected insect cells), untagged (pDEST8), N-terminal 6xHis tag (pDEST10), and N-terminal GST (pDEST20). (For purification of these tagged proteins see Purification of GST-tagged proteins or Purification of His-tagged proteins).

Different mammalian Flp-In cell lines are available from Invitrogen, only one of which allows for tetracycline-regulated expression of the GOI:

- Flp-In T-REx 293 Cell Line (human embryonic kidney cell line that also expresses high levels of the Tet repressor protein)
- Flp-In-293 Cell Line (human embryonic kidney cell line)
- Flp-In-CHO Cell Line (Chinese hamster ovary cell line)
- Flp-In-BHK Cell Line (baby hamster kidney cell line)
- Flp-In-CV-1 Cell Line (African Green Monkey kidney cell line)
- Flp-In-3 T3 Cell Line (mouse embryonic fibroblast cell line)
- Flp-In-Jurkat Cell Line (human T lymphocyte cell line)

The Flp-In T-REx 293 cell line contains a single integrated FRT site and was also selected to stably express the Tet repressor protein (TetR). Vectors equipped with two tetracycline operator 2 (TetO₂) sites (Hillen and Berens, 1994) in their CMV promoter region (Andersson et al., 1989) are subjected to TetR-mediated repression until TetR is released from the TetO₂ sites by the addition of tetracycline (or doxycycline) to the tissue culture medium. Examples of TetO₂ site-containing expression vectors (generally characterized by the suffix 'TO') include pcDNA4/TO (Invitrogen) or our pFRT_TO_DESTFLAGHA. To also obtain TetR-expressing stable lines for the Flp-In lines listed above, Invitrogen provides the plasmid

pcDNA6/TR, which can be transfected into cells followed by selection of stable clones.

The following additional TetR-expressing cell lines are available for generation of 'Flp-In T-REx' cell lines by transfection of pFRT/lacZeo vector (Invitrogen) and subsequent colony selection and Southern blot screening for FRT site integration (see Invitrogen's user manuals 'pFRT/lacZeo' and 'T-REx System'):

- T-REx-HeLa Cell Line (low levels of TetR expression resulting in limited range of induction)
- T-REx-Jurkat Cell Line
- T-REx-CHO Cell Line

Finally, the integration of the GOI within the Flp-In host cell line is accomplished by cotransfection of the destination vector containing GOI flanked by the FRT sites, pFRT_DESTFLAGHA_GOI or pFRT_TO_ DESTFLAGHA_GOI, together with the plasmid pOG44, encoding Flp recombinase, and followed by antibiotic selection of cell clones.

2. EQUIPMENT Major equipment 365-nm UV-transilluminator Agarose gel chambers Balances (e.g., 0.1 mg to 64 g and 0.1 g to 4.2 kg) Microcentrifuge CO_2 incubator (for mammalian cell culture) Hemacytometer High speed floor centrifuge (capable of at least 13 $000 \times g$) Incubator (for work with bacteria) Shaking incubator (37 °C) Microscope with fluorescence filter Multichannel pipet pH-meter Power supply PCR thermocycler Thermomixer

	Vortex mixer
	Water bath (42 °C)
	Water filter (e.g., MilliQBiocel water purification system)
Consumables	96-well plate, flat bottom
	96-well plate, conical bottom
	24-well plate
	12-well plate
	6-well plate
	10-cm tissue culture plate
	15-cm tissue culture plate
	0.2-ml PCR strips (Thermo Scientific, AB-0266)
	1.5-ml polypropylene tubes
	Cryogenic vials (e.g., Corning 2-ml internal threaded polypropylene cryogenic vial, 430488)
	50-ml conical tubes (e.g., Falcon)
	Scalpels (or razor blades)
	5-µm Supor membrane syringe filter (Pall Acrodisc)
	Syringes
	Syringes

3. MATERIALS

Reagents & chemicals

Agarose, electrophoresis grade

Boric acid (H₃BO₃)

Bromophenol blue

Calf intestinal alkaline phosphatase (CIP)

Dimethyl sulfoxide (DMSO; Sigma, D2438)

DNA ladder (1 kb)

EDTA disodium salt dihydrate (Sigma, E5134)

Ethidium bromide

	Ficoll type 400
	Agar
	Sodium chloride (NaCl)
	Sodium hydroxide (NaOH)
	Phosphate buffered saline (PBS; 10×, commercially available)
	Tris base (Fisher Scientific, BP152-1)
	Tris–HCl (Promega, PR-H5121)
	Tryptone
	Yeast extract
Antibiotics	Ampicillin sodium salt (Sigma, A9518-25G)
	Blasticidin S, cell culture grade (InvivoGen, ant-bl-1)
	Doxycycline, monohydrochloride hemiethanolate hemihydrate (Sigma, D9891-1G)
	Hygromycin B, cell culture grade (InvivoGen, ant-hg-1)
	Kanamycin sulfate (Sigma, K4000-25G)
	Penicillin–Streptomycin, cell culture grade (Invitrogen, 15140–122; $100 \times$, contains 10 000 units of penicillin (base) and 10 000 µg of streptomycin (base)/ml)
	Zeocin, cell culture grade (InvivoGen, ant-zn-1)
Cell culture media	D-MEM, Dulbecco's Modified Eagle Medium high glucose (1×), with L-glutamine, without sodium pyruvate (Invitrogen, 11965-092)
	Fetal bovine serum heat-inactivated (FBS, heat-inactivated)
	Opti-MEM, reduced-serum medium (1×), liquid (with GlutaMAX-I) (Invitrogen, 51985-034)
Commercial kits	QIAfilter Plasmid Midi Kit (Qiagen, 12243)
	QIAprep Spin Miniprep Kit (Qiagen, 27106)
	QIAquick PCR Purification Kit (Qiagen, 28104)
	QIAquick Gel Extraction Kit (Qiagen, 28704)

Competent cells	One Shot [®] ccdB Survival [™] 2 T1R Competent Cells (Invitrogen, A10460)		
	One Shot [®] TOP10 Chemically Competent <i>E. coli</i> (Invitrogen, C4040-10)		
Vectors	pENTR4 Dual Selection Vector (Invitrogen, A10465)		
	pOG44 (part of the Flp-In™ Core System by Invitrogen)		
	pFRT_DESTFLAGHA (www.addgene.com)		
	pFRT_TO_DESTFLAGHA (www.addgene.com)		
	pFRT_DESTFLAGHA_eGFP (www.addgene.com)		
Other reagents	AccuPrime Pfx DNA Polymerase (Invitrogen, 12344-024)		
	Flp-In TM Core System (Invitrogen, K6010-02)		
	Flp-In TM T-REx TM 293 Cell Line (Invitrogen, R780-07)		
	Gateway [®] LR Clonase [®] II enzyme mix (Invitrogen, 11791-100)		
	Lipofectamine 2000 (Invitrogen, 11668-019)		
	Protein-specific or anti-HA (Covance, MMS-101P) antibody		
	Restriction endonucleases (e.g., Sall and NotI from NEB)		
	T4 DNA Ligase (NEB, M0202M)		
	Trypsin-EDTA, 0.05% (Gibco, 25300)		

DNA oligonucleotides for sequencing	
pENTR4 forward primer	5' CTGTTAGTTAGTTACTTAAGCTC
pENTR4 reverse primer	5' AGACACGGGCCAGAGCTG
pFRT_DESTFLAGHA forward primer	5' CGCAAATGGGCGGTAGGCGTG
pFRT_DESTFLAGHA reverse primer	5' TAGAAGGCACAGTCGAGG
pFRT_TO_DESTFLAGHA forward primer	Same as pFRT_DESTFLAGHA forward primer
pFRT_TO_DESTFLAGHA reverse primer	Same as pFRT_DESTFLAGHA reverse primer

3.1. Solutions & buffers

Step 1.2.2.1 We use water purified by a Millipore water purification system DNA loading dye (5×)

Component	Final concentration	Stock	Amount
EDTA–NaOH, pH 8.0	50 mM	0.5 M	1 ml
Bromophenol blue	0.2% (w/v)	n/a	20 mg
Ficoll type 400	20% (w/v)	n/a	2 g
H_2O to 10 ml			

10 imes TBE

Final concentration	Stock	Amount
445 mM	n/a	53.9 g
445 mM	n/a	27.5 g
10 mM	0.5 M	20 ml
	445 mM 445 mM	445 mM n/a 445 mM n/a

 H_2O to 11

Step 1.5 LB medium		
Component	Amount	
Yeast extract	5 g	
Tryptone	10 g	
NaCl	10 g	
H ₂ O to 11		

Autoclave for 15 min at 121 °C. Prepare stock solutions of the selection antibiotics (100 mg ml⁻¹) and filter them through a 5-µm syringe filter. Add them fresh before each inoculation to a final concentration of 50 µg ml⁻¹ (kanamycin, 1:2000 dilution of stock solution) or 100 µg ml⁻¹ (ampicillin, 1:1000 dilution of stock solution).

LB agar plates

Component	Final concentration	Stock	Amount
Yeast extract	n/a	n/a	5 g
Tryptone	n/a	n/a	10 g
NaCl	n/a	n/a	10 g
Agar	n/a	n/a	15 g

NaOH	1 mM	1 M	1 ml
H_2O to 11			

Autoclave for 30 min at 121 $^{\circ}$ C. After cooling it down to 50 $^{\circ}$ C, add the desired selection antibiotic (for concentrations see above) and pour the plates (about 35 ml per plate).

Step 1.7	TE buffer,	pH 8.0	at room	temperature
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Component	Final concentration	Stock	Amount
Tris–HCl, pH 8.0	10 mM	1 M	5 ml
EDTA–NaOH, pH 8.0	2 mM	0.5 M	2 ml
H ₂ O			493 ml

Step 2 Maintenance medium for parental Flp-In T-REx HEK 293 cell line

DMEM supplemented with 10% FBS (final concentration), 100 U ml⁻¹ of penicillin and 100 μ g ml⁻¹ streptomycin, 15 μ g ml⁻¹ blasticidin, and 100 μ g ml⁻¹ zeocin.

Antibiotic free medium for transfection

DMEM supplemented with 10% FBS (final concentration).

Stable cell line selection medium

D-MEM supplemented with 10% FBS (final concentration), 100 U ml⁻¹ of penicillin and 100 μ g ml⁻¹ streptomycin, 100 μ g ml⁻¹ hygromycin, and 15 μ g ml⁻¹ blasticidin. Blasticidin is only added to maintain TetR expression for generating inducible constructs.

Blasticidin	Selects for the TetR gene-expressing cell, only in inducible constructs (stock concentration 10 mg ml ^{-1} ; diluted to 15 µg ml ^{-1} final concentration in medium)
Hygromycin	Selects for the pFRT vector-containing cell (stock concentration 100 mg ml^{-1} ; diluted to 100 µg ml^{-1} final concentration in medium)
Zeocin	Selects for the FRT recipient site-containing cell (stock concentration 100 mg ml ⁻¹ ; diluted to 100 μ g ml ⁻¹ final concentration in medium)

Freezing medium for Flp-In T-REx HEK 293 cell lines

90% FBS, 10% DMSO. Cool to 4 °C before using.

$1 \times \text{PBS}$

Component	Final concentration	Stock	Amount
PBS	$1 \times$	$10 \times$	100 ml
H ₂ O			900 ml



4. PROTOCOL

4.1. Preparation

I. Isolate the following plasmids using a midiprep or maxiprep kit (alternatively see Isolation of plasmid DNA from bacteria). Depending on the number of reactions, you will need 10–50 μg of each of them:

pENTR4 (or any other of the pENTR vector family): entry vector for the Gateway System

pOG44: expression vector for Flp recombinase

pFRT_DESTFLAGHA: plasmid with FRT site, hygromycin resistance, and the N-terminal double-epitope tag (FLAG and HA); receives the cDNA of the gene from the pENTR4 vector using LR recombination sites; used for stable expression of the protein of interest in either Flp-In cell lines or T-REx Flp-In cell lines

pFRT_TO_DESTFLAGHA: plasmid with FRT site, hygromycin resistance, and the N-terminal double-epitope tag (FLAG and HA); receives the cDNA of the gene of interest from the pENTR4 vector using LR recombination sites; used for tetracycline inducible expression of the protein of interest in T-REx Flp-In cell lines

pFRT_DESTFLAGHA_eGFP: plasmid for the stable expression of eGFP and hygromycin resistance; will be used to assess transfection efficiency

II. Maintain Flp-In T-REx HEK 293 cell line:

Culture Flp-In T-REx HEK 293 cells on plates in DMEM with 10% FBS, 1% penicillin/streptomycin, 15 μ g ml⁻¹ blasticidin, and 100 μ g ml⁻¹ zeocin. Cells are split 1:5 every 2–4 days.

III. Generate vector maps (e.g., pENTR4_GOI) in a suitable software (Vector NTI or MacVector):

This will simplify the choice of restriction enzymes in Step 1.5 and the sequence analysis in Step 1.6. The programs developed and licensed by Invitrogen are also useful for performing a virtual LR reaction and for a basic analysis of protein properties.

4.2. Duration

Step	Action	Duration
0	Preparation	1 day
1.1	Primer design	2 h + time of primer synthesis
1.2	PCR + PCR product purification	3.5 h
1.3	Restriction digestion (PCR product + pENTR4)	4 h
1.4	Ligation + transformation	3 h
1.5	Inoculation, miniprep and restriction digestion	Overnight + 4 h
1.6	Sequencing + sequence analysis	Overnight + 1 h
1.7	LR reaction + transformation	4 h
1.8	Inoculation, miniprep and restriction digestion	Overnight + 4 h
2.1	Preparation of transfection	1 h + overnight
2.2	Transfection	1 h
2.3–2.8	Colony isolation	12–14 days
2.9	Selection of individual colonies	2 h
2.10-2.11	Expansion of individual clones for cryopreservation	11–14 days
2.12-2.14	Protein detection by Western blotting, cryopreservation of stable cell lines for long- term storage	1–2 days

4.3. Tip

Remember to use ccdB survival cells (One Shot[®] ccdB Survival^M 2 T1R Competent Cells by Invitrogen) for pENTR4, pFRT_DESTFLAGHA, and pFRT_TO_DESTFLAGHA, since regular bacterial strains such as TOP10 are not suitable

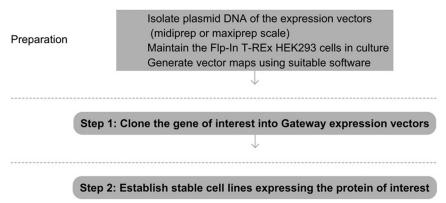


Figure 8.4 Flowchart of the complete protocol, including preparation.

for propagation of plasmids containing the ccdB (coupled cell division B; inhibits cell division) gene.

See Fig. 8.4 for the flowchart of the complete protocol, including preparation.



5.1. Overview

In the first step the cDNA of the GOI is cloned into the entry vector pENTR4 and subsequently transferred via LR cloning into the destination vector for mammalian expression.

5.2. Duration

Step	Action	Duration
1.1	Primer design	2 h + time of primer synthesis
1.2	PCR + PCR product purification	3.5 h
1.3	Restriction digestion (PCR product $+$ pENTR4)	4 h
1.4	Ligation + transformation	3 h
1.5	Inoculation, miniprep and restriction digestion	Overnight + 4 h
1.6	Sequencing + sequence analysis	$Overnight + 1 \ h$
1.7	LR reaction + transformation	4 h
1.8	Inoculation, miniprep and restriction digestion	Overnight + 4 h

1.1 Design primers specific to chosen cDNA of your gene of interest

1.1.1 PCR primers

If you already have the cDNA of your GOI cloned in frame in one of the Invitrogen entry vectors, continue with Step 1.7. If not, look up the multiple cloning site of pENTR4 regarding your choice of restriction endonucleases (http://tools.invi trogen.com/content/sfs/vectors/pentr4_dual_selection_vector_ mcs.pdf for cloning your GOI cDNA into this vector. Make sure that your GOI does not contain any internal restriction sites recognized by the chosen restriction endonucleases; use the program supported at http://www.restrictionmapper.org/.

We routinely use SalI/NotI for this purpose. For SalI/NotI cloning, adapt the following PCR primers:

Forward PCR primer:

5' ACGC**GTCGAC**xxxx

The SalI recognition site is in bold. xxxx represents the first 10–15 bases of your GOI starting from the ATG. The ATG start codon must follow the SalI site (at least in pENTR4) to maintain the reading frame after LR recombination into destination vectors.

Reverse PCR primer

5' ATAGTTTA**GCGGCCGC**xxxx

The NotI recognition site is in bold. xxxx represents the reverse complement of the last 10–15 bases of your gene of interest, ending with the stop codon.

Refer to the primer design chapter in this book series for general guidelines (see Explanatory chapter: PCR -Primer design). If you choose to use either a different entry vector or a different set of restriction endonucleases make sure to clone your GOI in the correct reading frame.

1.1.2 Sequencing primers

pENTR4 forward primer: 5' CTGTTAGTTAGTTACTTAA GCTC

pENTR4 reverse primer: 5' AGACACGGGCCAGAGCTG pFRT_DESTFLAGHA forward primer: 5' CGCAAATGGG CGGTAGGCGTG

pFRT_DESTFLAGHA reverse primer: 5' TAGAAGGCACA GTCGAGG

 $pFRT_TO_DESTFLAGHA$ forward primer is the same as $pFRT_DESTFLAGHA$ forward primer

pFRT_TO_DESTFLAGHA reverse primer is the same as pFRT_DESTFLAGHA reverse primer

Also design gene-specific internal sequencing primers such that they are about 700 bases apart from each other (assuming a sequencing read length of 900 bases). This is not needed if your GOI is smaller than 1200 bases (again assuming a sequencing read length of 900 bases).

- 1.2 PCR and PCR product purification
 - **1.2.1** PCR to amplify GOI for subcloning (see Reverse-transcription PCR (RT-PCR))

The number of PCR cycles and the amount of input cDNA depend on whether you amplify from an existing plasmid containing your GOI or from cDNA transcribed from total RNA.

To amplify a GOI from cDNA (obtained from total RNA extracted with TRIzol from Flp-In T-REx HEK 293 cells and reverse transcribed using SuperScript[®] III, both by Invitrogen), we use the following PCR conditions (using AccuPrime Pfx Polymerase, Invitrogen):

Process	Step	Temperature	Duration
Heat activation	1	95 °C	2 min
Denaturation	2	95 °C	45 s
Annealing	3	55–64 °C	30 s
Extension	4	68 °C	1 min per kb
Final extension	5	68 °C	5 min

Repeat Steps 2–4 20–35 times, depending on the expression level of your GOI and the amount of input cDNA (we usually use around 100 ng cDNA and 30 cycles of PCR). Include a negative control (blank).

- **1.2.2** PCR purification
 - **1.2.2.1** Cast a 1% agarose gel in $1 \times$ TBE containing 0.4 µg ml⁻¹ of ethidium bromide (see Agarose Gel Electrophoresis).
 - **1.2.2.2** Take an aliquot of 4 μ l of the PCR product and add 1 μ l 5× DNA loading dye.
 - **1.2.2.3** Load this mix next to a 1 kb DNA ladder on the agarose gel and run the gel in 1 × TBE at 150 V for about 45 min.

- **1.2.2.4** Visualize PCR products using a UV 365 nm transilluminator.
- 1.2.2.5 If a single band is visible at the predicted size, purify the remainder of the PCR reaction using the QIAquick PCR Purification kit. If there are multiple bands, gelpurify the remainder of the PCR reaction by cutting only the band at the predicted size. Follow the manufacturer's instructions using the QIAquick Gel Extraction kit.
- 1.3 Restriction digestion of PCR product and pENTR4 vector
 - **1.3.1** Digest the PCR product and the pENTR4 vector (about 1 μ g) using the restriction endonucleases present in your PCR primer sequence in the appropriate enzyme buffer. Include controls for single cuts and a negative control (uncut vector). NotI and Sall can be used simultaneously in buffer 3 (NEB). We usually use about 50% of the initial PCR product in this step in a 30 μ l reaction volume with 1 μ l of each enzyme.
 - **1.3.2** Incubate for 2 h at 37 °C (check specific enzyme temperature requirements).
 - **1.3.3** If the restriction endonucleases you are using create blunt ends on both sides of the cut vector, dephosphorylate the vector to avoid religation of the vector (e.g., using calf intestinal alkaline phosphatase, NEB).
 - **1.3.4** In the meantime cast a 1% agarose gel in $1 \times$ TBE containing 0.4 µg ml⁻¹ of ethidium bromide.
 - 1.3.5 Add the appropriate volume of 5× DNA loading dye to the reaction and gel-purify the reaction by running the gel in 1× TBE at 150 V for 1 h until a good separation of the digested fragments is achieved.
 - **1.3.6** Cut fragments of the predicted size from the gel with a scalpel and proceed with the gel extraction according to the manufacturer's instructions (QIAquick Gel Extraction kit).
- 1.4 Ligation and transformation (pENTR4_GOI)
 - **1.4.1** Choose the overall concentration of vector and insert such that it is between 1 and 10 ng μ l⁻¹ for efficient ligation. Molar ratios of insert to vector between 2 and 6 are optimal for single insertions. Use 1 μ l T4 DNA ligase in a reaction volume of 20 μ l. Incubate for 1 h at room temperature. Check for vector religation efficiency by including a reaction where no insert is added to the ligation reaction.

1.4.2 We routinely use One Shot[®] TOP10 chemically competent *E. coli* (Invitrogen) for transformations (alternatively see Transformation of Chemically Competent *E. coli* or Transformation of *E. coli* via electroporation).

To 50 μ l of competent cell suspension, add 1 μ l of the ligation reaction and incubate on ice for 30 min. Heat-shock the cells for 30 s at 42 °C without shaking. Place the vial on ice for 2 min. Add 250 μ l of prewarmed S.O.C. medium (supplied by Invitrogen) to each vial and incubate horizontally at 37 °C for 1 h, shaking at 225 rpm. Spread 20 and 200 μ l from each transformation on a prewarmed selective plate containing 50 μ g ml⁻¹ kanamycin and incubate overnight at 37 °C.

5.3. Tip

You can split the 50 μ l cell suspension into 2 \times 25 μ l to save competent cells (but remember to adjust the volumes accordingly).

- 1.5 Miniprep and restriction digestion (pENTR4_GOI)
 - 1.5.1 Inoculate a single colony in 5 ml LB medium containing 50 μg ml⁻¹ kanamycin. We typically process —two to three colonies per selective plate. Incubate overnight at 37 °C, shaking at 225 rpm. Isolate plasmid following the manufacturer's instructions for the plasmid DNA extraction (include the optional 0.5 ml buffer PB wash step if using Qiagen miniprep kit. Alternatively, see Isolation of plasmid DNA from bacteria). Elute in 50 μl Buffer EB.
 - **1.5.2** Determine the DNA concentration by measuring UV absorbance at 260 nm (see Explanatory Chapter: Nucleic Acid Concentration Determination).
 - **1.5.3** Digest 200 ng of each plasmid sample selecting one or two restriction endonucleases that only cut once in the vector and once in the GOI.
 - **1.5.4** Analyze the restriction pattern on a 1% agarose gel.
- 1.6 Sequencing reaction and sequence analysis (pENTR4_GOI)
 - **1.6.1** Prepare the sequencing sample according to the guidelines of your sequencing facility. Sequence one or two of the colonies that showed the expected restriction pattern.
 - **1.6.2** Verify the sequences.
- **1.7** LR reaction and second transformation (pFRT_DESTFLAGHA_ GOI, pFRT_TO_DESTFLAGHA_GOI)

1.7.1 LR reaction

1.7.1.1 Set up the following reaction mix (we routinely scale the LR reaction down by 50% compared to manufacturer's recommendations) in a 1.5-ml polypropylene tube at room temperature (RT):

pENTR4_GOI (75 ng)	\times µl
pFRT_DESTFLAGHA (75 ng) or pFRT_TO_DESTFLAGHA (75 ng)	\times µl
TE buffer, pH 8.0 (at RT)	to 4 µl

- **1.7.1.2** Thaw the LR Clonase II enzyme mix on ice for 2 min. Vortex the enzyme briefly twice.
- **1.7.1.3** Add 1 μl enzyme mix to each sample and mix by vortexing twice. Microcentrifuge briefly.
- 1.7.1.4 Incubate reactions at 25 °C for 2 h.
- 1.7.1.5 Add 0.5 μl proteinase K solution (supplied by Invitrogen) and vortex briefly. Incubate for 10 min at 37 °C.
- 1.7.2 Transformation
 - 1.7.2.1 To 50 μl of competent cell suspension, add 1.5 μl of the LR reaction (e.g., One Shot[®] TOP10 chemically competent *E. coli*, Invitrogen) and incubate on ice for 30 min.
 - **1.7.2.2** Heat-shock the cells for 30 s at 42 °C without shaking. Place the vial on ice for 2 min.
 - **1.7.2.3** Add 250 μl of prewarmed S.O.C. medium to each vial and incubate horizontally at 37 °C for 1 h, shaking at 225 rpm.
 - **1.7.2.4** Spread 20 and 200 μ l from each transformation on a prewarmed selective plate containing 100 μ g ml⁻¹ ampicillin and incubate overnight at 37 °C.
- **1.8** Minipreps and restriction digestion (pFRT_DESTFLAGHA_GOI, pFRT_TO_DESTFLAGHA_GOI)
 - **1.8.1** Inoculate a single colony in 5 ml LB medium containing $100 \ \mu g \ ml^{-1}$ ampicillin. We typically process two to three colonies per selective plate. Incubate overnight at 37 °C, shaking at 225 rpm. Follow the manufacturer's instructions for the plasmid DNA extraction (include the optional 0.5 ml buffer

PB wash step if using Qiagen miniprep kit). Elute in 50 μ l buffer EB.

- **1.8.2** Determine the DNA concentration by measuring UV absorbance at 260 nm.
- 1.8.3 Digest 200 ng of each plasmid sample with a restriction endonuclease that ideally cuts once in the vector and once in your GOI (or perform a double digest to the same effect).
- **1.8.4** Analyze the restriction pattern on a 1% agarose gel.
- **1.8.5** Optional: Check the plasmid once more by sequencing. We do not routinely perform this step if the restriction digestion of the miniprep shows the expected pattern on the agarose gel.

See Fig. 8.5 for the flowchart of Step 1.

6. STEP 2 ESTABLISHING STABLE CELL LINES EXPRESSING THE PROTEIN OF INTEREST

6.1. Overview

The destination vector is transfected into Flp-In T-Rex HEK 293 cells to generate cells stably expressing the protein or gene of interest (GOI). After the initial transfection, the cells are subjected to antibiotic selection until the appearance of individual clones. Cells from these clones are then isolated and expanded and their protein expression is tested by Western blot analysis (see Western Blotting using Chemiluminescent Substrates). Cells are then cryopreserved.

Step	Action	Duration
2.1	Preparation of transfection	1 h and overnight
2.2	Transfection	1 h
2.3–2.8	Colony isolation	12–14 days
2.9	Selection of individual colonies	2 h
2.10-2.11	Expansion of individual clones for cryopreservation	11–14 days
2.12-2.14	Protein detection by Western blotting, cryopreservation of stable cell lines for long-term storage	1–2 days

6.2. Duration

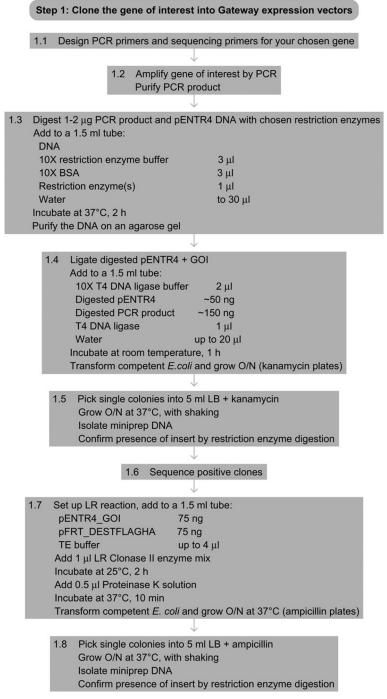


Figure 8.5 Flowchart of Step 1.

- **2.1** Aliquot Flp-In T-REx HEK 293 cells from the growing stock into antibiotic-free medium at a density of 50 000 cells per 1 ml medium per well of a 12-well plate. You will need one well per destination vector as well as one for the negative (pOG44 only) and one for the positive control (pFRT_DESTFLAGHA_eGFP). Make sure to spread the cells evenly by gentle swirling.
- **2.2** 24 h later cotransfect your Flp-In T-REx HEK 293 cells with a 9:1 ratio of pOG44: pFRT_DESTFLAGHA_GOI or pFRT_TO_ DESTFLAGHA_GOI destination vector. As suggested by the Invitrogen manual we use 0.9 μg pOG44 (maximum of 3 μl) and 0.1 μg of the destination vector (maximum of 3 μl).
 - **2.2.1** In a 1.5-ml polypropylene tube combine the destination vectors for each transfection with 50 μl of Opti-MEM. Pipette the mixture up and down twice to ensure proper mixing.
 - **2.2.2** Perform pOG44 only and pFRT_DESTFLAGHA_eGFP + pOG44 transfections to serve as a negative and positive control, respectively.
 - **2.2.3** In a separate 1.5-ml polypropylene tube combine $2 \mu l$ lipofectamine 2000 with 50 μl of Opti-MEM for each transfection and incubate for 5 min at room temperature. Invert the tube twice to ensure proper mixing.
 - **2.2.4** Add the Lipofectamine 2000/Opti-MEM mixture to the destination vector/Opti-MEM mixture. Mix combined solutions and incubate the mixture at room temperature for 20 min. Add the mixture to the cells and move the plate from side to side to distribute evenly across the wells.
- **2.3** 24 h after transfection, aspirate the medium and replace it with fresh antibiotic-free medium.
- 2.4 Check for the eGFP expression to assess the transfection efficiency.
- **2.5** 48 h after transfection split the cells into a 10-cm plate containing antibiotic-free medium. Incubate the cells at 37 °C for 12 h until they have completely attached to the culture dish.
- **2.6** Remove the medium and add selective medium containing the appropriate antibiotics (see above; use hygromycin alone for the eGFP cells).
- 2.7 Change the selective medium every 2–3 days until distinct foci can be identified. After about a week, there will be a noticeable selection of cells.
- 2.8 Continue changing the medium for a total of 10–14 days.
- **2.9** Mark 8–12 well separated colonies while looking at them under the microscope.

2.9.1 Prepare the following:

96-well plate (conical bottom) containing 20 μl of trypsin per well

96-well plate (flat bottom) containing 80 μl selection medium per well

Multichannel pipet

 $1 \times PBS$

- **2.9.2** Aspirate the medium and replace with 3 ml of 1X PBS. Set a P20 micropipettor to 3 μ l and gently scrape one colony off the plate. Once the colony is dislodged take it up into the pipet tip.
- 2.9.3 Place the cell suspension into 20 µl of trypsin in a conical bottom 96-well plate to prepare a single cell suspension. Pipette up and down several times. Incubate at room temperature for 5 min.
- **2.9.4** Add 20 μl of selective medium into each well and pipette up and down again several times to ensure that no cell aggregates remain. Avoid introducing bubbles.
- 2.9.5 Use a multichannel pipettor to transfer these 40 µl into a flat bottom 96-well plate already containing 80 µl/well of stable cell line selective medium.
- **2.9.6** Check the individual wells under the microscope to make sure that there are no cell clumps visible. If there are, pipette up and down several times to achieve a single cell suspension.
- **2.9.7** After picking the colonies, remove the PBS and add fresh media to maintain the original plates for a few days in case none of the newly isolated colonies survives. After you expand the colonies into a 24-well plate, induce the saved original plates with 1 μ g ml⁻¹ doxycycline for 12 h, wash the plates with 3 ml PBS, collect the remaining colonies in a 1.5-ml polypropylene tube, and solubilize in SDS sample buffer. Analyze the samples by Western blotting to ensure that your protein of interest is expressed.
- **2.10** After 2–3 days when the cells reach 80% confluence, split into a 24-well plate. Avoid letting the cells become overconfluent (usually the cells are ready to be split into the next larger plate after 2–3 days). After the cells have grown in the 24-well plate, sequentially expand them into a 12-well plate, a 6-well plate, and then a 10-cm plate. We usually expand up to five clones to the 10-cm plate stage and up to three clones to the 15-cm plate stage.

- 2.11 Once cells reach confluence on a 10-cm plate and are ready to be expanded onto a 15-cm plate, keep a fraction of the cells for Western blot analysis (if the cell line is constitutively expressing your protein of interest) or plate cells from each clone into separate wells of a 12-well plate and induce them with $1 \ \mu g \ ml^{-1}$ doxycycline overnight. Wash the cells with PBS, collect them in a 1.5-ml microcentrifuge tube, and solubilize them in SDS sample buffer. Make sure to assess protein expression in the individual colonies by Western blotting. If no protein-specific antibody is available, we routinely use an anti-HA antibody (see above).
- **2.12** Continue to split and expand cells until you have 3–5 15-cm plates of each cell line.
- **2.13** Once protein expression has been confirmed and the cells have reached the desired level of confluence, they are ready to cryopreserve.
- **2.14** We usually collect one 15-cm plate of 90% confluent cells per vial for cryopreservation.
 - 2.14.1 Remove culture medium from cells.
 - **2.14.2** Wash the cells with $1 \times PBS$.
 - 2.14.3 Add 3 ml trypsin to each plate and incubate for 5 min.
 - **2.14.4** Detach the cells by pipetting up and down.
 - **2.14.5** Add 15 ml of antibiotic-free culture medium to each plate to inactivate trypsin.
 - **2.14.6** Harvest the cells in 50-ml centrifuge tubes by centrifugation at $250 \times g$ for 7 min.
 - 2.14.7 Resuspend the cells in 1.5 ml cold freezing medium per initial 15-cm plate.
 - 2.14.8 Aliquot 1–1.5 ml of the cell suspension into cryogenic vials.
 - 2.14.9 Start the freezing process by placing the cryogenic vials at -20 °C for 2 h. Transfer the vials to -80 °C overnight. The next morning transfer the vials to liquid nitrogen storage. Check for cell viability by thawing one vial per batch some days after freezing.

6.3. Tip

Should you not be able to obtain colonies from inducible cell lines expressing a toxic protein of interest, you might wish to use Tet system-approved fetal bovine serum (e.g., Clontech, 631106). Regular FBS usually contains very low levels of tetracycline, which might be sufficient to induce the expression of the protein of interest.

See Fig. 8.6 for the flowchart of Step 2.

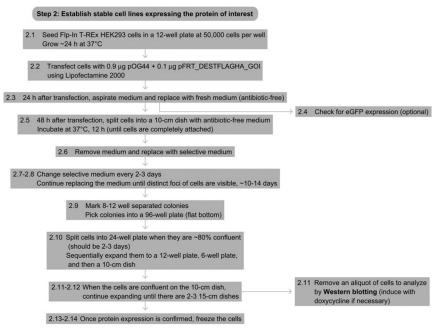


Figure 8.6 Flowchart of Step 2.

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Purification of His-tagged proteins. Affinity purification of a recombinant protein expressed as a fusion with the maltose-binding protein (MBP) tag Purification of GST-tagged proteins Protein Affinity Purification using Intein/Chitin Binding Protein Tags Immunoaffinity purification of proteins Strep-tagged protein purification Small-scale Expression of Proteins in E. coli Recombinant protein expression in baculovirus-infected insect cells Isolation of plasmid DNA from bacteria Explanatory chapter: PCR -Primer design Reverse-transcription PCR (RT-PCR) Agarose Gel Electrophoresis Transformation of Chemically Competent E. coli Transformation of E. coli via electroporation Explanatory Chapter: Nucleic Acid Concentration Determination Western Blotting using Chemiluminescent Substrates

CHAPTER NINE

Restrictionless Cloning

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Abstract

Many methods have been developed for the cloning of PCR products. These methods include blunt-end cloning, TA cloning, and using restriction sites incorporated into the PCR primers. The restrictionless cloning technique allows efficient directional cloning of PCR products into any cloning site within a vector regardless of whether the sites are contained within the insert to be cloned.

1. THEORY

A standard technique for cloning PCR inserts is to incorporate the restriction sites to be used into the amplification primers. Following amplification the PCR product is digested with the specific restriction enzymes to create the compatible ends for ligation into the digested vector. This method can be difficult because of the variable efficiency that restriction enzymes have when cleaving near the ends of a DNA molecule. Inefficient cleavage results in many inserts not containing the cohesive ends for ligation into the vector and therefore poor cloning efficiencies. Also, this standard method cannot be used if the PCR product to be cloned contains a recognition sequence for the restriction enzymes used to create the cohesive ends. The restrictionless cloning technique utilizes two PCR products to create almost identical inserts that when heat-denatured and annealed, result in a DNA insert containing the desired overhang sequences for ligation into a prepared vector. This protocol shows a specific example of restrictionless cloning although the logic behind the primer design is the same for all situations. Your unique circumstances would require specific modifications to the primer sequences to create your desired overhangs.

2. EQUIPMENT

PCR thermocycler Benchtop microcentrifuge Gel electrophoresis equipment UV/vis spectrophotometer Micropipettors Micropipettor tips 1.5-ml microcentrifuge tubes 0.2-ml thin-walled PCR tubes Gel extraction kit (Qiagen)

3. MATERIALS

Phusion DNA polymerase and buffer (New England Biolabs)10 mM dNTP mix (New England Biolabs)T4 DNA ligase and buffer (New England Biolabs)Calf Intestinal Alkaline Phosphatase (CIP) (New England Biolabs)

5' phosphorylated PCR primers Nonphosphorylated PCR primers Vector DNA Agarose Tris base Glacial acetic acid EDTA Ethidium bromide Competent bacteria of choice (e.g., DH5α)

3.1. Solutions & buffers

Component	Amount
Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA	18.6 g

Step 1 50 \times TAE agarose gel running buffer

Add water to 1 l

$1 \times TA$	E agarose	gel	running	buffer
---------------	-----------	-----	---------	--------

Component	Amount
$50 \times TAE$ buffer	10 ml
Water	490 ml

4. PROTOCOL

4.1. Preparation

Design and order oligonucleotides for PCR (see Step 2.2 for PCR primer design).

4.2. Duration

Preparation	About 2 days		
Protocol	About 3–5 days		

See Fig. 9.1 for the flowchart of the complete protocol.

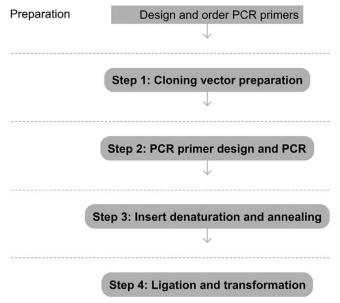


Figure 9.1 Flowchart of the complete protocol, including preparation.

5. STEP 1 CLONING VECTOR PREPARATION

5.1. Overview

Prepare vector by digesting with appropriate restriction enzymes to create cohesive ends for cloning. For example, this protocol discusses using pTYB1 (a bacterial expression vector from NEB) and NdeI and SapI as the cloning sites. Your specific cloning project would require modifications to the described protocols. Always follow the manufacturer's specific instructions for restriction enzyme use.

5.2. Duration

3–5 h

- **1.1** Choose cloning or expression vector and determine cloning sites within vector to be used. In this case the vector is pTYB1 and the cloning sites are NdeI and SapI.
- 1.2 Digest and dephosphorylate $1 \ \mu g$ of pTYB1 with NdeI, SapI, and alkaline phosphatase.
- **1.3** Incubate for 1 h at 37 $^{\circ}$ C.

1.4 Prepare a 1% agarose gel in 1× TAE with ethidium bromide. Run reaction on the 1% TAE agarose gel. Excise digested band and purify DNA with Gel Extraction kit (Qiagen) following recommended directions (see Agarose Gel Electrophoresis).

5.3. Tip

The restriction and dephosphorylation reactions can be done simultaneously in this situation. Check enzyme and buffer compatibility when using different restriction enzymes. Antarctic Phosphatase (NEB) can be used instead of CIP. Antarctic Phosphatase is active in NEB buffers 1–4. CIP is active in NEB buffers 2 and 4.

5.4. Tip

Dephosphorylation and gel purification of the vector are important for significantly reducing background colonies after ligation and transformation.

5.5. Caution

Ethidium bromide is a mutagen. Wear gloves and handle with care. Dispose gel and buffer in accordance with local regulations.

See Fig. 9.2 for the flowchart of Step 1.

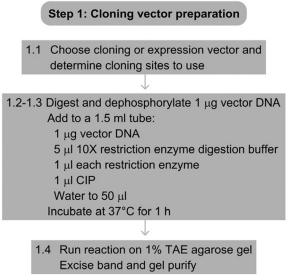


Figure 9.2 Flowchart of Step 1.

6. STEP 2 PCR PRIMER DESIGN AND PCR6.1. Overview

Two sets of primers (and two PCRs) are required to produce the correct cohesive ends on the final DNA insert that match the prepared vector (see Explanatory chapter: PCR -Primer design). The first PCR will create the top strand for the final insert and the second PCR will create the bottom strand for the final insert.

6.2. Duration

4–5 h

- **2.1** Determine the correct overhangs needed on the insert to match the ends of the prepared vector. Figure 9.3 shows an overview of expected overhang sequences on the example digested vector and final insert.
- 2.2 Design two sets of PCR primers that incorporate the desired overhang sequences. The first set of primers is used to create the top strand of the final insert. In this case the forward primer for PCR #1 will include 5'-phosphate-TATG followed by insert sequence to generate the NdeI overhang. The 5' phosphate is required for ligation into the dephosphorylated vector. Since no overhang needs to be created on the top strand of the insert, the reverse primer for PCR #1 is simply the reverse complement of the desired sequence to amplify. The second set of primers is used to create the bottom strand of the final insert. The forward primer for PCR #2 will include 5'-TG (part of NdeI site) followed by insert sequence. The reverse primer for PCR #2 will have 5'-phosphate-GCA followed by the reverse complement of the insert sequence to generate the SapI overhang.
- **2.3** After obtaining both sets of primers, amplify insert of interest (see General PCR) with Phusion DNA polymerase following recommended instructions.
- 2.4 Gel-purify both reactions as performed earlier with the prepared vector.
- 2.5 Quantitate DNAs after purification using a spectrophotometer at A_{260} (see Explanatory Chapter: Nucleic Acid Concentration Determination).

i. Cloning sites



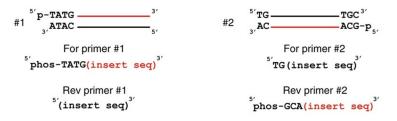
ii. Overhangs generated on vector after restriction digestion.



iii. Overhangs needed for insert to match vector.



iv. PCR products of insert that produce desired top and bottom strands.



v. Inserts produced after denaturing and annealing

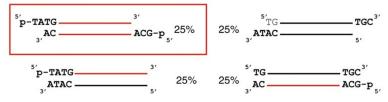


Figure 9.3 Overview of restrictionless cloning primer design and PCRs. The example cloning sites are shown in (i). The overhangs of the cloning vector after digestion are shown in (ii). In order to match the cohesive ends of the vector the insert will need to have the overhangs indicated in (iii). Two PCRs will be performed to generate the strands required to produce the desired overhangs for the insert (colored in red). PCR #1 will generate the top strand of the final insert and PCR #2 will generate the bottom strand of the final insert. The PCR products from these reactions and the primers used to generate them are shown in (iv). Following heat denaturing and annealing of the two PCR products \sim 25% of the dsDNA molecules will be the desired insert product (boxed) ready for ligation into the prepared vector (v).

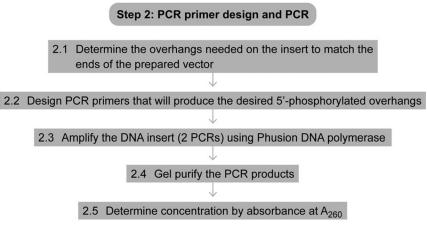


Figure 9.4 Flowchart of Step 2.

6.3. Tip

The primers not used to create the strands in the final insert do not need to be phosphorylated.

6.4. Tip

Do not use a DNA polymerase that adds bases to the ends of DNA such as Taq DNA polymerase as this will produce incorrect overhangs.

See Fig. 9.4 for the flowchart of Step 2.



7. STEP 3 INSERT DENATURATION AND ANNEALING

7.1. Overview

Heat denaturation will be used to separate the strands of DNA from one another in a mixture of the two insert PCRs. Slow cooling allows the formation of the correct final insert.

7.2. Duration

15 min

3.1 Mix equal amounts (~500 ng each) of insert PCR #1 and insert PCR #2 in a 0.2-ml PCR tube.

- 3.2 Heat mixture tube at 95 °C for 5 min in thermocycler.
- **3.3** Allow tube to cool in thermocycler for 10 min.

8. STEP 4 LIGATION AND TRANSFORMATION 8.1. Overview

The prepared vector and annealed final insert will be ligated together and transformed into the bacterial host of choice **(see Transformation of Chemically Competent *E. coli* or Transformation of *E. coli* via electroporation).

8.2. Duration

2-3 days

4.1 Ligate the annealed insert into the prepared vector as follows. Also prepare a no insert control reaction by leaving out the insert DNA in order to measure background colonies.

$10 \times$ T4 DNA ligase buffer	2 µl
Prepared vector	50–100 ng
Annealed insert	100–300 ng
T4 DNA ligase	1 µl
Water	up to 20 µl

Incubate at room temperature for 1–2 h or overnight at 16 °C.

- **4.2** Following the ligation reaction, transform electro-competent or chemically competent *E. coli* cells of your choice following recommended instructions. Plate cells on selective media and incubate overnight.
- 4.3 Pick transformed colonies and screen for desired clone.

8.3. Tip

Do not use excessive amounts of ligation reaction when doing transformations. $1-2 \mu l$ of ligation reaction per 30 μl of competent cells should be sufficient.

See Fig. 9.5 for the flowcharts of Steps 3 and 4.

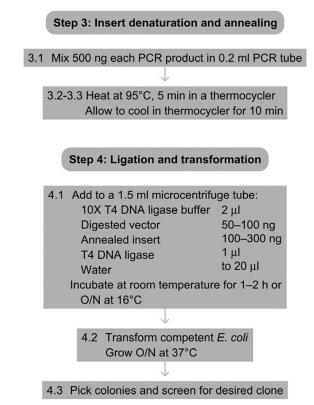


Figure 9.5 Flowchart of Steps 3 and 4.

REFERENCES

Referenced Protocols in Methods Navigator Explanatory chapter: PCR -Primer design. Agarose Gel Electrophoresis. General PCR. Explanatory Chapter: Nucleic Acid Concentration Determination. Transformation of Chemically Competent *E. coli*. Transformation of *E. coli* via electroporation.



Isolation of Plasmid DNA from Bacteria

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Abstract

The purpose of this protocol is the isolation of plasmid DNA from bacteria. The boiling method for isolating plasmids by Holmes and Quigley (1981) is presented here. This method is rapid and simple and it allows for a large number of samples to be processed simultaneously (up to 40 samples). Thus, it is appropriate for the preparation of bacterial plasmids in order to screen a large number of colonies or small cultures for the presence of recombinant DNA inserts. The protocol can be effectively scaled up for the preparation of plasmids from liter cultures.

1. THEORY

Plasmid DNA is used for a number of downstream applications such as transfection, sequencing, screening clones, restriction digestion, cloning, and PCR. A number of methods have been developed for the purification of plasmid DNA from bacteria. The most common method that is used is based on the alkaline lysis method (Birnboim and Doly, 1979). Nowadays, a variety of convenient and fast plasmid DNA purification kits are commercially available ensuring advanced quality and purity (see Explanatory Chapter: How Plasmid Preparation Kits Work). The size of the bacterial culture used (and effectively the plasmid DNA yield) defines the plasmid preparation as miniprep, midiprep, maxiprep etc. The small-scale mini preparation of plasmid DNA is commonly used to screen bacterial clones for the presence of recombinant DNA inserts. Here, we present a fast and simple protocol by Holmes and Quigley (1981) that allows the screening of a large number of colonies or small cultures for recombinant DNA inserts. Bacterial cells are collected and briefly boiled and the insoluble genomic DNA and cellular debris is removed by centrifugation. Plasmids are recovered by isopropanol precipitation and treated with RNase. The protocol works for plasmids ranging in size from 3.9 to 13.4 kb and yields enough plasmid DNA for several restriction digests (see Fig. 10.1). Multiple samples can be processed simultaneously (e.g., 20–40 samples).

2. EQUIPMENT

Shaking incubator Microcentrifuge or refrigerated microcentrifuge Boiling water bath (100 °C) -or-Heating block (e.g., Eppendorf thermomixer) Refrigerator (4 °C) Deep freezer (-80 °C) Freezer (-20 °C) Pipet-aid 5-ml disposable serological pipettes Micropipettors

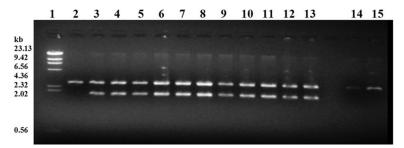


Figure 10.1 Restriction enzyme digestion of ligated plasmid DNA was electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Plasmids were prepared by the boiling protocol from microscale cultures of DH5 α cells that were transformed with plasmid pUCotsA that contains a trehalose gene insert from *Brevibacterium lactofermentum* (ATCC 21799) in plasmid pUC18. Transformation was carried out as described in Kushner et al. (1978). Restriction digestion of the ligated plasmid with the endonucleases *Kpn*I and *Xba*I yields a 2.7 kb and a 1.8 kb band corresponding to the plasmid vector and the insert, respectively. Lane 1: Lambda DNA marker (λ DNA/*Hind*III); lane 2: plasmid vector pUC18; lanes 3–13: plasmids isolated from white colonies; lanes 14 and 15: plasmids isolated from blue colonies (Andreou, 2005).

Pipettor tips 13-ml sterile polypropylene snap-cap tubes 1.5-ml microcentrifuge tubes Toothpicks

3. MATERIALS

LB broth Antibiotic (e.g., ampicillin, tetracycline) Sucrose Triton X-100 EDTA Tris base Lysozyme (Sigma) Sodium acetate (NaOAc) NaOH Isopropanol DNase-free RNase (Sigma)

3.1. Solutions & buffers

Step 2 STET Buffer

Component	Final concentration	Stock	Amount
Sucrose	8%		8 g
Triton X-100	0.5%	20%	2.5 ml
EDTA	50 mM	500 mM	10 ml
Tris–HCl, pH 8.0	10 mM	1 M	1 ml

Add purified water to 100 ml

Lysozyme (10 mg ml⁻¹)

Dissolve 10 mg of lysozyme in 1 ml of 10 mM Tris.Cl (pH 8.0)

ΤE

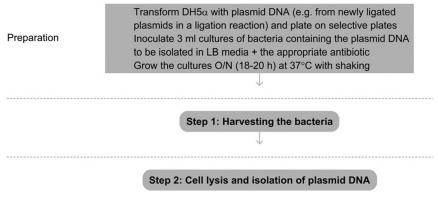
Component	Final concentration	Stock	Amount
Tris–HCl, pH 8.0	10 mM	1 M	1 ml
EDTA	0.1 mM	500 mM	20 µl

Add purified water to 100 ml

4. PROTOCOL 4.1. Duration	
Preparation	About 2 days
Protocol	About 2 h

4.2. Preparation

Transform DH5 α with the desired plasmid DNA, either a purified plasmid or a ligation reaction to be screened for the presence of inserts, and plate the bacteria on the appropriate selective plates. Pick individual colonies of the transformed *Escherichia coli* strain and inoculate 3 ml of LB broth supplemented with 25 µg ml⁻¹ ampicillin or another appropriate antibiotic in 13-ml sterile polypropylene snap-cap tubes (see also Growth Media for *E. coli*). Grow the bacterial cultures overnight (18–20 h) at 37 °C in a shaker for uniform growth to a density of about A_{650} =1.2.





See Fig. 10.2 for the flowchart of the complete protocol, including preparation.

5. STEP 1 HARVESTING THE BACTERIA

5.1. Overview

The bacterial cells are collected by centrifugation.

5.2. Duration

20 min

- **1.1** Transfer 1.5 ml of the bacterial culture to a 1.5-ml microcentrifuge tube and store the rest of the culture in the refrigerator (4 °C).
- 1.2 Pellet the bacteria at 8000 rpm for 5 min and remove the media.
- **1.3** Leave the bacterial pellet to air-dry.

See Fig. 10.3 for the flowchart of Step 1.

6. STEP 2 CELL LYSIS AND ISOLATION OF PLASMID DNA 6.1. Overview

The bacterial cells are boiled briefly at 100 °C in the presence of agents that weaken the cell wall and help prevent DNA degradation by nucleases. The partially denatured genomic DNA and denatured proteins are pelleted down by centrifugation. Plasmids are recovered by isopropanol precipitation and RNase degrades RNA into smaller components.

Step 1: Harvesting the bacteria

- 1.1 Transfer 1.5 ml of the culture to a 1.5 ml microcentrifuge tube Store the remainder of the culture at $4^{\circ}C$
- 1.2 Centrifuge the bacteria in a microcentrifuge at 8000 rpm, 5 min Discard the media (as biohazrdous waste)
 Spin briefly and remove last traces of liquid

1.3 Air-dry the bacterial pellet

Figure 10.3 Flowchart of Step 1.

6.2. Duration

1.5 h

- 2.1 Resuspend the bacterial pellet in 0.35 ml of STET Buffer.
- **2.2** Add 25 μ l of freshly prepared lysozyme (10 mg ml⁻¹) and mix by shaking gently for 3 s.
- **2.3** Place the tube in a boiling water bath $(100 \degree C)$ for 40 s.
- **2.4** Centrifuge immediately at 14 000 rpm for 10 min at room temperature (or alternatively at 4 °C). Remove the pellet from the tube using a toothpick.
- **2.5** Add 40 μl of 3 M sodium acetate, pH 5.2, and 420 μl isopropanol and mix by vortexing briefly.
- **2.6** Place the sample in a -80 °C freezer for 30 min.
- **2.7** Centrifuge the sample at 14 000 rpm for 15 min at 4 °C. Remove the supernatant and air-dry the pellet.
- **2.8** Resuspend the pellet in 50 μ l of TE that contains DNase-free RNase (50 μ g ml⁻¹) and incubate the sample for 10 min at 37 °C.
- 2.9 The plasmid DNA is now ready to use in downstream applications, for example, restriction enzyme digestion or PCR and agarose gel electrophoresis (see General PCR, Agarose Gel Electrophoresis). Store the miniprep plasmid DNA at -20 °C.

6.3. Caution

Isopropanol is highly flammable. It should be kept away from heat and open flames. Poisoning can occur from inhalation, ingestion, or skin absorption and it may act as an irritant. Use in well-ventilated areas and wear safety glasses and protective gloves.

6.4. Tip

Air-dry the pellet instead of using a vacuum in order to avoid over-drying the pellet as this will make the DNA hard to dissolve.

6.5. Tip

The DNase-free RNase treatment removes the RNA that may cover small DNA fragments in agarose gels.

6.6. Tip

Resuspend the pellet in TE for long-term storage or in Tris–HCl, pH 8.5 to avoid EDTA inhibition of downstream reactions.

See Fig. 10.4 for the flowchart of Step 2.

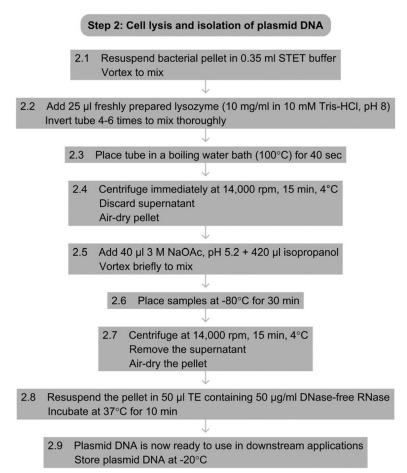


Figure 10.4 Flowchart of Step 2.

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Explanatory Chapter: How Plasmid Preparation Kits Work. Growth Media for *E. coli*. General PCR. Agarose Gel Electrophoresis.

Preparation of Genomic DNA from Bacteria

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Abstract

The purpose of this protocol is the isolation of bulk cellular DNA from bacteria (alternatively see Preparation of genomic DNA from *Saccharomyces cerevisiae* or Isolation of Genomic DNA from Mammalian Cells protocols).

1. THEORY

High-quality, purified DNA is crucial for a number of downstream applications such as PCR, cloning, and DNA library construction. The isolation of DNA is traditionally achieved using organic extraction of the soluble DNA while the insoluble cell debris is removed. The DNA is then purified from soluble proteins and RNA by ethanol precipitation. Here, chromosomal DNA from bacteria is obtained by the method of Lovett and Keggins (Lovett and Keggins, 1979), with the exception of achieving the lysis of the protoplasts with 1% sodium dodecyl sulfate instead of Sarkosyl. The method can be applied for the isolation of genomic DNA from Gram-negative and Gram-positive bacteria and is particularly effective for rod-shaped Gram-positive bacteria such as *Bacillus subtilis*. A variety of convenient and fast DNA purification methods are also commercially available nowadays.

2. EQUIPMENT

Shaking incubator Centrifuge UV spectrophotometer UV transparent cuvettes Large-bore pipette 15-ml polypropylene centrifuge tubes

3. MATERIALS

Bacterial strains (source of DNA) Lysogeny Broth (LB; or other appropriate bacterial liquid growth medium) Tris base Hydrochloric acid (HCl) EDTA Sodium chloride (NaCl) Lysozyme RNase Proteinase K Sodium dodecyl sulfate (SDS) Tris-saturated Phenol (Roche) Ethanol Sterile deionized water

3.1. Solutions & buffers

Step 1 TES

Component	Final concentration	Stock	Amount
Tris–HCl, pH 7.5	20 mM	50 mM	40 ml
EDTA	5 mM	250 mM	2 ml
NaCl	100 mM	1 M	10 ml

Add water to 100 ml

RNase: Pancreatic, 2 mg ml^{-1} in water, heat-shocked at $80 \degree \text{C}$ for 15 min

Proteinase K: 10 mg ml^{-1} in TES, predigested at 37 °C for 90 min 10% SDS: Dissolve 10 g SDS in 100 ml water (final volume)

Step 2 Ethanol: 95%, ice-cold TE

Component	Final concentration	Stock	Amount
Tris–HCl, pH 8.0	10 mM	50 mM	2 ml
EDTA	0.1 mM	250 mM	4 µl

Add water to 10 ml

4. PROTOCOL

4.1. Preparation

Inoculate 10 ml of liquid broth with bacteria and let it grow overnight (18–20 h) at 37 $^{\circ}$ C with shaking (see also Growth Media for *E. coli*).

4.2. Duration

Preparation	18–20 h
Protocol	5 h

See Fig. 11.1 for the flowchart of the complete protocol, including preparation.

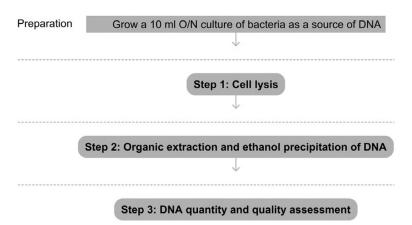


Figure 11.1 Flowchart of the complete protocol, including preparation.

5. STEP 1 CELL LYSIS

5.1. Overview

The cells are collected and washed. A lysozyme buffer is used to damage cell walls and the detergent SDS further aids in disrupting cell membranes and lysing the cells. DNA, protein, and other cell components are released. RNase degrades RNA into smaller components and Proteinase K digests protein contaminants.

5.2. Duration

2.5 h

- **1.1** Centrifuge the 10 ml cell culture at 8000 rpm at 4 °C for 10 min. Discard supernatant.
- 1.2 Resuspend the cells in 2.5 ml TES buffer.
- **1.3** Centrifuge the cell suspension at 8000 rpm at 4 °C for 10 min. Discard supernatant.
- 1.4 Wash cells a second time with 2.5 ml TES.
- **1.5** Resuspend the cells in 2.5 ml TES buffer. Treat the cell suspension with 2 mg lysozyme. Swirl the suspension gently and incubate it at 37 °C for 30 min.
- 1.6 Add the RNase solution to $100 \ \mu g \ ml^{-1}$, and incubate the suspension at 37 °C for 30 min.
- Dilute the lysate to twice its original volume with the addition of 2.5 ml TES buffer.

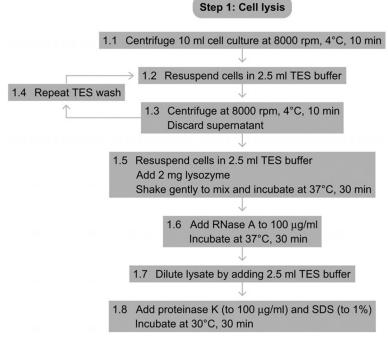


Figure 11.2 Flowchart of Step 1.

1.8 Treat the lysate with proteinase K (to 100 μ g ml⁻¹) and SDS (to 1%) at 30 °C for 30 min.

See Fig. 11.2 for the flowchart of Step 1.

6. STEP 2 ORGANIC EXTRACTION AND ETHANOL PRECIPITATION OF DNA

6.1. Overview

DNA is separated from other cellular contaminants by organic extraction and is then precipitated from the solution by ethanol.

6.2. Duration

1.5 h

- **2.1** Shake the lysate gently with an equal volume (5 ml) of TES-saturated phenol at room temperature for 10 min.
- **2.2** Centrifuge the sample for 10 min at room temperature to separate the phases.

- **2.3** Transfer the aqueous phase with a large-bore pipette to a new 15-ml centrifuge tube.
- **2.4** Add an equal volume of TES-saturated phenol, shake the sample gently for 1–2 min and centrifuge it at room temperature for 5 min.
- 2.5 Repeat the phenol extraction (Steps 2.3–2.4) twice.
- **2.6** Transfer the final aqueous phase (~ 2.5 ml) to two volumes of cold 95% ethanol and hold it at 4 °C for at least 30 min.
- **2.7** Centrifuge the sample at 12 000 rpm at 4 °C for 20 min and leave the precipitate to air-dry for 15 min.
- 2.8 Resuspend the precipitate in 0.5–1 ml of TE (can take up to 12–18 h).
- **2.9** Store the dissolved DNA sample at 4 °C.

6.3. Tip

After the phenol extraction the sample can be extracted with one volume of chloroform or chloroform: isoamyl alcohol to remove any trace of phenol.

6.4. Tip

Transfer the upper aqueous phase, which contains DNA, without disturbing the interface in order to avoid the transfer of debris from the interface.

6.5. Tip

Once ethanol is added to the sample, the chromosomal DNA becomes visible as white fibers.

6.6. Tip

In order to desalt the DNA sample, resuspend the precipitate in 2 ml of TES and dialyse against 1000 volumes of TES at $4 \degree C$ for 12-18 h. The sample could be washed with ethanol 70% to remove salts, but dialysis is recommended.

6.7. Tip

TE is suitable for long-term storage of DNA but the EDTA it contains can interfere with certain downstream applications. If this is the case, precipitate DNA and resuspend it in sterile water.

6.8. Caution

Phenol can cause chemical burns that may not be noticed until the following day since it numbs the skin as it burns. Wear gloves, goggles and a lab coat. Also, keep tubes capped tightly. Work in a fume hood.

See Fig. 11.3 for the flowchart of Step 2.

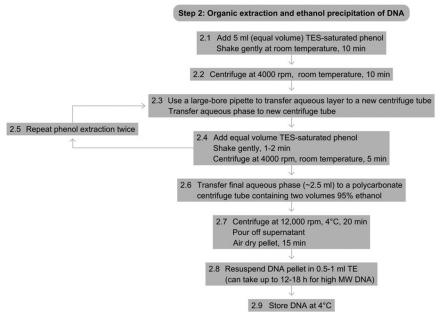


Figure 11.3 Flowchart of Step 2.

7. STEP 3 DNA QUANTITY AND QUALITY ASSESSMENT 7.1. Overview

Here you assess the quantity of DNA present in the sample by measuring the absorbance (A) at wave lengths of 260 and 280 nm and/or agarose gel electrophoresis (see also Explanatory Chapter: Nucleic Acid Concentration Determination and Agarose Gel Electrophoresis). Gel electrophoresis is less accurate in quantitation but offers the advantage of allowing a qualitative analysis to take place.

7.2. Duration

About 1 h

- **3.1** Scan a dilution of the DNA-containing solution in a UV spectrophotometer over the range of 240–320 nm to estimate DNA purity and DNA concentration.
- **3.2** Visualize the DNA present in the sample on a 0.8% agarose gel containing a DNA-intercalating dye such as ethidium bromide. Use a UV transilluminator to see the DNA band and to take a picture (Fig. 11.4).

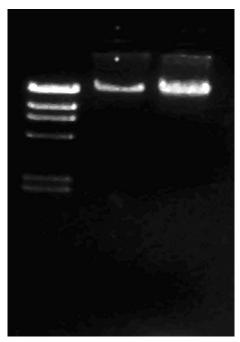


Figure 11.4 Agarose gel electrophoresis of microbial genomic DNA. Genomic DNA was electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Lane 1: Lambda DNA marker (λ DNA/*Hind*III); lane 2: Genomic DNA isolated from *Corynebacterium glutamicum* ATCC 21253; lane 3: Genomic DNA isolated from *Brevibacterium lactofermentum* ATCC 21799 (Andreou, 2005).

7.3. Tip

An A_{260}/A_{280} ratio of around 1.8 (between 1.7 and 2.0) suggests a high-quality DNA sample. Smaller ratios indicate protein or phenol contamination (ratios > 0.5). Once you have established that your sample contains pure DNA you can determine the DNA concentration. An A_{260} of 1.0 suggests a concentration of 50 µg m Γ^1 of dsDNA.

7.4. Tip

The presence of a single band near the wells is indicative of whole, uncut genomic DNA. A slight smear is acceptable but is indicative of partial degradation.

7.5. Caution

Ethidium bromide is a known mutagen and a carcinogen so it should be handled with extreme care. Wear gloves and a lab coat. Do not contaminate the surrounding lab bench area and equipment with EtBr. Dispose of EtBr waste in designated containers.

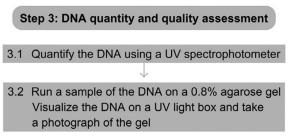


Figure 11.5 Flowchart of Step 3.

7.6. Caution

UV presents a safety hazard. Protect your eyes and skin from exposure to UV light. See Fig. 11.5 for the flowchart of Step 3.

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Preparation of genomic DNA from *Saccharomyces cerevisiae*. Isolation of Genomic DNA from Mammalian Cells. Growth Media for *E. coli*. Explanatory Chapter: Nucleic Acid Concentration Determination. Agarose Gel Electrophoresis.

CHAPTER TWELVE

Preparation of Genomic DNA from Saccharomyces cerevisiae

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Abstract

The ability to isolate genomic DNA rapidly and effectively for analysis by PCR, Southern blotting, or other methods is an essential skill. This protocol provides a fast and efficient method for obtaining genomic DNA from *S. cerevisiae*.

1. THEORY

This method to extract genomic DNA from yeast is fast and reliably generates ample DNA for multiple applications. If high molecular weight genomic DNA is required, an alternative method employing enzymatic lysis, rather than mechanical lysis, should be employed (Boeke et al., 1985).

2. EQUIPMENT

Refrigerated centrifuge Refrigerated microcentrifuge Vortex mixer Micropipettors Micropipettor tips 15-ml polypropylene centrifuge tubes 1.5-ml microcentrifuge tubes Glass beads, 425–600 µm, acid-washed

3. MATERIALS

YPD media Tris base EDTA Hydrochloric acid (HCl) Sodium chloride (NaCl) Triton X-100 Sodium dodecyl sulfate (SDS) Phenol/chloroform/isoamyl alcohol, 25:24:1 (v/v/v) Ethanol Ammonium acetate (NH₄OAc) RNase A Sterile H₂O

3.1. Solutions & buffers

Step 1 Lysis Buffer

Component	Final Concentration	Stock	Amount
Tris–HCl, pH 8.0	10 mM	1 M	10 ml
EDTA, pH 8.0	1 mM	0.5 M	2 ml

NaCl	100 mM	5 M	20 ml
Triton X-100	2% (v/v)	100%	20 ml
Sodium dodecyl sulfate	e (SDS) 1% (v/v)	10% (w/v)	100 ml
Add water to 11			
TE Buffer			
Component	Final concentration	Stock	Amount
Tris–HCl, pH 8.0	10 mM	1 M	10 ml
EDTA, pH 8.0	1 mM	0.5 M	2 ml
Add water to 11			

4. PROTOCOL

4.1. Preparation

Inoculate a single yeast colony into 10 ml YPD and grow, shaking, at 30 °C overnight.

4.2. Duration

Preparation	1 day
Protocol	3–4 h

See Fig. 12.1 for the flowchart of the complete protocol.

5. STEP 1 HARVESTING CELLS FROM THE OVERNIGHT CULTURE

5.1. Overview

Isolate yeast cells from the overnight culture and remove trace media.

5.2. Duration

15 min

- **1.1** Transfer the overnight culture into a 15-ml centrifuge tube.
- **1.2** Centrifuge at 3600 rpm at 4 °C for 5 min.
- 1.3 Resuspend the cell pellet in 1 ml H_2O . Transfer cells to a 1.5-ml microcentrifuge tube.
- 1.4 Centrifuge at 3600 rpm at 4 °C for 5 min. Pour off the supernatant.

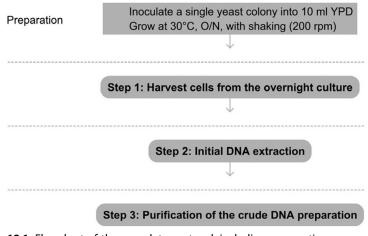


Figure 12.1 Flowchart of the complete protocol, including preparation.

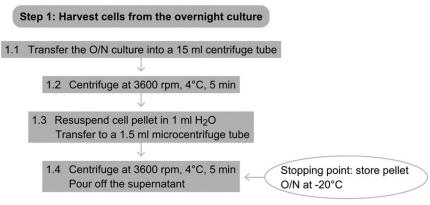


Figure 12.2 Flowchart of Step 1.

5.3. Tip

The protocol may be temporarily halted at this point; store the cell pellet at -20 °C overnight.

See Fig. 12.2 for the flowchart of Step 1.



Cells are lysed and a crude DNA preparation is extracted.

6.2. Duration

35–45 min

- 2.1 Resuspend the cell pellet in 200 µl Lysis Buffer.
- **2.2** Add 200 μl glass beads and 400 μl phenol/chloroform/isoamyl alcohol (25:24:1).
- **2.3** Vortex for 2 min (single tube vortexer) or 10 min (multitube vortexer).
- 2.4 Add 400 µl TE Buffer and mix by vortexing briefly.
- 2.5 Centrifuge 10 min at maximum speed at room temperature.
- **2.6** Transfer 400 μ l of the aqueous layer to a new microcentrifuge tube.
- 2.7 Add 1 ml ice-cold ethanol. Mix by inverting.
- 2.8 Centrifuge for 5 min at maximum speed at room temperature.
- 2.9 Wash the pellet with 70% ethanol.
- **2.10** Dry the pellet at room temperature for ~ 5 min.
- **2.11** Resuspend the pellet in 500 μ l TE Buffer.

6.3. Tip

Remove glass beads from the top of the tube. Incomplete closure of the microcentrifuge tube will cause phenol/chloroform contamination of equipment and dissolution of tube labels.

6.4. Tip

Cell lysis can be confirmed by examining the resulting cell slurry under a microscope. If lysis is incomplete, continue vortexing in short intervals until lysis is nearly complete.

6.5. Tip

This preparation may be used for robust PCR applications, although the DNA will not be stable for long periods of time. For extremely stable genomic DNA, complete Step 3.

6.6. Tip

The protocol may be temporarily halted here. Store the pellet in TE at $4 \degree C$ overnight. See Fig. 12.3 for the flowchart of Step 2.

7. STEP 3 PURIFICATION OF THE CRUDE DNA PREPARATION

7.1. Overview

Contaminating RNA and cellular debris are removed.

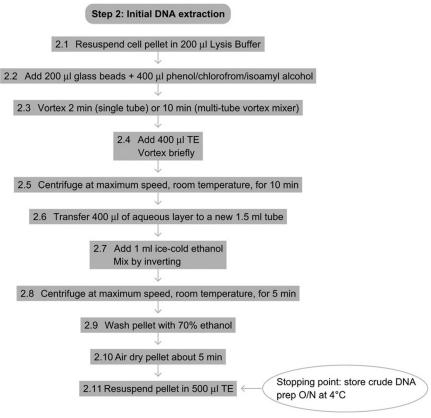


Figure 12.3 Flowchart of Step 2.

7.2. Duration

1–2 h

- **3.1** Add 15 μ l 2 mg ml⁻¹ RNase A.
- **3.2** Incubate at 37 °C for 30 min.
- 3.3 Add 500 μl phenol/chloroform/isoamyl alcohol (25:24:1). Vortex for 30 s (single tube vortexer) or 5 min (multitube vortexer) to mix.
- 3.4 Centrifuge for 5 min at maximum speed at room temperature.
- 3.5 Transfer 400 µl of the aqueous layer to a clean microcentrifuge tube.
- **3.6** Add 1 ml ice-cold ethanol, and 10 μl 4 M ammonium acetate. Invert to mix.
- 3.7 Centrifuge for 10 min at maximum speed at room temperature.
- **3.8** Wash the cell pellet with 70% ethanol.

3.9 Dry the pellet at room temperature for ~ 5 min.

3.10 Resuspend the pellet in $100 \ \mu$ l TE Buffer.

7.3. Tip

If a very high yield of genomic DNA is required, incubate at $-20 \degree C$ for at least 1 h in Step 3.6.

7.4. Tip

If a very high yield of genomic DNA is required, perform the centrifugation for Step 3.7 at $4 \,^{\circ}C$.

See Fig. 12.4 for the flowchart of Step 3.

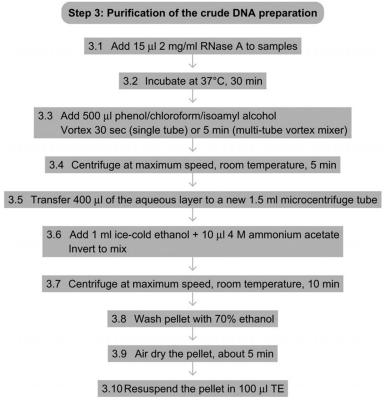


Figure 12.4 Flowchart of Step 3.

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CHAPTER THIRTEEN

Isolation of Genomic DNA from Mammalian Cells

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Abstract

The isolation of genomic DNA from mammalian cells is a routine molecular biology laboratory technique with numerous downstream applications. The isolated DNA can be used as a template for PCR, cloning, and genotyping and to generate genomic DNA libraries. It can also be used for sequencing to detect mutations and other alterations, and for DNA methylation analyses.

1. THEORY

In general, isolation of genomic DNA from mammalian cells involves cell lysis, removal of proteins and other cellular contaminants, and organic extraction, followed by recovery of DNA. Typically, mammalian cells are lysed using a detergent-based buffer, which solubilizes lipids, thus disrupting the integrity of cell membranes. This releases cellular components into solution. Proteinase K is then added to facilitate the digestion and removal of proteins from the cell lysates. Next, organic extraction is carried out, in which a mixture of phenol, chloroform and isoamyl alcohol is added. DNA separates into the aqueous phase, while most other contaminants separate into the organic phase. An optional treatment with RNase A ensures that the sample is free from RNA contamination. DNA is then recovered by ethanol precipitation.

2. EQUIPMENT

Centrifuge Incubator (50 °C) Microcentrifuge Vortex mixer Magnetic stir plate Micropipettors Pipet-aid 15-ml Conical centrifuge tubes 1.5-ml Microcentrifuge tubes Micropipettor tips Beaker, 1 1 Magnetic stir bars 10-ml pipettes pH indicator paper Aluminum foil

3. MATERIALS

Sterile deionized water Tris base Hydrochloric acid (HCl) Sodium chloride (NaCl) EDTA disodium (Na₂EDTA.2H₂O)

Sodium dodecyl sulfate (SDS) Proteinase K Phenol 8-hydroxyquinoline Chloroform Isoamyl alcohol Ammonium acetate (NH₄OAc) Ethanol Phosphate buffered saline (PBS) RNase A (optional)

3.1. Solutions & buffers

Step	2	Lysis	Buffer
------	---	-------	--------

Component	Final Concentration	Stock	Amount
NaCl	100 mM	1 M	1 ml
Tris–HCl, pH 8.0	10 mM	50 mM	2 ml
EDTA, pH 8.0	25 mM	250 mM	1 ml
SDS	0.5%	10%	0.5 ml
Proteinase K	0.1 mg ml^{-1}	1 mg ml^{-1}	1 ml

Add sterile water to 10 ml. Add proteinase K fresh before use

Step 3 Buffered Phenol

Component	Stock	Amount
Phenol		500 ml
8-hydroxyquinoline		0.5 g
Tris–HCl, pH 8.0	50 mM	Variable

See preparation step for instructions to make it up

Phenol Extraction Buffer	
Component	Amount
Buffered phenol	25 ml
Chloroform	24 ml
Isoamyl alcohol	1 ml

Mix well. Wrap container in aluminium foil to protect from light

Step 4 7.5 M ammonium acetate

Dissolve 57.8 g ammonium acetate in 100 ml (final volume) of sterile deionized water.

70% Ethanol

Mix 70 ml 100% ethanol and 30 ml sterile deionized water.

TE Buffer

Component	Final concentration	Stock	Amount
Tris–HCl, pH 8	10 mM	50 mM	2 ml
EDTA	1 mM	250 mM	40 µl

Add sterile deionized water to 10 ml

4. PROTOCOL

4.1. Preparation

Make the buffered phenol. Add 0.5 g 8-hydroxyquinoline to a glass beaker containing a stir bar. Add 500 ml phenol and 500 ml 50 mM Tris–HCl, pH 8.0.

Cover with aluminium foil to protect light-sensitive reagents from oxidation.

Stir for 10 min at room temperature, allowing the phases to separate.

Decant most of the upper aqueous phase into an appropriate waste container. Carefully remove the remainder with a 10-ml pipette. Add another 500 ml 50 mM Tris–HCl, pH 8.0. Stir and decant aqueous phase as before. Check the pH of the lower phenol phase with pH paper. Repeat equilibrations with 50 mM Tris–HCl, pH 8.0 until the pH of the phenol phase reaches 8.0.

Add 250 ml of 50 mM Tris–HCl, pH 8.0. Store at 4 °C in either a brown glass bottle or a clear glass bottle wrapped in aluminium foil to protect from light.

Have cells ready to extract DNA.

4.2. Duration

Preparation	Variable
Protocol	About 2 days

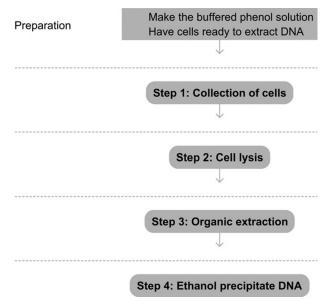


Figure 13.1 Flowchart of the complete protocol, including preparation.

4.3. Caution

Phenol is highly corrosive. It should be handled with care and should be opened only in a fume hood. Used phenol should be disposed appropriately according to chemical safety regulations, not down the sink.

See Fig. 13.1 for the flowchart of the complete protocol.

5. STEP 1 COLLECTION OF CELLS

5.1. Overview

Cells grown in culture are harvested and washed.

5.2. Duration

 $20 \min$

- **1.1** Collect cells in a 15-ml conical centrifuge tube. If starting with tissue culture cells in suspension, directly collect suspension in a conical tube. If starting with adherent cells in culture, trypsinize and collect cells in a conical tube.
- **1.2** Determine the number of cells collected.

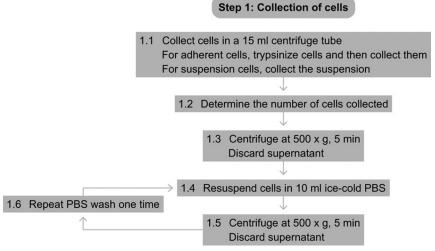


Figure 13.2 Flowchart of Step 1.

- **1.3** Centrifuge at $500 \times g$ for 5 min. Discard supernatant.
- **1.4** Resuspend in 10 ml ice-cold PBS.
- **1.5** Centrifuge at $500 \times g$ for 5 min. Discard supernatant.
- 1.6 Repeat PBS wash (Steps 1.4 and 1.5).

See Fig. 13.2 for the flowchart of Step 1.

6. STEP 2 CELL LYSIS

6.1. Overview

A detergent-based buffer is used to disrupt cell membranes and release DNA, protein, and other cell components. Proteinase K removes protein contaminants (see also Lysis of mammalian and Sf9 cells).

6.2. Duration

12–18 h

- **2.1** Resuspend cells in a suitable amount of Lysis Buffer. In general, use 1 ml of buffer per 10^8 cells.
- 2.2 Transfer to a 1.5-ml microcentrifuge tube. Vortex the samples.
- **2.3** Incubate at 50 °C for 12–18 h.
- See Fig. 13.3 for the flowchart of Step 2.

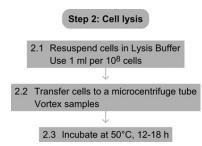


Figure 13.3 Flowchart of Step 2.

7. STEP 3 ORGANIC EXTRACTION

7.1. Overview

Organic extraction is used to separate DNA from other cellular contaminants.

7.2. Duration

 $10 \min$

- **3.1** Add an equal volume of phenol extraction buffer to the lysed cell suspension.
- **3.2** Vortex for 10 s.
- **3.3** Centrifuge at $2000 \times g$ for 5 min in a microcentrifuge at room temperature.
- **3.4** Transfer the upper aqueous phase, which contains DNA, to a new microcentrifuge tube. Determine the volume of the aqueous phase obtained.
- **3.5** Optional: Add RNase A to a final concentration of 20 μ g ml⁻¹. Incubate sample at 37 °C for 20 min.

See Fig. 13.4 for the flowchart of Step 3.

8. STEP 4 ETHANOL PRECIPITATION

8.1. Overview

Ethanol is used to precipitate DNA from solution.

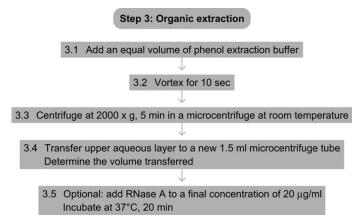


Figure 13.4 Flowchart of Step 3.

8.2. Duration

 $40 \min$

- **4.1** Add 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of 100% ethanol. Vortex.
- **4.2** Centrifuge at $2000 \times g$ for 10 min in a microcentrifuge at room temperature.
- 4.3 Remove supernatant carefully, without disrupting DNA pellet.
- **4.4** Add 1 ml of 70% ethanol and invert tube several times to wash the DNA pellet.
- **4.5** Centrifuge at $1700 \times g$ for 5 min in a microcentrifuge at room temperature.
- **4.6** Remove supernatant. Uncap the tube and allow the pellet to air-dry for 10–15 min.
- 4.7 Resuspend DNA in TE buffer or sterile water.

8.3. Tip

Resuspension in TE buffer stabilizes the DNA for long-term storage. However, the EDTA in the TE buffer may interfere with certain downstream applications, in which case, resuspension in water is preferred.

See Fig. 13.5 for the flowchart of Step 4.

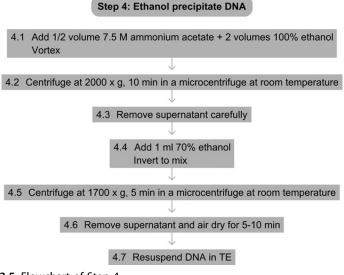


Figure 13.5 Flowchart of Step 4.

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Lysis of mammalian and Sf9 cells



Sanger Dideoxy Sequencing of DNA

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Abstract

While the ease and reduced cost of automated DNA sequencing has largely obviated the need for manual dideoxy sequencing for routine purposes, specific applications require manual DNA sequencing. For instance, in studies of enzymes or proteins that bind or modify DNA, a DNA ladder is often used to map the site at which an enzyme is bound or a modification occurs. In these cases, the Sanger method for dideoxy sequencing provides a rapid and facile method for producing a labeled DNA ladder.

1. THEORY

Sequencing of DNA has been revolutionized by the advent of dideoxynucleotides and the Sequenase enzyme. Dideoxynucleotide triphosphates are readily incorporated into a growing DNA chain, but lack the 3' hydroxyl group necessary to allow the chain to continue, and effectively terminate polymerization. Sequenase is a T7 DNA polymerase that has been chemically (Sequenase 1.0) or genetically (Sequenase 2.0) modified to remove its 3'-5' exonuclease activity (Fuller et al., 1996). Sequencing DNA using Sequenase and dideoxynucleotides takes place in four steps. First a primer is annealed to single-stranded DNA. Single-stranded DNA can be synthesized by a bacteriophage, or as in the more frequently used method described below, by denaturing double-stranded plasmid DNA. Next, the annealed primer is extended to form various lengths of labeled DNA. Label can be incorporated in the form of a radiolabeled primer used in the annealing step, or, as described below, a radioactive nucleotide can be incorporated during the extension step. We choose to use $[\alpha^{-32}P]$ deoxythymidine, as it is less costly than other radioactive nucleotides, but any $[{}^{35}S]$ -, $[\alpha - {}^{33}P]$ -, or $[\alpha - {}^{32}P]$ -deoxynucleotide can be used. Finally, the extension reaction is divided between four different termination reactions, each containing either dideoxy-adenosine, -cytosine, -guanosine, or -thymidine. These four reactions are then resolved on a denaturing polyacrylamide gel and visualized by autoradiography.

2. EQUIPMENT

Microcentrifuge Heating block (68 and 95 °C) Incubator (42 °C) Sequencing gel apparatus (e.g., BRL Model S2, available from Labrepco or Lab-X, or equivalent)

Power supply (capable of 65 W) Sequencing gel plates (Labrepco for BRL Model S2 plates) Sharktooth comb (CBS scientific, $0.2 \text{ mm} \times 62$ well comb) 0.2 mm spacers with foam ears, slightly longer than plates 0.4 mm spacers (CBS scientific) Large binder clips (or other gel clamps) Gel dryer Phosporimager (or film processor) Phosphor screen (or autoradiography film) Micropipettors Micropipettor tips 1.5-ml microcentrifuge tubes 50-ml conical tubes 200 ml beaker 500 ml Sidearm vacuum flask Buchner funnel Microtiter plate (10 μ l wells) Glass fiber prefilter Whatman 3MM chromatography paper Syringe, 60 ml Needle, 18 gauge

3. MATERIALS

DNA to be sequenced Sequencing primer dNTP stocks (dATP, dCTP, dGTP, dTTP, 10 mM) $[\alpha^{-3^2}P]$ -dTTP (800 Ci mmol⁻¹, 10 mCi ml⁻¹) ddNTP stocks (ddATP, ddCTP, ddGTP, ddTTP, 10 mM) N-tris(hydroxy-methyl)methyl-2-aminoethanesulfonic acid (TES) Sodium chloride (NaCl) Magnesium chloride (MgCl₂) Hydrochloric acid (HCl) Formamide EDTA Xylene cyanol FF Bromphenol blue Sodium hydroxide (NaOH) Dithiothreitol (DTT) Sequenase (USB Biochemicals) 40% Acrylamide:bisacrylamide solution (19:1) Tris base Boric acid (H₃BO₃) Urea Ammonium persulfate (APS) Tetramethylethylenediamine (TEMED) 95% Ethanol Siliconizing agent (e.g., Sigma-Cote or a nonsilicone smoothing agent)

3.1. Solutions & buffers

Step 1 TMN	
------------	--

Component	Final concentration	Stock	Amount
TES	560 mM	2.8 M	1 ml
NaCl	400 mM	2 M	1 ml
MgCl ₂	160 mM	0.8 M	1 ml
HCl	1.21 M	12.1 M (37%)	1 ml
H ₂ O			1 ml

Step 2 Termination Mixes (ddA, ddC, ddG, ddT)

Component	Final concentration	Stock	Amount
dATP	80 µM	10 mM	8 µl
dCTP	80 µM	10 mM	8 µl
dGTP	80 µM	10 mM	8 µl
dTTP	80 µM	10 mM	8 µl
Particular ddNTP	8 μΜ	10 mM	0.8 µl
H ₂ O			967 µl

-dT mix

Component	Final concentration	Stock	Amount
dATP	1.5 μM	10 mM	0.75 μl
dCTP	1.5 μM	10 mM	0.75 μl
dGTP	1.5 μM	10 mM	0.75 μl
H ₂ O			5 ml

Component	Final concentration	Stock	Amount
Formamide	95%		47.5 ml
EDTA, pH 8.0	20 mM	500 mM	2 ml
Xylene cyanol FF	0.05%		2.5 mg
Bromophenol blue	0.05%		2.5 mg

Step 3 STOP dye

Add water to 50 ml

Step 4 $10 \times TBE$

Component	Final concentration	Amount
Tris base	89 mM	108 g
Boric acid	89 mM	55 g
EDTA	2 mM	7.44 g

Add water to 11

10% APS

Dissolve 5 g ammonium persulfate in 50 ml H₂O in a 50-ml conical tube.

4. PROTOCOL

4.1. Preparation

Isolate plasmid DNA (see Isolation of plasmid DNA from bacteria) using either a standard miniprep kit or CsCl density gradient centrifugation for best results.

Order or synthesize an oligonucleotide primer (see Explanatory chapter: PCR -Primer design) designed to anneal \sim 30 nucleotides from where you wish to start reading the DNA sequence, as you would for PCR, but use only one primer for each sequence. Both plasmid and oligonucleotides should be dissolved in water or a low salt solution without EDTA.

4.2. Duration

Preparation	About 1 day
Protocol	5-9 h + overnight

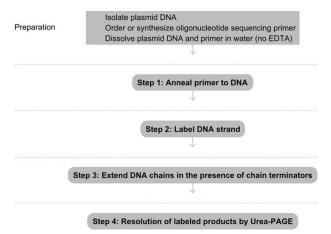


Figure 14.1 Flowchart of the complete protocol, including preparation.

4.3. Caution

Consult your institute Radiation Safety Officer for proper ordering, handling, and disposal of radioactive materials.

See Fig. 14.1 for the flowchart of the complete protocol.

5. STEP 1 ANNEAL PRIMER TO DNA

5.1. Overview

Anneal a complementary primer ~ 30 nucleotides from where you wish to start reading the DNA sequence.

5.2. Duration

30 min

- **1.1** Mix the following in a 1.5-ml tube:
 - 1–10 µg DNA
 - 1 μ l primer at 3 pmol μ l⁻¹
 - 1 µl 1 M NaOH
 - H_2O to bring volume to 10 µl
- **1.2** Incubate for 10 min at 68 $^{\circ}$ C.
- 1.3 Add 2 μ l of TMN buffer as each tube is removed from the water bath or heat block.
- **1.4** Transfer tubes to a rack at room temperature for 10 min.

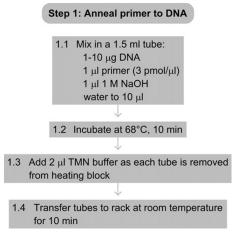


Figure 14.2 Flowchart of Step 1.

5.3. Tip

Make labeling mix (for Step 2) and aliquot termination mixes (for Step 3) during incubations (Steps 1.2 and 1.4) for best use of time.

See Fig. 14.2 for the flowchart of Step 1.

6. STEP 2 LABELING

6.1. Overview

Extend the annealed primer, incorporating ³²P-labeled TTP to label each strand.

6.2. Duration

5 min

- 2.1 Aliquot 2.5 μ l of termination mixes to four wells of a microtiter dish for each reaction. Add ddA to one column, ddC to the next, ddG to the third, and ddT to the fourth column, with as many rows as you have reactions.
- **2.2** Make a labeling master mix for 10–12 reactions plus 2 extra for pipetting loss.

For each reaction include:

2 μl – dT mix 1 μl 100 mM DTT 0.5–1 μl [α-³²P]-TTP

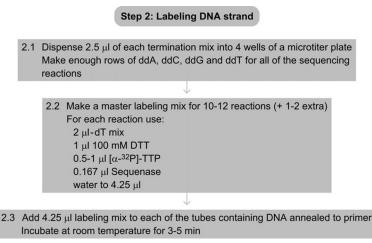


Figure 14.3 Flowchart of Step 2.

0.167 µl Sequenase

 H_2O to make 4.25 μ l

2.3 Add 4.25 μl labeling mix to each tube from Step 1 (containing DNA annealed with primer) and leave at room temperature for 3–5 min.

6.3. Tip

If a 10- μ l microtiter dish is not available, PCR plates or tubes also work, and can be placed in a thermal cycler to incubate.

6.4. Tip

Use 0.5 μ l [α -³²P]-TTP if less than 2-weeks old or 1 μ l if older. See Fig. 14.3 for the flowchart of Step 2.

7. STEP 3 EXTENSION/TERMINATION

7.1. Overview

Labeled DNAs will be extended and terminated by the incorporation of dideoxynucleotides.

7.2. Duration

30 min

3.1 Remove 3.5 μl each labeling reaction and add to each of the four termination mixes in the microtiter dish.

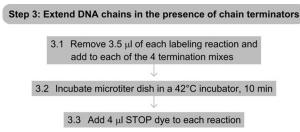


Figure 14.4 Flowchart of Step 3.

3.2 Incubate microtiter dish in a 42 °C incubator for 10 min.

3.3 Add 4 μ l STOP dye to each reaction.

See Fig. 14.4 for the flowchart of Step 3.

8. STEP 4 RESOLUTION OF LABELED PRODUCTS BY UREA-PAGE

8.1. Overview

Pour a sequencing gel and resolve the labeled DNA products by electrophoresis (see Analysis of RNA by analytical polyacrylamide gel electrophoresis).

8.2. Duration

4-8 h+overnight

- **4.1** Place sequencing plates on two stable racks. Wipe plates with 95% ethanol until dry.
- **4.2** Treat plates with a siliconizing or nonsilicone smoothing agent. Do not use excessive amounts (>1 ml per plate) as this can allow the gel to migrate upwards during electrophoresis.
- **4.3** Assemble plate sandwich as shown in Fig. 14.5. Clamp together with five large binder clips on each side on the center of the spacers.
- 4.4 Make gel solution:

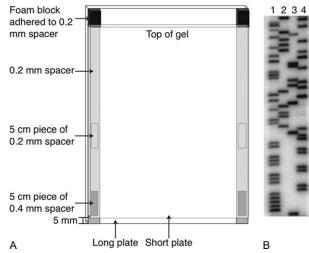
42 g Urea

 $10 \text{ ml} 10 \times \text{TBE}$

15 ml 40% acrylamide:bisacrylamide solution (19:1)

 H_2O to make 100 ml

Solution will get cold. Heat in a microwave for 10 s or on a hot plate at high temperature to bring solution just to room temperature. Do not allow the acrylamide solution to get warmer than room



temperature as this can cause premature polymerization. Invert several times or stir to dissolve urea. Filter solution through a glass fiber filter in a Buchner funnel attached to a vacuum flask and degas briefly.

4.5 Transfer the filtered gel solution to a 250 ml beaker, and add 500 μ l 10% APS and 50 μ l of TEMED. Quickly stir solution with a 60 ml syringe, and draw solution into the syringe. Pour gel from bottom to top, by placing the syringe outlet at the center of the gel sandwich and slowly pushing the gel solution between the plates. Allow gel to move across the bottom section, and then tilt the plates slightly to

facilitate gradual movement of the gel front into the plates until the syringe is almost empty. Lower the plates, refill the syringe, and continue tilting and pushing gel into plates until the gel front reaches the top of the short plate.

- **4.6** Insert the flat side of a sharktooth comb into the sandwich to make a large well ~ 0.5 cm into the gel (Combs from CBS scientific have a line marking how far to insert comb). Clamp the comb in place with three evenly spaced binder clips.
- 4.7 Rinse the syringe for future use. Allow the gel to polymerize for \sim 30 min to 1 h.
- **4.8** Remove comb and rinse well with a gentle stream of water to remove any strings of polyacrylamide that may be present.
- **4.9** Place the gel in sequencing apparatus without disturbing spacers. Fill the upper and lower reservoirs with 1× TBE and use a 60 ml syringe with a bent 18-gauge needle to rinse the well and underneath the gel to remove any bubbles.
- **4.10** Prerun gel at 65 W for 15 min to 1 h.
- 4.11 Place the microtiter dish (from Step 3.3) on a heat block at 95 °C for 3 min to denature DNA, and then place on ice. Wash well just before loading to remove any urea that has seeped out of gel, and insert sharktooth comb with teeth into the gel to ~1 mm deep.
- **4.12** Load 2 μ l per lane in the wells between the teeth of the comb. After loading the first set of four sequencing lanes, load one lane with stop dye and then continue loading the remaining sets in adjacent lanes so that loading is asymmetrical for later analysis.
- **4.13** Run the gel at 65 W for \sim 1 h 45 min to 2 h to run bromphenol blue dye near the bottom of the gel in order to resolve the first \sim 100–150 nucleotides. Run the gel longer to see sequence further from the primer, keeping in mind that Sequenase extends only \sim 600–800 nucleotides.
- **4.14** Drain buffer from the upper chamber, and lay gel sandwich on a covered area of bench.
- **4.15** Remove spacers and use a metal spatula or wedge separator to carefully pry plates apart. If the gel is stuck to both plates, leave it until it cools completely and then attempt to pry apart.
- **4.16** Place Whatman 3MM paper on the gel and press gloved hands firmly around entire surface to stick the gel to Whatman paper. Carefully remove gel/paper from plate, starting from top. Cover the gel with

plastic wrap and trim any areas that were not loaded to speed up the drying process.

- **4.17** Place the gel on a second piece of Whatman 3MM paper on the gel dryer and dry at 85 °C for 1 h, or longer, under vacuum.
- **4.18** Expose to X-ray film or phosphor screen for autoradiography overnight, and process (Fig. 14.5).

8.3. Tip

Handle the sequencing plates very carefully as they are much more easily broken than most electrophoresis plates. The slightest bump into the side of a sink or a benchtop will chip the plates, which can render the plates unusable.

8.4. Tip

Reactions may be stored at $-20 \degree C$ for several days to several weeks, but the quality of sequence may deteriorate over time. It is recommended that samples be run on a gel as soon as possible after sequencing.

8.5. Tip

To maximize time, the sequencing gel can be poured before setting up annealing reactions (Step 1), and set up and prerun during extension/termination (Step 3).

8.6. Tip

To get more information from one set of sequencing reactions, simply load the reactions multiple times. Load a set of samples and run the gel for 1-2 h, pause running and load the samples again in empty lanes, and run the bromphenol blue from these samples to the bottom of the gel. The first set of samples loaded will have sequence further from the primer, while the samples run last will have sequence starting from the primer.

8.7. Tip

Drying times may vary depending on the pump. Allow the gel to cool before removing vacuum to prevent cracking if you are unsure that gel is dry.

See Fig. 14.6 for the flowchart of Step 4.

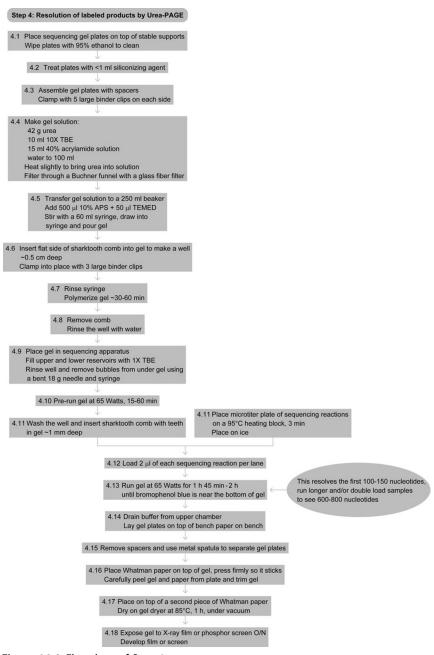


Figure 14.6 Flowchart of Step 4.

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Referenced Protocols in Methods Navigator

Isolation of plasmid DNA from bacteria Explanatory chapter: PCR -Primer design Analysis of RNA by analytical polyacrylamide gel electrophoresis CHAPTER FIFTEEN

Preparation of Fragment Libraries for Next-Generation Sequencing on the Applied Biosystems SOLiD Platform

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Abstract

The primary purpose of this protocol is to prepare genomic DNA libraries that can then be analyzed by massively parallel next-generation sequencing on the Applied Biosystems SOLiD platform. This protocol can be adapted to next-generation sequencing workflows to ultimately generate up to 1 billion 50 bp sequence tags from the ends of each of the DNA molecules in the library in a single next-generation sequencing run.

1. THEORY

The term next-generation sequencing refers to technologies that have enabled the massively parallel analysis of DNA sequence facilitated through the convergence of advancements in molecular biology, biochemistry and nucleic acid chemistry, electrical and mechanical engineering, and computational biology. Commercialized next-generation sequencing technologies are capable of generating massively parallel sequence data for tens to hundreds of millions of DNA templates simultaneously and generate more than four gigabases of sequence in a single day. These technologies have largely started to replace or significantly augment high-throughput Sanger sequencing for large-scale genomic projects, a trend that is likely to expand as the technologies continue to evolve and advance (Metzker, 2010). These technologies have created significant enthusiasm for the advent of a new era of individualized medicine.

The majority of the existing next-generation sequencing technologies employ massively parallel 'short-read' sequencing in which a short sequence (between 25 and 100 bp, depending on the technology and user options) at one or both ends of each of many hundreds of millions of DNA molecules are sequenced simultaneously. These reads are then aligned back to a reference genome assembly to allow measurement of genomic variation such as mutations and single-nucleotide polymorphisms compared to that reference genome assembly. In contrast, classical Sanger sequencing produces long reads of a single DNA molecule at a time, and high-throughput instruments allowing parallel sequencing of hundreds to thousands of sequences in parallel are in routine use. The advantage of the next-generation sequencing platforms is that the cost and throughput of sequencing is significantly lower (estimated to be 100-fold or more cheaper depending on the platform used). The major disadvantage of next-generation sequencing is the inability to generate long reads at this sequence throughput. However, this disadvantage is quickly diminishing due to increasing read-lengths and the use of matepaired libraries that can circumvent much of the need for long sequence reads.

Because these technologies produce sequence from the ends of each molecule, it is common to fragment genomic DNA to produce DNA libraries with random ends to facilitate whole genome sequence coverage. Such a library, commonly referred to as a fragment library, is the most common type of library sequenced with Next-Generation Sequencing technologies. For each of the existing Next-Generation Sequencing technologies, the protocols used to generate fragment libraries compatible with massively parallel sequencing on that platform are different, but the basic principles are the same. In each case, genomic DNA is randomly fragmented by shearing, sonication, or nebulization. These fragments are modified with adaptor sequences that contain universal amplification and/or sequencing primer sequences. The resulting library of DNA molecules represents the fragment library. In this chapter we describe in detail the procedures used to generate fragment libraries for one of the commercially available next-generation sequencing platforms - the SOLiD platform marketed by Applied Biosystems. In the accompanying explanatory chapter (see Explanatory Chapter: Next Generation Sequencing), we review concepts and procedures for this platform as well as the other commercially available ones, covering the principles behind preparing the fragment libraries for massively parallel sequencing and the sequencing chemistries.

2. EQUIPMENT

Covaris S2 Sonication System Microcentrifuge (e.g., Eppendorf model 5417R) 96-well plate PCR thermal cycler (e.g., GeneAmp[®] PCR System 9700) NanoDrop[®] ND-1000 Spectrophotometer E-Gel iBase and E-Gel Safe Imager (Invitrogen, G6466) Vortex mixer PicoFuge[®] (e.g., Stratagene) Micropipettors (2, 20, 200, and 1000 µl) Aerosol barrier pipet tips Covaris microTUBEs 0.5-ml Eppendorf LoBind centrifuge tubes 1.5-ml Eppendorf LoBind centrifuge tubes PCR strip tubes Agilent bioanalyzer 2100 instrument (optional)

3. MATERIALS

Note: Several of the components below should be purchased from New England Biolabs (NEB) and/or from Life Technologies/Applied Biosystems using the indicated catalog numbers. All oligonucleotide sequences for adaptors and primers can be ordered from commercial vendors such as Integrated DNA Technologies (IDT) or Life Technologies.

- SOLiD Library P1 adaptor plus strand (5'-CCACTACGCCTCCG CTTTCCTCTCTATGGGCAGTCGGTGAT-3')
- SOLiD Library P1 adaptor minus strand (5'-ATCACCGACTGCCC ATAGAGAGGAAAGCGGAAGGCGTAGTGGTT-3')
- SOLiD Library P2 adaptor plus strand (5'-AGAGAATGAGGAACCC GGGGCAGTT-3')
- SOLiD Library P2 adaptor minus strand (5'-CTGCCCCGGGTTCC TCATTCTCT-3')
- SOLiD Library PCR primer 1 (5'-CCACTACGCCTCCGCTTTCC TCTCTATG-3')
- SOLiD Library PCR primer 2 (5'-CTGCCCCGGGTTCCTCATT CT-3')
- NEBNext End Repair Reaction Buffer Mix $(10\times)$ (NEB, E6062A or E6062AA, or as part of E6060S/L, or see recipe below)
- NEBNext End Repair Enzyme Mix (NEB, E6061A or E6061AA, or as part of E6060S/L)
- SOLiD Library Column Purification Kit (Applied Biosystems, 4443744)
- NEBNext Quick Ligation Reaction Buffer (NEB, E6064A or E6064AA, or as part of E6060S/L, or see recipe below)
- T4 DNA Ligase (NEB, E6063A or E6063AA or as part of E6060S/L)
- LongAmp Taq 2× Master Mix (NEB, E6065A or E6065AA or as part of E6060S/L)
- E-Gels compatible with E-Gel iBase system
- Tris base
- Hydrochloric acid (HCl)
- EDTA

Nuclease-free water Isopropyl alcohol Ethylene glycol 50-bp DNA ladder (e.g., Invitrogen 10416-014)

3.1. Solutions & buffers

Step 1 $1 \times$ Low TE Buffer

Component	Final concentration	Stock	Amount
Tris–HCl (pH 8.0)	10 mM	1 M	10 ml
EDTA (pH 8.0)	0.1 mM	0.5 M	200 µl

Add water to 11

Step 2 1	$10 \times End$	Repair	Reaction	Buffer	Mix
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Component	Final concentration	Stock	Amount
Tris–HCl (pH 7.5)	500 mM	1 M	2.5 ml
MgCl ₂	100 mM	1 M	500 µl
Dithiothreitol	100 mM	1 M	500 µl
ATP	10 mM	100 mM	500 µl
dATP	4 mM	100 mM	200 µl
dCTP	4 mM	100 mM	200 µl
dGTP	4 mM	100 mM	200 µl
dTTP	4 mM	100 mM	200 µl

Add water to 5 ml

Note: This buffer can be purchased from New England Biolabs as part of the NEBNext Master Mix 3 (cat# E6060S/L) or as an individual buffer (cat# E6062A/AA)

Step 3 and also used in Preparation of Adaptor Oligos $5 \times$ NEBNext Quick Ligation
Reaction Buffer

Component	Final concentration	Stock	Amount
Tris–HCl (pH 7.6)	330 mM	1 M	1.65 ml
MgCl ₂	50 mM	1 M	250 µl
Dithiothreitol	5 mM	1 M	25 µl
ATP	5 mM	100 mM	250 µl
Polyethylene glycol (PEG 6000)	30%	100%	1.5 ml

Add water to 5 ml

Note: This buffer can be purchased from New England Biolabs as part of the NEBNext Master Mix 3 (cat# E6060S/L) or as an individual buffer (cat# E6062A/AA)

4. PROTOCOL

4.1. Preparation

Isolate relatively high-molecular-weight (>5 kb) genomic DNA to use as starting material. One to five microgram of genomic DNA is needed for this protocol (see Preparation of Genomic DNA from Bacteria, Preparation of genomic DNA from *Saccharomyces cerevisiae* or Isolation of Genomic DNA from Mammalian Cells).

Prepare double-stranded P1 and P2 adaptors at $50\,\mu\text{M}$ stock concentration.

Mix equal volumes of 125 μ M P1 adaptor plus and minus strand oligos. Add 1 part of 5× NEBNext Quick Ligation Reaction Buffer to 4 parts of the plus and minus strand oligo mixture. This will give a final concentration of 50 μ M of each oligo and 1× Quick Ligation Buffer. Hybridize oligos by running the following program in a thermal cycler:

Temperature (°C)	Time (min)
95	5
72	5
60	5
50	3
40	3
30	3
20	3
10	3
4	Hold

Store the hybridized oligos at -20 °C until ready for use.

4.2. Duration

Preparation	1–5 h
Protocol	1–2 days

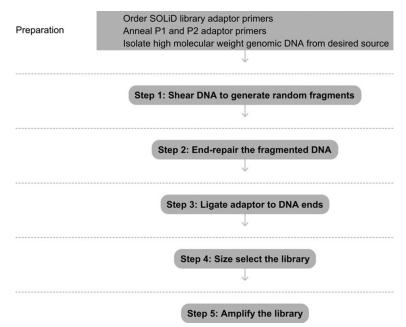


Figure 15.1 Flowchart of the complete protocol, including preparation.

4.3. Caution

Consult your institute's BioSafety Officer for proper handling of potentially infectious DNA samples and of potentially harmful reagents.

See Fig. 15.1 for the flowchart of the complete protocol.

5. STEP 1 SHEAR THE DNA TO GENERATE RANDOM FRAGMENTS

5.1. Overview

Fragment the input genomic DNA to make small DNA molecules with a modal size of $\sim 100-200$ bp with random ends. This is done by sonication in a Covaris S2 System.

5.2. Duration

30 min

1.1 Dilute the desired amount of DNA to $100 \ \mu l \text{ in } 1 \times \text{Low TE Buffer in a}$ 1.5-ml LoBind centrifuge tube.

- **1.2** Slowly transfer the DNA to a microTUBE loaded into the Covaris S2 through the presplit septa, being careful not to introduce any bubbles into the bottom of the tube.
- **1.3** Shear the DNA using the following Covaris S2 System conditions:

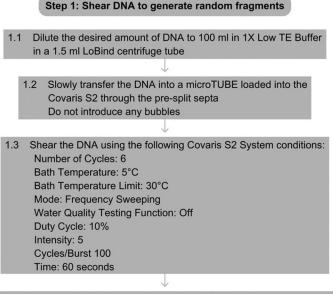
Number of cycles: 6 Bath temperature: 5 °C Bath temperature limit: 30 °C Mode: frequency sweeping Water quality testing function: off Duty cycle: 10% Intensity: 5 Cycles/burst: 100 Time: 60 s

1.4 Insert a pipette tip through the presplit septa, and slowly transfer the sheared DNA from the microTUBE to a new 1.5-ml LoBind centrifuge tube.

5.3. Caution

Follow Covaris S2 System instructions for proper setup, operation, and maintenance of the instrument.

See Fig. 15.2 for the flowchart of Step 1.



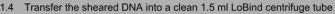


Figure 15.2 Flowchart of Step 1.

6. STEP 2 END-REPAIR THE FRAGMENTED DNA 6.1. Overview

DNA fragments generated by sonication in Step 1 will be end polished to produce a library of DNA fragments with blunt, 5'-phosphorylated ends that are ready for ligation. The end polishing is accomplished by using the T4 DNA polymerase, which can fill in 5' overhangs via its polymerase activity and recess 3' overhangs via its $3' \rightarrow 5'$ exonuclease activity. The phosphorylation of 5' ends is accomplished by T4 polynucleotide kinase.

6.2. Duration

 $\sim 1 \text{ h}$

2.1 Mix the following components in a sterile LoBind centrifuge tube:

Fragmented DNA	$\sim 100 \ \mu l$ (recovered from sonicator)
10× End Repair Reaction Buffer Mix	20 µl
End Repair Enzyme Mix	10 µl
Sterile water	to 200 µl

2.2 Incubate at 20 °C for 30 min.

- 2.3 Purify DNA using SOLiD Library Column Purification Kit:
 - **2.3.1** Add 4 volumes (in this case add 800 μ l since the reaction is 200 μ l) of Binding Buffer (B2-S) with 55% isopropanol to the reaction from above.
 - **2.3.2** Add 700 μ l of this mixture to the provided column sitting in the collection tube and let column stand for 2 min.
 - 2.3.3 Centrifuge the column at > 10 000 × g for 1 min and discard the flow-through.
 - **2.3.4** Repeat Steps 2.3.2 and 2.3.3 until all of the sample has been loaded onto the column.
 - 2.3.5 Add 650 µl of Wash Buffer (W1) to the column.
 - 2.3.6 Centrifuge the column at >10 000×g for 2 min and discard flow through. Repeat centrifugation again without adding any additional W1 buffer to remove the residual Wash Buffer.
 - **2.3.7** Air-dry the column for 2 min and transfer the column from the collection tube to a clean 1.5-ml LoBind centrifuge tube.
 - **2.3.8** Add 50 μl of Elution Buffer (E1) to the column and let stand for 2 min.

- **2.3.9** Centrifuge the column at $> 10\ 000 \times g$ for 1 min.
- **2.3.10** Add the eluate from Step 2.3.9 back to the column, and let stand for 2 min. Centrifuge the column at $> 10\ 000 \times g$ for 1 min.
- 2.3.11 Calculate the final concentration using the NanoDrop ND-1000 spectrophotometer according to manufacturer's instructions (alternatively see Explanatory Chapter: Nucleic Acid Concentration Determination).

6.3. Tip

Read the kit manual before first use to make sure all buffers are properly prepared and to get familiarized with the kit components and procedures.

6.4. Tip

The best yields are achieved when 5 μ g or less of genomic DNA were initially used. If more than this amount was used in the above steps, it is best to use multiple columns.

6.5. Tip

This is a stopping point and purified DNA can be stored at $4 \degree C$, or can be taken directly for Adaptor Ligation.

See Fig. 15.3 for the flowchart of Step 2.

7. STEP 3 ADAPTOR LIGATION

7.1. Overview

The primary goal of this step is to ligate adaptors to the DNA library. These adaptors are necessary for subsequent amplification and sequencing of the DNA library on the SOLiD instrument. Ligation of double-stranded DNA adaptors is accomplished by use of T4 DNA ligase. The double-stranded adaptors do not have 5' phosphates and contain a 5' overhang on one end to prevent ligation in the incorrect orientation. The lack of 5' phosphate leads to generation of a nick on one strand of the ligated DNA. This will be nick translated and removed during an upcoming amplification step.

7.2. Duration

30-60 min

3.1 Calculate the amount of 50 μ M stock P1 and P2 adaptors needed for ligation. The goal is to have a 30:1 ratio of each adaptor to DNA molecules in the library. Assuming a modal size of ~165 bp using the

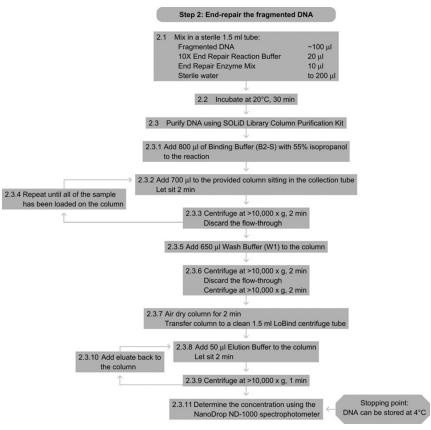


Figure 15.3 Flowchart of Step 2.

sonication conditions listed above and 660 pg per base pair of DNA, one would have 9.2 pmol μg^{-1} of DNA. Therefore, for a 30:1 ratio, we would need 5.5 μ l of the 50 μ M P1 and P2 adaptor stocks per 1 μ g of DNA library.

3.2 Mix the following components in a sterile LoBind centrifuge tube:

End repaired DNA from Step 2	Variable
5× NEBNext Quick Ligation Reaction Buffer	40 µl
P1 DNA adaptor (50 μM)	Variable
P2 DNA adaptor (50 μM)	Variable
T4 DNA ligase	10 µl
Sterile water	to 200 µl

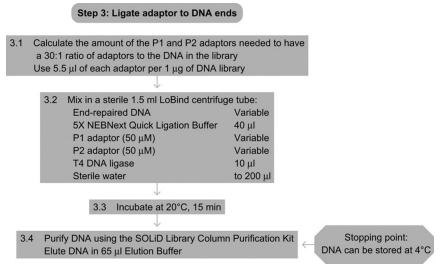


Figure 15.4 Flowchart of Step 3.

- **3.3** Incubate at 20 $^{\circ}$ C for 15 min.
- **3.4** Purify DNA as described in Step 2.3, except that the DNA should be eluted in 65 μ l instead of 50 μ l of Elution Buffer (E1).

7.3. Tip

This is a stopping point and purified DNA can be stored at $4 \degree C$, or can be taken directly for size selection.

See Fig. 15.4 for the flowchart of Step 3.

8. STEP 4 SIZE SELECTION OF LIBRARY

8.1. Overview

In this step, the adaptor-ligated library will be size-selected to $\sim 200-250$ bp size range. By doing this, we remove unligated adaptors and also select the optimal size-range for subsequent emulsion PCR and sequencing. In the procedure described below, we will use the Invitrogen E-Gel system for convenient loading, running, and elution of the size selected DNA.

8.2. Duration

1 h

4.1 Remove the combs from the E-Gel and load onto the E-Gel iBase system linked to the E-gel Safe Imager Real-Time Transilluminator according to the manufacturer's instructions.

- 4.2 Load 20 µl of the ligated, purified DNA from Step 3 into each of three wells in the top row for each sample. When loading, skip the center well, the wells to the right and left of the center well, and the two outer most wells.
- **4.3** Load 10 µl of the 50-bp ladder (0.1 µg µl⁻¹) to the center top well. Add 7 µl of water to fill the well.
- 4.4 Fill empty wells in the top row with $20 \ \mu l$ of nuclease-free water.
- **4.5** Fill the middle row of wells in the gel with 20 μ l of nuclease-free water. Add 20 μ l of nuclease free water to the center middle row of wells.
- **4.6** Run the gel with iBase program SizeSelect 2% (preprogrammed in iBase) for a total of precisely 11 min and 40 s. Monitor the gel in real-time using the real-time transilluminator. If needed, the wells in the middle row should be filled with nuclease-free water. When the 200 bp band from the ladder is at the bottom of the middle row center well but still within the well, if the run has not stopped already, then stop the run.
- **4.7** Collect the solutions from the wells, which should now contain DNA in the range of 200–250 bp, and pool them according to samples.
- **4.8** Wash each of the middle row collection wells with 25 μl of nuclease-free water and collect the wash solution and add to the retrieved material collected in Step 4.7.

8.3. Tip

Alternatively, the user could run the library on a standard 1% agarose gel along with a 50-bp DNA ladder (see Agarose Gel Electrophoresis). After running, the portion of the DNA library corresponding to 200–250 bp could be excised using a sterile blade. This excised gel fragment can be purified with a standard DNA gel extraction procedure such as those available in commercialized kits (e.g., Qiagen Gel Extraction kit). However, the procedure described above will allow more standardized generation of fragment libraries.

See Fig. 15.5 for the flowchart of Step 4.

9. STEP 5 AMPLIFICATION OF THE LIBRARY

9.1. Overview

In this step, the size-selected fragment library is amplified to a small extent to prepare for downstream emulsion PCR and sequencing steps. The initial phase of amplification involves a step for nick translation of the nick

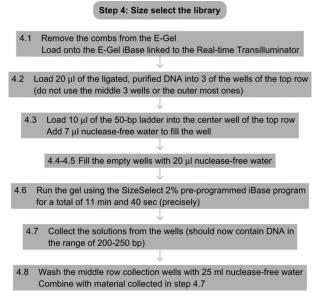


Figure 15.5 Flowchart of Step 4.

remaining at the 5' ligation junction of the adaptors. This nick will get translated to the end of the adaptor to remove the nick completely. The next steps use standard PCR with primers specific to the P1 and P2 adaptors to amplify the library. The polymerase used for amplification can also carry out the nick translation step, and therefore only one enzyme is needed.

9.2. Duration

 $\sim\!1\text{--}2$ h

5.1 Mix the following components in a sterile LoBind centrifuge tube:

DNA from size selection step	Variable
P1 adaptor primer (50 μM stock)	10 µl
P2 adaptor primer (50 µM stock)	10 µl
2× LongAmp Taq Master Mix	250 µl
Sterile water	to 500 µl

- **5.2** Alliquot $125 \,\mu$ l into each of four PCR tubes.
- **5.3** Place in a thermal cycler and carry out the following cycling program:

Cycle process	Temperature	Time	Cycles
Nick translation	72 °C	20 min	1
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	15 s	2-10 ^a
Annealing	62 °C	15 s	
Extension	70 °C	1 min	
Final extension	70 °C	5 min	1
Hold	4 °C	∞	1

^aAdjust the number of cycles based on starting amount of DNA as follows:

100 ng to 1 µg: 6-8 cycles

1-2 µg: 4-6 cycles

2-5 µg: 2-3 cycles

- 5.4 Clean up amplified library according to protocols described in Step 2.3.
- **5.5** Quantitate and quality control the generated library using an Agilent Bioanalyzer 2100 (optional), according to the manufacturer's recommendations. Samples should then be ready for sequencing workflows including emulsion PCR, bead enrichment, and sequencing. Refer to Applied Biosystems web site or consult a SOLiD Next-Generation Sequencing service facility for protocols on these subsequent steps.

9.3. Tip

These cycling conditions represent starting guidelines. It may be necessary to optimize the number of cycles to avoid overamplification while obtaining enough library material to proceed to downstream emulsion PCR steps. Amplified products should be tested for quality by analysis with an Agilent Bioanalyzer or agarose gel to ensure that the predominant products (>75%) are within the size selected range. Otherwise, it is likely that the product was overamplified producing PCR artifacts that will compromise sequence real estate.

See Fig. 15.6 for the flowchart of Step 5.

	Step 5: Amplify the libra	ігу
5.1	Mix in a sterile 1.5 ml LoBind cen Size-selected DNA P1 adaptor (50 μM) P2 adaptor (50 μM) 2X LongAmp Taq Master Mix Sterile water	trifuge tube: Variable 10 μl 10 μl 250 μl to 500 μl
	5.2 Aliquot 125 μ l into each of fou \downarrow	r PCR tubes
5.3	Perform PCR using the following 72°C 20 min 95°C 5 min 2-10 cycles of: 95°C 95°C 15 sec 62°C 15 sec 70°C 1 min 70°C 5 min 4°C Hold	cycling program
5.4 Clean up a	amplified library using the SOLiD Lib	prary Column Purification Kit
	↓ 	
	and quality control the library using a	an Agilent Bioanalyzer 2100

Figure 15.6 Flowchart of Step 5.

REFERENCES

Referenced Literature

Metzker, M. L. (2010). Sequencing technologies – The next generation. *Nature Reviews Genetics*, 11, 31–46.

Related Literature

These protocols were adapted from the Applied Biosystems protocols in the Applied Biosystems SOLiD[™] 4 System Library Preparation Guide (www.appliedbiosystems.com), as well as the protocols supplied with the NEBNext DNA Sample Prep Master Mix Set 3 (NEB E6060S/L) kit.

Referenced Protocols in Methods Navigator

Explanatory Chapter: Next Generation Sequencing Preparation of Genomic DNA from Bacteria Preparation of genomic DNA from *Saccharomyces cerevisiae* Isolation of Genomic DNA from Mammalian Cells Explanatory Chapter: Nucleic Acid Concentration Determination Agarose Gel Electrophoresis CHAPTER SIXTEEN

Explanatory Chapter: Next Generation Sequencing

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Abstract

Technological breakthroughs in sequencing technologies have driven the advancement of molecular biology and molecular genetics research. The advent of highthroughput Sanger sequencing (for information on the method, see Sanger Dideoxy Sequencing of DNA) in the mid- to late-1990s made possible the accelerated completion of the human genome project, which has since revolutionized the pace of discovery in biomedical research. Similarly, the advent of next generation sequencing is poised to revolutionize biomedical research and usher a new era of individualized, rational medicine.

The term next generation sequencing refers to technologies that have enabled the massively parallel analysis of DNA sequence facilitated through the convergence of advancements in molecular biology, nucleic acid chemistry and biochemistry, computational biology, and electrical and mechanical engineering. The current next generation sequencing technologies are capable of sequencing tens to hundreds of millions of DNA templates simultaneously and generate >4 gigabases of sequence in a single day. These technologies have largely started to replace high-throughput Sanger sequencing for large-scale genomic projects, and have created significant enthusiasm for the advent of a new era of individualized medicine.

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1. THEORY 1.1. Overview of commercialized next generation sequencing platforms

Given the promise of and the demand for next generation sequencing technologies, there has been intense competition for the development of NGS platforms. 454 life technologies, later acquired by Roche, was the first to commercially release an NGS platform. Solexa, now part of Illumina, released the next platform, with Applied Biosystems marketing the third commercialized platform, which was acquired from Agencourt. Helicos was the first company to release a single-molecule sequencing NGS platform, and more recently several new companies have entered the arena, including Complete Genomics, Pacific Biosciences, and Ion Torrents, with others likely to follow in the near future.

The major steps involved in next generation sequencing technologies that are generically applicable to all of the current technologies are library choice/construction, preparation of libraries for sequencing, and massively parallel sequencing. We first discuss some of the terminology used in Next Generation Sequencing experiments and then discuss each of these steps below and highlight the broad similarities and differences between platforms.

1.2. Terminology in next generation sequencing

- Read: refers to a single contiguous stretch of sequence returned from the instrument.
- Fragment read: a read generated from a fragment library; these reads are generated from a single end of a small DNA fragment that is typically in the order of 100–500 bps depending on the sequencing platform.
- Fragment paired-end reads: two reads generated from each end of a DNA fragment from a fragment library.
- Mate-paired read: two reads generated from each end of a large DNA fragment with a predefined size-range.
- Coverage: the average number of times each base pair in the target genome was covered by reads. For example, 30× coverage implies that each base pair in the reference genome was covered by 30 reads on average.

Two major types of libraries can be employed depending on the application: fragment libraries, and mate-paired libraries. For fragment libraries, genomic DNA from a sample is randomly fragmented to a small modal size, typically just 1–5 times the size of the sequencing platform's read length. Sequencing adaptors are then attached to these library molecules to allow sequencing from a single end of each DNA fragment in the library. The protocols used to generate such fragment libraries for the Applied Biosystems SOLiD platform are described in detail in the accompanying protocols chapter (see Preparation of fragment libraries for Next Generation Sequencing on the Applied Biosystems SOLiD platform). More recently, it has become possible to sequence from both ends of such library DNA fragments using a process referred to as fragment paired-end sequencing. Fragment libraries are ideal for analysis of single-nucleotide substitutions/variations. Each DNA fragment in the library produces a single read and multiple overlapping fragments are sequenced for each position in the genome. A coverage of $>30\times$ is usually needed to confidently distinguish true variation from sequencing errors and for robustly distinguishing homozygous and heterozygous SNPs. Additionally, fragment libraries can also provide information on genomic copy number. This can be done by taking all of the fragment library reads within fixed genomic bins and carrying out analyses to assess whether the number of reads observed is different from the number expected by random chance (e.g., Xie and Tammi, 2009), representing an extension of digital karyotyping analyses (Wang et al., 2002). Fragment libraries can also be target-enriched with microarray- or solution-based hybrid capture strategies for targeted resequencing (Albert et al., 2007; Gnirke et al., 2009). In these analyses, first, a fragment library is prepared. Next, the library is subjected to target sequence enrichment by hybridization to target-complementary oligonucleotides, called 'baits.' The oligonucleotide 'baits' can be immobilized on the surface of a microarray. Agilent and Nimblegen, among other companies, offer this as a standard or custom design product. More recently, the 'bait' oligonucleotides are synthesized in situ on microarrays, then released by cleavage from the microarray, amplified, and modified with biotin and immobilized on magnetic beads to allow solution-based capture of targets (Gnirke et al., 2009). Agilent markets this as their SureSelect solution-capture-based target enrichment process, and kits have been released for use with the SOLiD and Illumina NGS platforms.

Such approaches have allowed targeted resequencing of any portion of the genome, such as all exons in the human genome (Maher, 2009).

A mate-paired library is constructed by first randomly shearing or fragmenting genomic DNA to a modal size that is typically >1000 bps, which significantly exceeds the read lengths produced by most of the currently commercialized platforms. This library is then size-separated on a gel, and the part of the library corresponding to a specific size range, for example, 2-3 kbp, is excised and purified. These fragments are then circularized via ligation of an adapter under conditions that promote circularization of library molecules with the adaptor separating the two ends, as opposed to ligation of different library molecules together. This geometry allows generation of a library consisting of DNA fragments comprised of subfragments from the two ends of the original size-selected DNA library. The two mate-paired subfragments are then sequenced to reveal the sequences at the two ends of each 2-3 kbp library template. Because we know a priori the possible distances between the two sequences comprising the mate-paired read, after alignment to the reference genome, we can assess whether there was likely to be an amplification, deletion, or translocation between the mate-paired sequences. Similarly, the orientation of the sequences can be used to detect potential inversions. Therefore, mate-paired libraries not only provide information on single nucleotide substitutions, but also on genomic structural variation, as has been demonstrated in several recent reports (Korbel et al., 2007; McKernan et al., 2009).

With the advent of more recent NGS platforms, other library types are also possible. Pacific Biosciences, for instance, has developed ultra-long read lengths of >1000 base pairs. They have deployed these highly processive reads to generate repeated serial reads of both strands of double-strand DNA after circularization of a fragment library with hairpin adaptors ligated to each end of the fragments. The resulting 'SMRT Bell' libraries allow high-fidelity sequencing where the accuracy increases with the number of times the polymerase traverses the circularized SMRT Bell fragments (http://www.pacificbiosciences.com/). This company is also developing strobe-sequencing, where the progress of the processive polymerase in copying long template DNA is followed in an on-off periodic fashion as a way to generate several mate-tags of sequence from a long DNA template, with all tags oriented in the same direction. Complete Genomics has introduced highly complex library generation strategies involving serial cutting and circularization to fabricate DNA nanoballs for unchained ligation-based sequencing (Drmanac et al., 2010). This strategy has been used for

resequencing of whole human genomes (Roach et al., 2010). Other library configurations and geometries are likely to be introduced as the diversity of NGS platforms increases.

1.4. Preparation of libraries for sequencing on different NGS platforms

The steps involved in preparing libraries for sequencing on a specific NGS platform are usually tailor-made. For the Roche 454 and Applied Biosystems SOLiD systems, this involves emulsion PCR (Dressman et al., 2003) to amplify individual template DNA molecules clonally on the surface of a bead. In emulsion PCR, individual DNA templates are sequestered along with PCR reagents, such as nucleotide triphosphates, primers, and Taq polymerase, and a primer-coated bead within an aqueous droplet surrounded by a hydrophobic shell within an oil-in-water emulsion. Subjecting these droplets to PCR allows clonal amplification of each template DNA molecule on the surface of the bead. In the case of Roche 454, the beads are then deposited in picoliter wells of a plate. These beads serve as the substrate for sequencing on the instrument (Margulies et al., 2005). In the case of Applied Biosystems, the clonally amplified DNA molecules on the surface of the bead are end-modified and covalently and randomly attached to the surface of a glass slide (http://www.appliedbiosystems.com). This glass slide is then loaded for sequencing on the instrument. Recent improvements in the automation of the emulsion PCR process have streamlined these otherwise cumbersome steps. For the Illumina/Solexa Genome Analyzer and HiSeq platforms, DNA libraries are clonally bridge amplified to generate clonal clusters of each DNA template in situ on the surface of lanes in a flow cell (http://www.illumina.com). These flow cells are then subjected to massively parallel sequencing as described in the following section. For Helicos, library generation is simpler and does not require any clonal amplification steps. In their true single-molecule sequencing (tSMS) platform, library fragments are tailed with poly-adenosine using the terminal deoxynucleotidyl transferase (TdT) enzyme and hybridized onto oligodT primer-conjugated flow cells, which are then subjected to sequencing via extension from the oligo-dT primers (Harris et al., 2008).

1.5. Massively parallel sequencing of libraries on different NGS platforms

Each of the currently commercialized NGS platforms uses distinct chemistries to allow massively parallel sequencing of many millions to billions of template DNA molecules. The differences in chemistries confer various strengths and weaknesses to each platform. Because these technologies are rapidly evolving, we focus our discussion on the broad characteristics of the chemistries that are likely to remain stable for the currently commercialized platforms and only touch briefly on up-and-coming platforms that have not yet seen widespread adoption.

The Roche 454 system (http://www.454.com/) uses a sequenceby-synthesis strategy in which DNA templates on the surface of a bead are copied by a DNA polymerase which is forced to add a single-nucleotide species one at a time by cycling the flow of each nucleotide in turn and repeating these cycles for several iterations (Margulies et al., 2005). The pyrophosphates released by the polymerase are converted to light by a pyrophosphatase-based pyrosequencing process in which the amount of light emitted can be used to calculate the number of a specific nucleotide added at each cycle. One somewhat persistent problem with this method is that mononucleotide repeat tracks (e.g., a run of 12 adenines in a row) can lead to errors. This method allows sequencing read lengths of 400 or more base pairs in current implementations. However, the overall throughput is limited by the number of picoliter wells on a plate that can be sequenced, and this platform currently has the lowest sequence capacity per time or per dollar compared to other commercialized platforms.

Illumina (http://www.illumina.com/) and Helicos (http://www.heli cosbio.com/) also use a sequence-by-synthesis strategy, but avoid errors associated with mononucleotide runs by using fluorescently labeled reversible chain terminator nucleotides allowing controlled addition of only a single nucleotide at a time, even in stretches of mononucleotide repeats. Because these platforms halt at the addition of every single nucleotide, the coupling efficiencies become limiting and read lengths are typically less than 100 bp. In the case of Helicos, which uses tSMS technology, there appears to be a persistent issue of 'dark bases' in which the nucleotide incorporation is not associated with fluorescence generation. This will probably be an issue with other emerging tSMS platforms as well.

The Applied Biosystems (http://solid.appliedbiosystems.com) (now Life Technologies) SOLiD platform uses a sequence-by-ligation approach in which a DNA ligase, instead of a DNA polymerase, is used to assess sequence via sequential ligation of fluorescently labeled oligonucleotide probes that can interrogate each combination of two adjacent bases (16 combinations possible). However, there are only four different fluorescent dyes, and each one must interrogate one of four possible dinucleotide combinations.

Because of this, an individual ligation reaction does not uniquely identify the corresponding dinucleotide combination. Each base in the sequence is interrogated twice in this degenerate fashion and the combined data across an entire read can be deconvoluted to decipher the final sequence. The first step of the sequencing reaction is to anneal a sequencing primer to the P1 adaptor on the library template (see accompanying fragment library preparation chapter for details) and then to add a mixture of the 16 possible labeled probes. The appropriate di-base probe binds to the first and second base of the template and is ligated to the sequencing primer only if there is a perfect match. The fluorophore associated with this probe is then registered and the probe is enzymatically processed to allow sequential ligation of another probe to query the sixth and seventh bases. This process is carried out a total of ten times for the first primer. After the last ligation step, the reaction is 'reset' by denaturing and washing away the newly synthesized DNA strand from the template DNA that is covalently linked to the bead (see emulsion PCR description above). A new sequencing primer designed to hybridize to a sequence that is offset by one base from the first primer is then annealed so that the first ligation reaction stemming from this sequencing primer interrogates the last base of the adaptor sequence (position 0) and the first base of the template. This primer also goes through a total of ten ligation steps. There are a total of five different sequencing primers that each undergo ten ligation steps. This results in each base being interrogated twice and a sequencing length of 50 base pairs per read.

1.6. The near- and long-term horizon

Each of the platforms described above is routinely making advancements in sequencing throughput in terms of both time and cost per gigabase pairs of sequence output. In the meanwhile, other platforms such as Complete Genomics (http://www.completegenomics.com/), Pacific Biosciences (http://www.pacificbiosciences.com/), Ion Torrents (http://www. iontorrent.com/), and possibly several other players are preparing to enter the market. As a result of this intense competition, cost and time per gigabase pair of sequence produced is rapidly declining. Another consequence of this is that currently commercialized platforms may lose or fail to gain market share and be in danger of folding.

Nonetheless, with the rapid declines in cost, it will be possible to carry out large-scale genomic projects to elucidate novel biology at an unprecedented scale. Additionally, it may become routine to sequence entire human genomes in the context of health and disease and apply such technologies to entire populations and not just individuals. This information can serve as a source of individualized biomarkers that can provide individualized guidance for therapeutic decision-making. The key will be to develop, in parallel, the computational, biostatistical, and bioinformatics solutions to harness the power of these increasingly cost-effective technologies and deploy them not only to understand novel biology, but also to improve the practice and delivery of health care.

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Referenced Protocols in Methods Navigator

Sanger Dideoxy Sequencing of DNA

Preparation of fragment libraries for Next Generation Sequencing on the Applied Biosystems SOLiD platform



Generating Mammalian Stable Cell Lines by Electroporation

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Abstract

Expression of functional, recombinant mammalian proteins often requires expression in mammalian cells (see Single Cell Cloning of a Stable Mammalian Cell Line). If the expressed protein needs to be made frequently, it can be best to generate a stable cell line instead of performing repeated transient transfections into mammalian cells. Here, we describe a method to generate stable cell lines via electroporation followed by selection steps.

This protocol will be limited to the CHO *dhfr* – Urlaub et al. (1983) and LEC1 cell lines, which in our experience perform the best with this method.

1. THEORY

Electroporation is a popular technique to introduce foreign DNA into host cells. The DNA enters the cells following a quick electric pulse that generates temporary openings in the cell membrane. After electroporation, selection steps must be followed to allow for cells containing only the target DNA to grow.

While outside the scope of this protocol, we have listed some other cell lines and appropriate selection markers in Table 17.1. Stable cell lines also can be generated by other transfection methods (e.g., using lipid-like transfection reagents or calcium phosphate transfection. See also Rapid creation of stable mammalian cell lines for regulated expression of proteins using the Gateway[®] Recombination Cloning Technology and Flp-In T-REx[®] lines) followed by appropriate selection steps.

Two different selection methods are described here for the first round of selection immediately following electroporation. The first selection method relies on co-transfection with the pcDNA3.1 vector, which carries the *neo* gene that confers resistance to geneticin, an aminoglycoside antibiotic (Southern and Berg, 1982). It is generally not necessary for the gene of

Cell type	Transfection method	Selection markers	Round 1 selection	Days until die-off begins	Round 2 selection
CHO dhfr—	Electroporation	pSV2- <i>dhfr</i>	Alpha MEM, 5% D-FBS	12–14	Alpha MEM, 5% D-FBS, MTX (0.1–0.4 μM)
LEC1	Electroporation	pcDNA3.1, pSV2- <i>dhfr</i>	Alpha MEM, 5% FBS, 0.5 mg ml ⁻¹ geneticin	15–17	Alpha MEM, 5% D-FBS, MTX (0.025– 0.1 μM)
HEK 293 GnTi-	PEI	pcDNA3.1	DMEM:F12, 5% FBS, 2 mg ml ⁻¹ geneticin	7-10	N/A
CHO- S	PEI	pcDNA3.1	DMEM:F12, 5% FBS, 0.5 mg ml ⁻¹ geneticin	7–10	N/A

Table 17.1 Summary of selection method

interest and the selectable marker to be on the same plasmid. Positively transfected cells are selected by growth in the presence of geneticin. The second selection method relies on dihydrofolate reductase (DHFR) activity (Wigler et al., 1980) and its inhibitor methotrexate (MTX). DHFR selection works best with the cells lacking DHFR activity, such as CHO *dhfr*–. But cells having wild-type DHFR activity can also be selected with high MTX concentrations (Table 17.1). The DHFR/MTX selection step can be repeated with higher concentrations of MTX until a desirable level of protein expression is achieved.

Stable cell lines often lose their protein expression with time as a result of a heterogeneity in the transfected population of cells. A more homogeneous population of cells can be obtained by limiting dilution cloning or picking individual colonies of drug-resistant cells.

2. EQUIPMENT

Laminar flow hood CO₂ incubator Centrifuge Electroporator (BIO-RAD Gene Pulser II) Sterile 2 mm gap cuvettes Water bath (37 °C) Inverted microscope Hemacytometer 0.22-µm sterile filters T75 tissue culture flasks Sterile 50-ml polypropylene conical tubes Sterile 1.5-ml polypropylene tubes Sterile 1.5-ml polypropylene tubes Sterile 150-mm tissue culture dishes Sterile 100-mm tissue culture dishes Sterile pregreased glass cloning cylinders (Sigma C1059) Sterile pipette tips Sterile disposable pipettes

3. MATERIALS

Plasmid DNA expressing gene of interest Cell line (CHO *dhfr*-, LEC1) (ATCC) Fetal bovine serum (FBS, Invitrogen) Dialysed FBS (D-FBS, Invitrogen) Geneticin[®] (Invitrogen) Methotrexate (MTX, Sigma) Hanks Balanced Salt Solution (HBSS w/o Ca, Mg; Invitrogen 14170) HT supplement, $100 \times$ (Invitrogen) TrypLETM Express (Invitrogen) MEM α (containing Earl's Salts and L-glutamine, but no ribonucleosides, deoxyribonucleosides, NaCO₃; Invitrogen 12000) DMEM/F12 (with L-glutamine, but HEPES, NaHCO₃; no Invitrogen 12500) Freestyle[™] 293 medium (Invitrogen 12338-026) Hybridoma SFM (Invitrogen 12045) pcDNA 3.1 (Invitrogen) pSV2-dhfr (ATCC)

Note Some of the stock solutions come with the pH indicator phenol red. This supplement does not affect the application and might be useful if the researcher wishes to visualize any pH changes that can occur in the solutions over time. In the case of non- CO_2 incubators (e.g., when scaling-up the production of adherent cells in roller bottles), HEPES-buffered media can be used to keep the pH stable.

Note Catalog numbers are from the US website of Invitrogen and may differ on other local websites.

3.1. Solutions & Buffers

Step 2 Lec1 Growth Medium: Alpha MEM + 5% FBS

Add 50 ml FBS to 11 of Alpha MEM

CHO *dhfr*- Growth Medium: Alpha MEM + 5% FBS + HT solution

Add 50 ml FBS and 10 ml of 100× HT solution to 11 of Alpha MEM

Steps 4–6 50 mg ml⁻¹ Geneticin[®] (active)

Add enough active Geneticin to 50 ml of water to make a 50 mg ml⁻¹ stock solution. Each lot of Geneticin will have a different active concentration. For example, if a 5 g bottle of Geneticin has an active concentration of 750 μ g mg⁻¹ of powder, it contains an active weight of 3.75 g Geneticin. Therefore dissolve the 5 g of powder in 75 ml of water to obtain an active concentration of 50 mg ml⁻¹. Mix to dissolve, pass through a 0.2 mm filter to sterilize, and dispense aliquots into sterile tubes. Store short term at 4 °C or long term at -20 °C

Lec1 Selection Medium

Add 50 ml FBS and 10 ml of 50 mg ml⁻¹ Geneticin to 11 of Alpha MEM

CHO dhfr- Selection Medium

Add 50 ml D-FBS to 11 of Alpha MEM

Step 6 30 mM MTX

Component	Final Concentration	Stock	Amount
Methotrexate	30 mM		0.136 g
Alpha MEM			9.5 ml
NaOH	50 mM	1 M	0.5 ml

Mix well until MTX is dissolved and pass through a 0.2 μm filter to sterilize. Store in sterile aliquots at $-20~^\circ C$

1 mM MTX

Add 33.3 µl of 30 mM MTX to 1 ml Alpha MEM

Component	Final concentration	Stock	Amount
MTX	25 nM	1 mM	12.5 µl
MTX	50 nM	1 mM	25 µl
MTX	75 nM	1 mM	37.5 µl
MTX	100 nM	1 mM	50 µl
MTX	200 nM	1 mM	100 µl
MTX	300 nM	1 mM	150 µl
MTX	400 nM	1 mM	200 µl
MTX	500 nM	1 mM	250 µl
MTX	1 μΜ	1 mM	500 µl

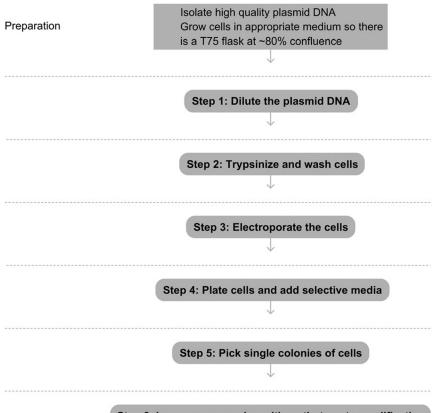
MTX selection concentrations

Add the indicated amount of MTX to 500 ml Alpha MEM containing 5% FBS

4. PROTOCOL 4.1. Preparation

Before transfection, sterile high-quality DNA must be prepared. The vector containing the appropriate expression promoter (see Molecular Cloning) and the gene of interest should be transformed into a recA- strain of *E. coli* (see Transformation of Chemically Competent *E. coli* or Transformation of *E. coli* via electroporation) and then the plasmid DNA isolated (see Isolation of plasmid DNA from bacteria). Commercially available, endotoxin-free kits for large-scale plasmid DNA isolation produce sufficiently high-quality DNA. High-quality DNA is characterized as having an OD260/280 ratio between 1.88 and 1.92, an OD260/230 ratio of 2.1–2.2, and a concentration above 0.5 mg ml⁻¹ (see Explanatory Chapter: Nucleic Acid Concentration Determination).

All steps are carried out using sterile technique in a laminar flow hood. Solutions should be sterile filtered through 0.22-µm filters. All plastic and glassware, if not purchased as sterile, should be autoclaved twice. Cell growth media are warmed to 37 °C prior to contact with cells. Before electroporating, cells should have undergone at least five passages from thawing. Grow LEC1 or CHO *dhfr*- cells to 80% confluency in a T75 flask. One T75 flask at 80% confluence yields enough cells for 1–2 electroporations. No residual trypsin can be present during electroporation.



Step 6: Increase expression with methotrexate amplification

Figure 17.1 Flowchart of the complete protocol, including preparation.

4.2. Duration

Preparation	1 week
Protocol	\sim 1–3 months

See Fig. 17.1 for the complete protocol, including preparation.

5. STEP 1 DILUTE PLASMID DNA 5.1. Overview

The proper amount of each high-quality plasmid DNA is diluted into the appropriate media for electroporation.

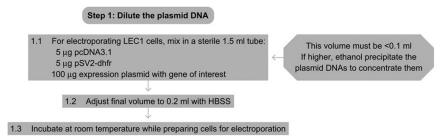


Figure 17.2 Flowchart of Step 1.

5.2. Duration

5 min

- **1.1** In a sterile tube, combine 5 μg pcDNA3.1, 5 μg pSV2-*dhfr*, and 100 μg of the expression plasmid containing the gene of interest.
- 1.2 Adjust the final volume to 0.2 ml with HBSS.
- 1.3 Incubate at room temperature while preparing cells.

5.3. Tip

These plasmids contain the selection markers for LEC1 cells. See Table 17.1 for the selection markers for other cell lines.

5.4. Tip

The total volume of the plasmid DNA mixture must be <0.1 ml, (i.e., half of the final volume). If the volume in Step 1.1 is larger than 0.1 ml, the electroporation efficiency will decrease. If this occurs, increase the starting concentrations of each DNA stock.

See Fig. 17.2 for the flowchart of Step 1.

6. STEP 2 PREPARE CELLS FOR ELECTROPORATION 6.1. Overview

Cells are trypsinized from a T75 flask, counted, and then washed into the appropriate media for electroporation.

6.2. Duration

30 min

- **2.1** Aspirate the media from the flask.
- 2.2 Add 4 ml TrypLETM Express.

- **2.3** Lay flask flat to ensure that the entire surface is covered with the liquid and incubate for 1 min at room temperature.
- 2.4 Remove TrypLETM Express solution.
- **2.5** View cells under microscope and wait until cells have released from the bottom of flask, appearing round in the microscope.
- **2.6** Immediately add 20 ml of the appropriate growth media to the flask (Table 17.2).
- **2.7** Pipette gently up and down against the cell growth surface of the flask to resuspend cells.
- 2.8 Transfer cell suspension to a 50-ml conical centrifuge tube.
- **2.9** Aliquot a small amount of suspension and count the number of cells using a hemacytometer.
- **2.10** Centrifuge the cell suspension at $200 \times g$ for 5 min at room temperature.
- 2.11 Aspirate the supernatant.
- **2.12** Add 20 ml HBSS to wash the cells. Gently pipette up and down to resuspend the cell pellet.
- **2.13** Centrifuge the cell suspension at $200 \times g$ for 5 min at room temperature. Aspirate the supernatant.
- **2.14** Add HBBS so that the final cell concentration is 20×10^6 cells ml⁻¹.
- 2.15 Gently resuspend the cell pellet with serological pipette.

Cell line	ldeal growth condition	Optimal media	Cell source
HEK293S Gnti-	Suspension	Freestyle293 'completed'	ATCC CRL3022
HEK293S Gnti-	Adherent	DMEM/F12, 5% FBS	ATCC CRL3022
HEK293T/17	Adherent	DMEM/F12, 5% FBS	ATCC CRL11268
CHO-S	Suspension	Hybridoma, 1% FBS	INVITROGEN R800-07
CHO-S	Adherent	DMEM/F12, 5% FBS	INVITROGEN R800-07
CHO dhfr-	Adherent	Alpha MEM, 5% FBS, HT	ATCC CRL-9096
LEC1	Adherent	Alpha MEM, 5% FBS	ATCC CRL-1735
COS7	Adherent	DMEM/F12, 5% FBS	ATCC CRL1651

Table 17.2 Cell lines and growth conditions

6.3. Tip

Adding the growth media at Step 2.6 serves to inactivate the enzyme in $TrypLE^{TM}$ Express.

6.4. Tip

All traces of trypsin must be removed prior to electroporation or the efficiency will be negatively affected. Do not skip the wash steps.

See Fig. 17.3 for the flowchart of Step 2.

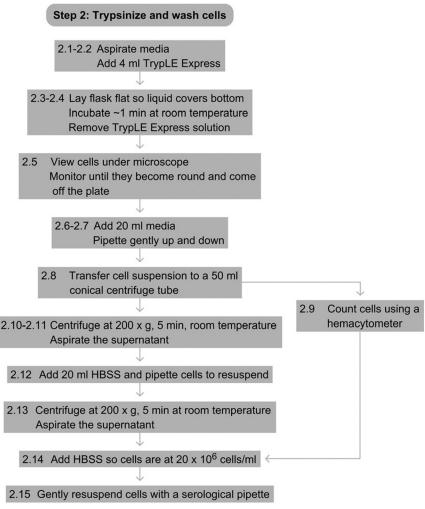


Figure 17.3 Flowchart of Step 2.

7. STEP 3 ELECTROPORATE THE CELLS

7.1. Overview

Cells are transfected using an electroporation device.

7.2. Duration

1–2 min

- **3.1** Add 0.2 ml of the cell suspension (from Step 2.15) to the diluted DNA (from Step 1.3).
- **3.2** Mix gently and transfer the mixture to a 2-mm sterile electroporation cuvette.
- **3.3** Cap the cuvette and transfer to the electroporator.
- 3.4 Electroporate cells according to the manufacturer's instructions.

7.3. Tip

If multiple samples are being processed, place the cell/DNA mixture in the cuvette on ice until all samples are ready to electroporate.

7.4. Tip

We find the optimal settings for CHO dhfr– cells and LEC1 cells to be at high capacitance, 0.174 kV, and 400 μ F. These variables may need to be optimized for each cell line.

See Fig. 17.4 for the flowchart of Step 3.

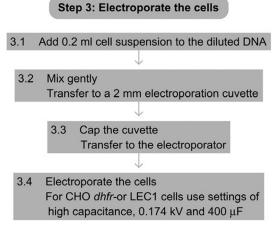


Figure 17.4 Flowchart of Step 3.

8. STEP 4 PLATING ELECTROPORATED CELLS

8.1. Overview

Electroporated cells are plated into first selection medium and returned to the incubator. Untransfected cells typically start to die off between day 12 and day 17 in culture.

8.2. Duration

 $20 \min$

12-17 days for cells to die off and resistant cells to grow.

- **4.1** Transfer the cells to a fresh sterile 1.5-ml microcentrifuge tube.
- 4.2 Adjust the total cell volume to 1 ml with HBSS and mix gently.
- **4.3** Transfer 0.1 ml of the cell suspension to a 150-mm dish containing 25 ml of appropriate round 1 selection medium (Table 17.1).
- **4.4** Place the dish in a CO_2 incubator at 37 °C.
- **4.5** Seven days after electroporation, aspirate media from the cells and refeed with 25 ml of the appropriate round 1 selection medium.
- **4.6** Aspirate and add fresh selection media every 3–4 days until there is an obvious die-off of cells as judged by the disappearance of the monolayer of cells. Viable cells will remain in selected areas, giving the appearance of individual colonies.

8.3. Tip

If all cells die or none of the cells dies, then either the quality of cells or DNA is nonideal. Thaw and culture new cells and/or prepare a higher quality batch of DNA.

8.4. Tip

Die-off is usually observed between 14 and 17 days after electroporation but that varies for different cell lines. See *Table* 1 for approximate times for several cell lines.

8.5. Tip

Multiple 150-mm dishes can be prepared by plating different amounts of the cell/ DNA mix. We usually plate 0.10, 0.15, 0.25, and 0.5 ml of the cell/DNA suspension, respectively, into four separate dishes containing 25 ml each of the appropriate first round selection media.

See Fig. 17.5 for the flowchart of Step 4.

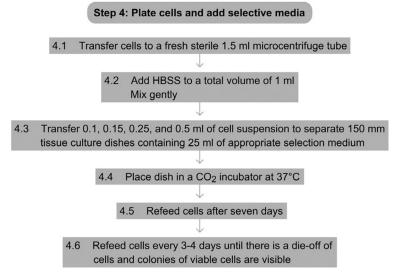


Figure 17.5 Flowchart of Step 4.

9. STEP 5 PICKING SINGLE COLONIES OF CELLS

9.1. Overview

After most cells die off, single colonies of transfected cells are isolated.

9.2. Duration

20 min active, plus time for cells to grow

- **5.1** With media still in the dish, carefully hold it above eye level and note visible colonies. They appear as round opaque areas against a clear background.
- **5.2** Mark a circle around each colony on the bottom of the dish with a black marker. Do not circle colonies that are too close together, or the cloning rings will not be able to fit around a single colony. Choose at least 24 colonies per transfection plate.
- **5.3** View the cells under the microscope to confirm that the areas you have circled are colonies of viable cells.
- 5.4 Carefully tilt the dish and remove all media with a pipette.
- **5.5** Using forceps, grease a cloning ring and place it around a colony, so as to make a well around each colony.
- **5.6** Pipette 0.2 ml TrypLETM Express into each cloning ring.

- 5.7 Cover the plate and place on microscope stage.
- **5.8** Watch for cells to round up and pull away from the plate. They will become round and highly refractile. This usually takes 30–60 s.
- **5.9** Return plate to hood. Using a pipette, gently remove TrypLETM Express from all 24 cloning rings.
- **5.10** Add 0.2 ml appropriate cell selection medium to six rings. The remaining rings remain empty.
- **5.11** Beginning with the first cylinder containing selection medium, gently pipette up and down to dislodge the cells from the surface.
- **5.12** Transfer the cell suspension to one well of a 24-well dish containing 1 ml of the appropriate selection media.
- 5.13 Transfer the cells from the next five rings containing selection media.
- **5.14** Add 0.2 ml media to another six cloning rings and transfer cells. Continue until the cells have been removed from all 24 cloning rings and transferred to a well of the 24-well plate.
- **5.15** Put the 24-well plate in the CO_2 incubator. Replace the appropriate selection media every 3 days. Assay for expression of your protein when the cells are 100% confluent.
- **5.16** Choose at least three of the highest expressing colonies. Expand cells for freezing stocks and for growth in a T75 flask to be used in a second round of selection with methotrexate.

9.3. Tip

Try to transfer the cells from all 24 cloning rings within 20 min to avoid cell death due to long exposure to TrypLETM Express or due to drying out. Alternatively, you can begin by filling all 24 cloning rings with appropriate selection media. Proceed to remove media from 6 wells and treat them with TrypLETM Express. Resuspend each well in selection media and transfer each into a separate well of a 24-well plate containing 1 ml appropriate selection media. Repeat for the next group of 6 wells until all 24 wells are collected.

9.4. Tip

Dispense 1 ml of the appropriate selection media in each well of a 24-well plate prior to placing the cloning rings on the transfected plate.

9.5. Tip

At this stage, if the level of protein expression is satisfactory, no further selection is needed and the stable cell line can be used for protein production.

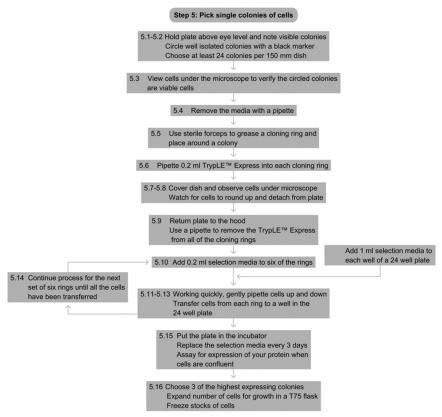


Figure 17.6 Flowchart of Step 5.

9.6. Tip

Once the cells in the 24-well plate are growing, the 150 mm master plate on which the cloning rings were placed can be discarded.

See Fig. 17.6 for the flowchart of Step 5.

10. STEP 6 METHOTREXATE AMPLIFICATION

10.1. Overview

For cells transfected with the pSV2-*dhfr* vector, target protein expression can be increased with further rounds of methotrexate selection.

10.2. Duration

Variable (weeks)

- **6.1** Grow the three highest expressing colonies in a T75 flask until they are 80% confluent.
- 6.2 Trypsinize and count the cells.
- 6.3 Resuspend the cells at a concentration of 1×10^6 cells ml⁻¹ in second round selection media without MTX (Table 17.1).
- **6.4** Prepare four 100-mm plates by adding 10 ml of second round selection media containing MTX spanning the concentration range of MTX suggested in Table 17.1.
- 6.5 Add 0.3 ml of cell suspension to each plate.
- 6.6 Gently swirl the plate to evenly distribute the cells.
- 6.7 Return dishes to the incubator and grow for 3 days.
- **6.8** Remove media from plate and replace with 10 ml of fresh second round selection media with MTX. Change the media every 3 days until cells die off. Typical times are listed in Table 17.1.
- **6.9** After most of the cells have died and colonies are visible, pick colonies as before.
- **6.10** Assay for the best expressing clones as appropriate for your protein of interest.
- 6.11 Expand and freeze the three highest expressing colonies.

10.3. Tip

Each cell type and expressed protein will have different optimal MTX concentrations. We typically plate cells at four concentrations of MTX and pick colonies from each concentration.

10.4. Tip

If no amplification is seen at the first round of MTX selection, MTX selection can be stopped.

10.5. Tip

Several vials (at least three) of positive clones should be frozen at each round of MTX selection.

10.6. Tip

If the first round of MTX selection results in colonies with higher levels of protein expression, two more rounds of MTX selection can be performed. Each round should be performed at four concentrations of MTX but using MTX concentrations that are two- to tenfold higher than those used in the previous round of selection.

See Fig. 17.7 for the flowchart of Step 6.

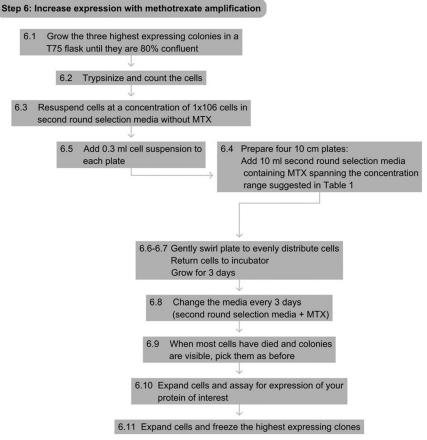


Figure 17.7 Flowchart of Step 6.

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Single Cell Cloning of a Stable Mammalian Cell Line

Rapid creation of stable mammalian cell lines for regulated expression of proteins using the Gateway[®] Recombination Cloning Technology and Flp-In T-REx[®] lines

Molecular Cloning Transformation of Chemically Competent *E. coli* Transformation of *E. coli* via electroporation Isolation of plasmid DNA from bacteria Explanatory Chapter: Nucleic Acid Concentration Determination

Transient Mammalian Cell Transfection with Polyethylenimine (PEI)

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Abstract

Standard protein expression systems, such as *E. coli*, often fail to produce folded, monodisperse, or functional eukaryotic proteins (see Small-scale Expression of Proteins in *E. coli*). The expression of these proteins is greatly benefited by using a eukaryotic system, such as mammalian cells, that contains the appropriate folding and posttranslational machinery. Here, we describe methods for both small- and large-scale transient expression in mammalian cells using polyethylenimine (PEI). We find this procedure to be more cost-effective and quicker than the more traditional route of generating stable cell lines. First, optimal transfection conditions are determined on a small-scale, using adherent cells. These conditions are then translated for use in large-scale suspension cultures. For further details on generating stable cell lines please (see Rapid creation of stable mammalian cell lines for regulated expression of proteins using the Gateway[®] Recombination Cloning Technology and FIp-In T-REx[®] lines or Generating mammalian stable cell lines by electroporation).

1. THEORY

DNA can be introduced into a host cell by transfection with polyethylenimine (PEI), a stable cationic polymer (Boussif et al., 1995). PEI condenses DNA into positively charged particles that bind to anionic cell surfaces. Consequently, the DNA:PEI complex is endocytosed by the cells and the DNA released into the cytoplasm (Sonawane et al., 2003). Our laboratory uses PEI over other cell transfection reagents because of its low cost.

This protocol is appropriate for two suspension cell lines, CHO-S and HEK 293 GnTi-. Many cell lines can be transfected successfully with PEI but in our experience these two cell lines express the highest level of protein compared to other cells.

2. EQUIPMENT

Laminar flow hood CO₂ incubator Platform shaker Centrifuge Water bath (37 °C) Inverted microscope Hemacytometer Sterile 0.22 µm filters Sterile 250-ml polypropylene centrifuge tubes Sterile 50-ml polypropylene conical tubes Sterile 50-ml polypropylene tubes Sterile 1.5-ml polypropylene tubes Sterile 6-well tissue culture plates micropipettors Sterile micropipettor tips Sterile disposable serological pipettes Sterile square polypropylene bottles

3. MATERIALS

Plasmid DNA directing your protein of interest Fetal bovine serum (FBS, Invitrogen) Polyethylenimine 'Max' (linear, MW 25 000) (Polysciences, Inc.) L-Glutamine $100 \times$ (Invitrogen) Sodium hydroxide (NaOH) MEM α (containing Earl's Salts and l-glutamine, but no ribonucleosides, deoxyribonucleosides, NaCO₃; Invitrogen 12000) DMEM/F12 (with L-glutamine, but HEPES, NaHCO₃; no Invitrogen 12500) Freestyle[™] 293 medium (Invitrogen 12338-026) FreeStyle[™] CHO-S (Invitrogen R800-07) Hybridoma SFM (Invitrogen 12045) Opti-MEM[®] (Invitrogen) HEK293S GnTI- (ATCC# CRL-3022) HEK293T/17 (ATCC# 11268)

Note Some of the stock solutions come with the pH indicator phenol red. This supplement does not affect the application and might be useful if the researcher wishes to visualize any pH changes that can occur in the solutions over time. In the case of non- CO_2 incubators (e.g., when scaling-up the production of adherent cells in roller bottles), HEPES-buffered media can be used to keep the pH stable.

Note Catalog numbers are from the US website of Invitrogen and may differ on other local websites.

3.1. Solutions & buffers

PEI 'Max'

Dissolve 1 g PEI 'Max' in 900 ml distilled water. Adjust the pH to 7.0 with 1 N NaOH. Add distilled water to 1 l

Note: Stable at least 9 months at 4 °C

Make smaller volumes depending on how much is needed. PEI 'Max' cannot be frozen!

FreeStyle[™] 293 'Completed'

Component	Stock	Amount	
FreeStyle™ 293 medium		11	
FBS	100%	10 ml	
L-Glutamine	200 mM	10 ml	

DMEM:F12, 5% FBS

Add 50 ml FBS to 11 of DMEM:F12

Alpha MEM, 5% FBS

Add 50 ml FBS to 11 of Alpha MEM

Hybridoma SFM, 1% FBS

Add 10 ml FBS to 11 of Hybridoma SFM

4. PROTOCOL 4.1. Preparation

Before transfection, sterile high-quality DNA must be prepared. The vector containing the appropriate expression promoter (see Molecular Cloning) and the gene of interest should be transformed into a recA- strain of *E. coli* (see Transformation of Chemically Competent *E. coli* or Transformation of *E. coli* via electroporation) and then the plasmid DNA isolated (see Isolation of plasmid DNA from bacteria). Commercially available, endotoxin-free kits for large-scale plasmid DNA isolation produce sufficiently high-quality DNA. High-quality DNA is characterized as having an OD260/280 ratio between 1.88 and 1.92, an OD260/230 ratio of 2.1–2.2, and a concentration above 0.5 mg ml⁻¹ (see Explanatory Chapter: Nucleic Acid Concentration Determination).

Cells must be greater than five passages from liquid nitrogen, adapted to media, free of mycoplasma contamination, and single cells if in suspension culture. All steps are carried out using sterile technique in a laminar flow hood. Solutions should be sterile-filtered through $0.22-\mu m$ filters. All plastic and glassware, if not purchased as sterile, should be double autoclaved. Cell growth media should be warmed to 37 °C prior to contact with cells. Different growth media are needed for each cell line and growth condition. These media are listed in Table 18.1. Each time the protocol says to use 'media,' use the appropriate media as outlined in Table 1 for the specific cell line and growth conditions. Cell type used depends on specific needs of protein of interest. In our laboratory, optimized protein expression conditions determined for adherent cells translate well into large-scale suspension conditions for the same cell line.

Cell line	Growth type	Ideal medium	Cell source
HEK293S GnTI-	Suspension	Freestyle™ 293 'completed'	ATCC# CRL-3022
HEK293S GnTI-	Adherent	DMEM:F12, 5% FBS	ATCC# CRL-3022
HEK293T/17	Adherent	DMEM:F12, 5% FBS	ATCC# CRL-11268
CHO-S	Suspension	Hybridoma SFM, 1% FBS	Invitrogen R800-07
CHO-S	Adherent	DMEM:F12, 5% FBS	Invitrogen R800-07

Table 18.1 Cells: Growth characteristics and medium

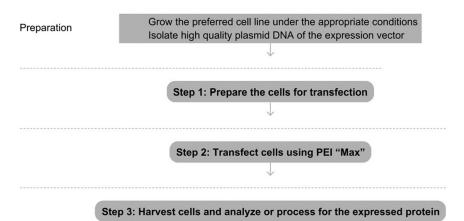


Figure 18.1 Flowchart of the complete protocol, including preparation.

4.2. Duration

Preparation	1 week
Protocol	1-2 weeks

See Fig. 18.1 for the flowchart of the complete protocol.

5. STEP 1 SMALL-SCALE TRANSIENT TRANSFECTION

5.1. Overview

This step will prescreen a variety of transfection conditions including media, cell type, ratio of PEI to DNA, and expression time to maximize protein expression before scaling up to a large-scale transfection.

5.2. Duration

5 days

Note

Transfection must be done in the absence of antibiotics.

6. STEP 1.1 SEED ADHERENT CELLS FOR TRANSFECTION 6.1. Overview

The appropriate amount of cells are transferred to a 6-well dish and allowed to become adherent.

6.2. Duration

1.5 days

- 1.1.1 Wash the cells with PBS, trypsinize, add 10 ml DMEM:F12+5% FBS, and gently pipet the cells several times to ensure an even suspension before counting. Transfer the cells to a 15-ml sterile centrifuge tube. Count the cells using a hemacytometer.
- **1.1.2** For each transfection condition to be tested, aliquot 3×10^5 cells into a sterile centrifuge tube.
- **1.1.3** Spin the cells at 200 × g at room temperature for 5 min. Aspirate supernatant.
- **1.1.4** Add DMEM:F12 + 5% FBS to the cell pellet to a final concentration of 3×10^5 cells ml⁻¹.
- **1.1.5** Resuspend the cells with a serological pipette.
- **1.1.6** Add 2 ml of DMEM:F12+5% FBS to each well of a 6-well plate.
- 1.1.7 Transfer 1 ml of the cell suspension to each well.
- **1.1.8** Place dish in the 37 $^{\circ}$ C incubator, 5% CO₂.
- 1.1.9 After 24 h, remove the media from each well.
- **1.1.10** Add 2.7 ml of fresh DMEM:F12+5% FBS to each well.
- **1.1.11** Return the dish to incubator.

See Fig. 18.2 for the flowchart of Step 1.1.

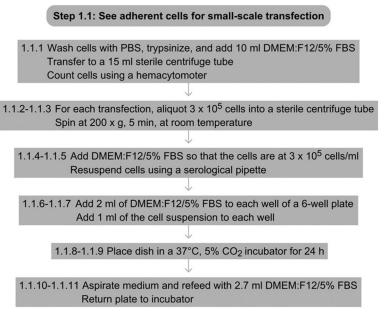


Figure 18.2 Flowchart of Step 1.1.

7. STEP 1.2 TRANSIENTLY TRANSFECT CELLS

7.1. Overview

Cells are transfected by adding DNA and PEI 'Max' to the cells. The PEI-DNA mixture is prepared and added to the cells on the same day as changing the media.

7.2. Duration

45 min active time; 4 days total

- **1.2.1** Dilute 9 μg of PEI 'Max' into a total volume of 150 μl of Opti-MEM. The amount of PEI can be varied.
- **1.2.2** Dilute 3 µg of DNA into a total volume of 150 µl of Opti-MEM.
- **1.2.3** Add the diluted PEI 'Max' to the diluted DNA.
- **1.2.4** Incubate the mixture at room temperature for 30 min.
- **1.2.5** Carefully add the PEI-DNA mixture to a well of adherent cells. Take care to gently pipette the solution down the side of the well and not on top of the cells, so as not to disrupt the adherent cells.
- **1.2.6** Return the dish to the 5% CO_2 incubator.

7.3. Tip

Opti-MEM can be replaced with Hybridoma Media without serum.

7.4. Tip

The protocol outlined here uses a 3:1 ratio of PEI to DNA (w/w). We have found this ratio to be optimal for most genes we have expressed. However, this ratio should be screened for each gene tested. We routinely screen ratios between 1:1 and 5:1.

7.5. Tip

In general, use $1 \mu g$ of DNA per 1 ml of culture to be transfected. PEI and DNA should each be diluted into 1/20 of the total culture volume before being combined.

7.6. Tip

Small-scale transfections can be performed with suspension-adapted cells. The protocol for small scale is essentially the same. For suspension culture, we use square plastic bottles designed to hold 125 ml; however, we add only 5–12 ml of cell medium for optimal aeration and agitation.

See Fig. 18.3 for the flowchart of Step 1.2.

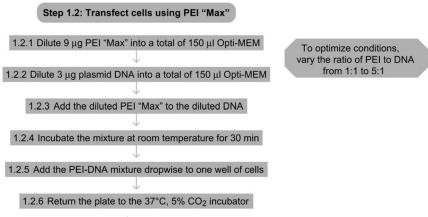


Figure 18.3 Flowchart of Step 1.2.

8. STEP 1.3 HARVEST CELLS AND ANALYZE PROTEIN EXPRESSION

8.1. Overview

Harvest and lyse cells (see Lysis of mammalian and Sf9 cells). Analyze protein expression by Western blotting (see Western Blotting using Chemiluminescent Substrates).

8.2. Duration

1-2 days

- **1.3.1** 96 h after transfection, collect samples to be analyzed. For secreted proteins collect and save the media. For membrane or intracellular proteins, remove media. Wash the cells with PBS and lyse the cells.
- **1.3.2** Analyze protein expression by Western blotting or ELISA as appropriate.

8.3. Tip

HEK293 GnTT cells can be resuspended by pipetting gently up and down with a serological pipette. CHO-S cells adhere more tightly to the dish and need to be manually resuspended with a cell scraper.

8.4. Tip

Some protocols call for trypsin digestion to detach the cells from the dish. This can be avoided by manual scraping of the cells. Trypsin could degrade the expressed protein if it is a membrane protein.

Step 1.3: Harvest cells and analyze for the expressed protein

```
1.3.1 After 96 h, harvest and lyse the cells
(for an intracellular or membrane protein)
Collect the medium for a secreted protein
```

After optimizing the ratio of PEI to DNA, vary the time from 24 to 96 h

1.3.2 Analyze for protein expression by Western blotting Figure 18.4 Flowchart of Step 1.3.

8.5. Tip

Once a cell type, media, and optimal ratio of PEI to DNA are established, this protocol can be repeated and samples taken between 24 and 96 h posttransfection to optimize the length of expression.

See Fig. 18.4 for the flowchart of Step 1.3.

9. STEP 2 LARGE-SCALE TRANSIENT TRANSFECTION OF SUSPENSION CELLS

9.1. Overview

Preparative scale expression of protein in suspension culture. For this protocol, the parameters optimized in Step 1 are expanded to larger volume cultures. You will need 400 ml of cells at a density of $2-3 \times 10^6$ cells ml⁻¹.

9.2. Duration

4-8 days

10. STEP 2.1 PREPARE THE CELLS TO BE TRANSFECTED 10.1. Overview

Harvest and count suspension cells to ensure that they are at the proper density. Centrifuge the cells and resuspend them in a total of 360 ml of fresh suspension growth medium.

10.2. Duration

30 min

- **2.1.1** Grow 400 ml of cells in the appropriate suspension growth medium to a density between 2 and 3×10^6 cells ml⁻¹.
- **2.1.2** Transfer the cell suspension to sterile centrifuge bottles.

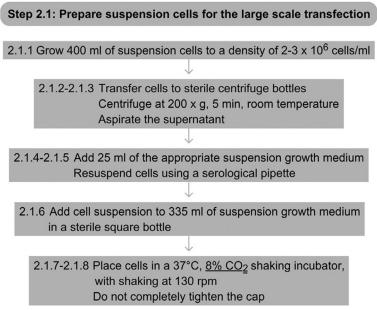


Figure 18.5 Flowchart of Step 2.1.

- **2.1.3** Spin the cells at $200 \times g$ at room temperature for 5 min. Aspirate the supernatant.
- 2.1.4 Add 25 ml of the appropriate fresh suspension growth medium.
- 2.1.5 Gently resuspend the cells with a serological pipette.
- **2.1.6** Add the cells to 335 ml of fresh suspension growth medium in a sterile square bottle.
- **2.1.7** Do not tighten the bottle cap all the way.
- **2.1.8** Place the cells into a 37 °C incubator shaker set at 8% CO₂, with shaking at 130 rpm.

See Fig. 18.5 for the flowchart of Step 2.1.

11. STEP 2.2 TRANSFECT CELLS

11.1. Overview

Transfect the cells using the optimal ratio or PEI to DNA as determined above.

11.2. Duration

45 min active time, expression time as determined above

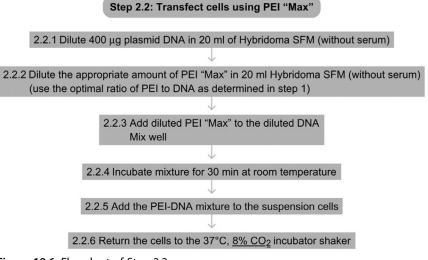


Figure 18.6 Flowchart of Step 2.2.

- **2.2.1** Dilute 400 μg of DNA in a total volume of 20 ml of Hybridoma SFM (without serum).
- **2.2.2** Dilute the appropriate amount of PEI 'Max,' as determined in Step 1, into a total volume of 20 ml of Hybridoma SFM (without serum).
- 2.2.3 Add the diluted PEI 'Max' to the diluted DNA and mix.
- **2.2.4** Incubate the mixture at room temperature for 30 min.
- 2.2.5 Add the PEI-DNA mixture to the suspension cells from Step 2.1.8.

2.2.6 Return the cells to the 37 °C incubator shaker, shaking at 130 rpm. See Fig. 18.6 for the flowchart of Step 2.2.

12. STEP 2.3 HARVEST CELLS AND PROCESS PROTEIN AS NEEDED

12.1. Overview

Harvest the cells (or medium for a secreted protein). Purify the protein or process as needed for downstream applications (see Salting out of proteins using ammonium sulfate precipitation, Using ion exchange chromatography to purify a recombinantly expressed protein, Gel filtration chromatography (Size exclusion chromatography) of proteins, Use and Application of Hydrophobic Interaction Chromatography for Protein Purification or Hydroxyapatite Chromatography: Purification Strategies for Recombinant Proteins, or look up the chapters on affinity purification if tags have been added to the protein: Purification of His-tagged proteins, Affinity purification of a recombinant protein expressed as a fusion with the maltose-binding protein (MBP) tag, Purification of GST-tagged proteins, Protein Affinity Purification using Intein/Chitin Binding Protein Tags, Immunoaffinity purification of proteins or Strep-tagged protein purification).

12.2. Duration

About 1 h

- **2.3.1** After the appropriate amount of time, as determined in Step 1, centrifuge the cells at $200 \times g$ for 5 min at room temperature.
- **2.3.2** If the protein is secreted, collect the medium. Centrifuge the cells at $200 \times g$ for 5 min at room temperature and sterile-filter the medium through a 0.22- μ m filter. Add sodium azide to 0.02%. The medium can be stored at 4 °C for months until needed.
- **2.3.3** If the protein is to be purified, the cell pellet should be flash-frozen in liquid nitrogen and stored at -80 °C until needed.

12.3. Tip

In suspension cultures, single cells are transfected more efficiently than cells that have clumped together during growth. Growth conditions may need to be optimized for single cell growth.

12.4. Tip

Square bottles should be autoclaved in two consecutive dry cycles (45 min each, dry 15 min each) with the lids as loose as possible without falling off. They should be allowed to cool completely in the laminar flow hood before tightening the lids. If the bottles collapse inward, cells will not grow well.

12.5. Tip

To generate more protein, we have found that 24 h posttransfection the cells can be diluted between 1:2 and 1:5 in the appropriate media. The effect of dilution and optimal dilution ratios should be determined empirically.

See Fig. 18.7 for the flowchart of Step 2.3.

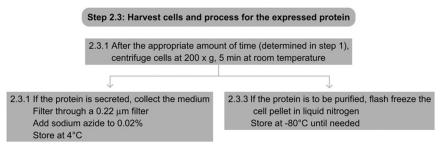


Figure 18.7 Flowchart of Step 2.3.

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Strep-tagged protein purification



Site-Directed Mutagenesis

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Abstract

Site-directed mutagenesis is a PCR-based method to mutate specified nucleotides of a sequence within a plasmid vector. This technique allows one to study the relative importance of a particular amino acid for protein structure and function. Typical mutations are designed to disrupt or map protein–protein interactions, mimic or block posttranslational modifications, or to silence enzymatic activity. Alternatively, noncoding changes are often used to generate rescue constructs that are resistant to knockdown via RNAi.

1. THEORY

Forward and reverse primers are centered on the desired base change(s) and overlap completely in both directions. Extension of these primers around the entire plasmid creates a copy of the template with the inserted mutation. This mutated plasmid then serves as a template for further PCR amplification. Since plasmids grown in *E. coli* are *dam* methylated, the restriction endonuclease Dpn I, which is specific to methylated and hemimethylated DNA, is used to digest the original parent template. The remaining plasmid is then transformed into *E. coli* for selection and DNA isolation.

2. EQUIPMENT

PCR Thermocycler Water bath (37 and 42 °C) Shaking incubator Incubator Micropipettors Micropipettor tips 0.2-ml thin-walled PCR tubes 1.5-ml polypropylene tubes 15-ml sterile snap-cap tubes Petri plates

3. MATERIALS

PCR primers Plasmid DNA (with desired sequence to be mutated) dNTPs (100 mM each of dATP, dCTP, dGTP, and dTTP) 10× High-fidelity DNA polymerase buffer High-fidelity *Pfu*-based DNA polymerase Ultrapure water DpnI Chemically competent cells (e.g., XL 10-Gold ultracompentent cells, Stratagene) Lysogeny Broth (LB media) Petri plates

3.1. Solutions & buffers

Component	Final Concentration	Stock	Amount	
dATP	10 mM	100 mM	100 µl	
dCTP	10 mM	100 mM	100 µl	
dGTP	10 mM	100 mM	100 µl	
dTTP	10 mM	100 mM	100 µl	

Step 1 10 mM dNTP mix

Add purified water to 1 ml

4. PROTOCOL

4.1. Preparation

PCR primers must be designed and synthesized prior to starting this protocol (see Explanatory chapter: PCR -Primer design).

Design forward and reverse primers containing the desired mutation(s). Primers should be 25–45 nucleotides long leaving at least 10–15 nucleotides of perfect matching sequence at both ends. In general, more point mutations being made will require longer primers. For example, a single base change probably requires only 25-mers. If three bases are to be mutated, the primers should be about 35–38 bases in length. Ideally, terminate primers with C or G and maintain a minimum GC content of 40%. Additional primers are needed for sequencing through the insert in order to verify mutations made and check for nonspecific mutations.

4.2. Duration

Preparation	Variable
Protocol	5–6 h+overnight incubation
	2 h+DNA sequencing

See Fig. 19.1 for the flowchart of the complete protocol.

5. STEP 1 SETTING UP AND RUN THE PCR 5.1. Overview

Set up and run the PCR using the primers containing the mutation(s) and a high-fidelity thermostable DNA polymerase.

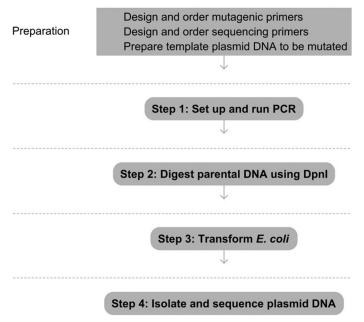


Figure 19.1 Flowchart of the complete protocol, including preparation.

5.2. Duration

about 2 h

- **1.1** Set up the reaction mixture on ice. Add to a 0.2-ml thin-walled PCR tube:
 - 1 μ l template (10 ng μ l⁻¹)
 - 5 μ l 10× Polymerase Buffer
 - 2.5 μ l Forward primer (50 ng μ l⁻¹)
 - 2.5 μ l Reverse primer (50 ng μ l⁻¹)
 - $1 \mu l dNTP mix (10 mM)$
 - 37.5 μ l purified water
 - 0.5 µl DNA Polymerase
- **1.2** Run the PCR with the following cycling parameters:
 - At 98 °C for 30 s (Initial denaturation of the template)
 - Repeat for 18 cycles:
 - At 98 °C for 10 s (Denature)
 - At 60 °C for 30 s (Anneal)
 - At 68 °C for 1 min per kb length of entire plasmid (Extension)

5.3. Tip

Add the enzyme last, immediately before starting the thermocycler. If setting up more than one reaction, make a master mix and dispense into tubes already containing the specific primers and/or template.

5.4. Tip

The annealing temperature can vary from 55 to 60 $^{\circ}C$ according to the primer $T_{\rm m}$.

5.5. Tip

The extension temperature for Taq DNA polymerase is $72 \,^{\circ}C$. However, the recommended extension temperature for many high-fidelity polymerases is $68 \,^{\circ}C$. Check the product information sheet of the high-fidelity thermostable DNA polymerase being used.

See Fig. 19.2 for the flowchart of Steps 1 and 2.

	Step 7	1: Set up and run PCR	
1.1	Template D 10X Polym Primer 1 (5 Primer 2 (5 dNTP mix (Ultrapure w	0 ng/µl) (10 mM)	1 μl 5 μl 2.5 μl 2.5 μl 1 μl 37.5 μl 0.5 μl
		\downarrow	
	Run PCR usi 98°C 18 cycles of: 98°C 60°C 68°C	ing the following cycling co 30 sec 10 sec 30 sec 1 min per kb of plasmid lei	
		est parental DNA using D ld 1 μl Dpnl (10-20 units)	pnl

2.2 Incubate at 37°C, 1-2 h

Figure 19.2 Flowchart of Steps 1 and 2.

6. STEP 2 DIGESTION OF TEMPLATE DNA

6.1. Overview

Parental strands of DNA will be digested using DpnI, which cuts methylated and hemimethylated DNA.

6.2. Duration

1–2 h

- **2.1** Add 1 µl of Dpn I restriction enzyme (10–20 units).
- **2.2** Incubate at 37 $^{\circ}$ C for 1–2 h.

7. STEP 3 TRANSFORMATION INTO CHEMICALLY COMPETENT E. COLI

7.1. Overview

The mutated plasmid is transferred into chemically competent E. coli via heat-shock (see Transformation of Chemically Competent E. coli, or alternatively see Transformation of E. coli via electroporation if you are using electrocompetent cells). The transformed bacteria are then grown on media containing the appropriate antibiotic for the plasmid.

7.2. Duration

2 h+overnight incubation

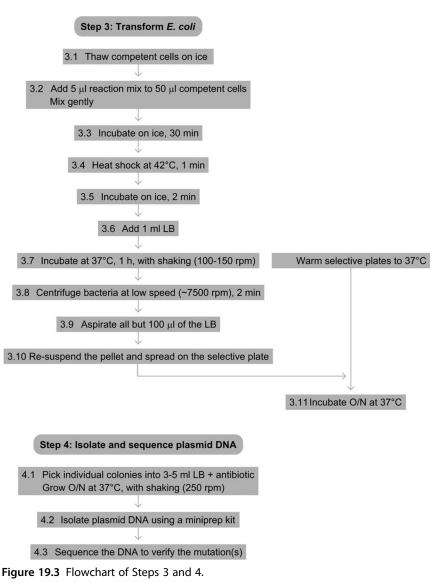
- **3.1** Let the competent cells thaw on ice.
- **3.2** Add 5 μ l of the reaction to 50 μ l of chemically competent cells. Mix gently to avoid damaging the cells (do not pipette up and down).
- **3.3** Incubate on ice for 30 min.
- **3.4** Heat shock at $42 \degree C$ for 1 min.
- **3.5** Incubate tube on ice for 2 min.
- 3.6 Add 1 ml of room temperature LB (or other medium, e.g., SOC).
- **3.7** Incubate at 37 $^{\circ}$ C for 1 h with shaking (100–150 rpm).
- 3.8 Centrifuge bacteria at low speed (~7500 rpm) for 2 min.
- **3.9** Aspirate all but $100 \ \mu l$ of the LB.
- **3.10** Carefully resuspend the pellet and spread all the 100 μl evenly onto a selective plate.
- 3.11 Incubate overnight at 37 °C with the plate turned upside down.

7.3. Tip

XL 10-Gold ultracompetent cells are recommended for their high transformation efficiency.

7.4. Tip

Check the supplier's instructions for the optimal length of time for the heat shock. See Fig. 19.3 for the flowchart of Steps 3 and 4.



8. STEP 4 COLONY SCREENING

8.1. Overview

Pick single colonies, grow overnight, and isolate plasmid DNA using a miniprep kit (alternatively see Isolation of plasmid DNA from bacteria). Sequence the DNA to verify that the mutations have been made.

8.2. Duration

15 min+overnight growth

- 1 h+DNA sequencing
- **4.1** Pick single colonies into 3–5 ml LB + antibiotic in a 14-ml snap-cap tube. Grow at 37 °C overnight with shaking (250 rpm).
- **4.2** Isolate plasmid DNA using a plasmid miniprep kit according to the manufacturer's instructions.
- **4.3** Sequence the DNA to verify that appropriate base changes have been made.

8.3. Tip

Since PCR is used to amplify the whole plasmid, it is important to sequence the entire insert in order to check for PCR-induced mutations. Using a high-fidelity polymerase will minimize this possibility. Alternatively, one could sequence the smaller region containing the desired mutations and subclone this region back into the original vector.

REFERENCES

Referenced Protocols in Methods Navigator Explanatory chapter: PCR -Primer design Transformation of Chemically Competent *E. coli* Transformation of *E. coli* via electroporation Isolation of plasmid DNA from bacteria

PCR-Based Random Mutagenesis

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CHAPTER TWENTY

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Abstract

Random PCR mutagenesis enables the rapid and inexpensive construction of a library of mutant genetic elements.

1. THEORY

PCR is routinely performed with standard polymerases, yielding a final PCR product with few errors. Although polymerases vary in fidelity, the inherent mutation rate of PCR is insufficient to generate the high frequency of mutations necessary to generate a mutant library. The addition of manganese to the PCR reaction reduces polymerase fidelity, resulting in error-prone PCR and an increased substitution frequency in the PCR products. By performing error-prone PCR on a genetic element of interest and cloning PCR products, a mutant library can be constructed and characterized by sequencing. Manganese-induced error-prone PCR favors transitions (purine-to-purine and pyrimidine-to-pyrimidine mutations); should a broad spectrum of mutations be desired, the relative concentrations of the dNTPs may be altered (Cadwell and Joyce, 1992). Additionally, error-prone PCR may generate no or multiple substitutions in a given PCR product, and additional screening and/or subcloning may be required to isolate single mutations.

2. EQUIPMENT

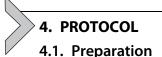
PCR thermocycler Micropipettors Micropipettor tips 0.2-ml thin-walled PCR tubes or plates

3. MATERIALS

PCR Primers Template DNA Thermostable DNA polymerase 10× Polymerase Buffer (supplied with enzyme) dNTP mix (or dATP, dTTP, dCTP, and dGTP) Manganese chloride (MnCl₂) Sterile water Step 1 dNTP mix

Component	Final concentration	Stock	Amount
dATP	2.0 mM	100 mM	20 µl
dTTP	2.0 mM	100 mM	20 µl
dCTP	2.0 mM	100 mM	20 µl
dGTP	2.0 mM	100 mM	20 µl
H ₂ O			920 µl
Step 3 MnCl ₂			
Component	Final concentration	Stock	Amount
MnCl ₂	500 μM	1 M	50 µl
H ₂ O			9.95 ml

3.1. Solutions & buffers



Prepare template DNA to be mutagenized. Appropriate templates include genomic DNA, plasmids, PCR products, etc.

PCR primers should be designed and optimized prior to beginning this protocol (see Explanatory chapter: PCR -Primer design). The primers should flank only the region to be mutagenized as mutations will occur with equal frequency throughout the PCR product. A nonmutagenic PCR should be performed to optimize annealing temperatures and extension times for each oligo pair to be used in this protocol.

4.2. Duration

Preparation	About 1 day
Protocol	About 2–3 h

See Fig. 20.1 for the flowchart of the complete protocol.

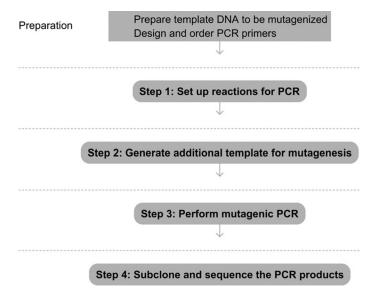


Figure 20.1 Flowchart of the complete protocol, including preparation.



The PCR reaction is assembled.

5.2. Duration

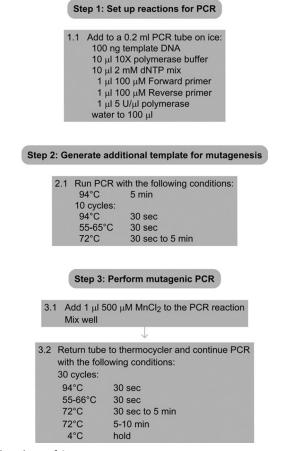
5 min

1.1 Place a PCR tube on ice. Add the following to the PCR tube: 100 ng Template DNA (in a volume of 69 μl or less) 10 μl 10× Polymerase buffer 10 μl 2 mM dNTP Mix 1 μl 100 μM Forward primer 1 μl 100 μM Reverse primer 1 μl 5 u μl⁻¹ Polymerase H₂O to 100 μl

5.3. Tip

If the volume of template DNA is large, it should be resuspended in H_2O , not TE, to avoid significantly altering the concentration of salt and EDTA in the reaction.

See Fig. 20.2 for the flowchart of Steps 1–3.





6. STEP 2 GENERATION OF ADDITIONAL TEMPLATE FOR MUTAGENESIS

6.1. Overview

The template DNA is amplified in a normal PCR to generate a large pool of template DNA for mutagenesis.

6.2. Duration

15 min – 1 h

2.1 Put the PCR mixture in the thermocycler and run the following program:

At 94 °C for	5 min
10 cycles:	
At 94 °C	for 30 s
At 55–65 °C for	30 s
At 72 °C	for 30 s – 5 min

6.3. Tip

The annealing temperature $(55-65 \,^{\circ} C)$ must be empirically determined prior to the mutagenic PCR. The extension time (72 $^{\circ}C$ incubation) is specific to each enzyme, but 1 min kb⁻¹ is a good estimate; extension times should not fall below 30 s regardless of the amplicon size.

7. STEP 3 MUTAGENIC PCR

7.1. Overview

The PCR is continued in the presence of manganese to increase the mutation rate.

7.2. Duration

1–4 h

3.1 Add 1 μ l 500 μ M MnCl₂ to the PCR reaction; mix well.

3.2 Put the mixture in the thermocycler and run the following program:

30 cycles:	
At 94 °C	for 30 s
At 55–65 °C	for 30 s
At 72 °C	for 30 s – 5 min
At 72 °C	for 5–10 min
4 °C	hold

7.3. Tip

The conditions for the two sets of PCR should be the same.

8. STEP 4 SUBCLONE AND SEQUENCE THE PCR PRODUCTS

8.1. Overview

The PCR products from the mutagenic PCR will be subcloned into a plasmid vector (see Molecular Cloning), transformed into *E. coli* (see Transformation of Chemically Competent *E. coli* or Transformation of *E. coli* via electroporation), and DNA from individual colonies will be sequenced (see Isolation of plasmid DNA from bacteria).

8.2. Duration

3-6 days

- **4.1** Run 10 μl of the PCR products on an agarose gel to determine the purity of the products (see Agarose Gel Electrophoresis).
- **4.2** If there is a single band of the correct size, use a PCR purification kit to clean it up. If not, run the remainder of the reaction on an agarose gel and isolate the correct band using a DNA gel extraction kit.
- **4.3** Digest the purified PCR product and vector DNA with the appropriate restriction enyzmes. Add to a 1.5-ml microcentrifuge tube:

DNA	1–2 µg
$10 \times$ restriction enzyme buffer	5 µl
$10 \times BSA$ (if required)	5 µl
Restriction enzyme(s)	1 µl
Water	up to 50 µl

Incubate at 37 °C (or appropriate temperature) for 1–2 h.

4.4 Run the digested DNAs on an agarose gel and isolate them using a DNA gel extraction kit.

4.5 Ligate the digested PCR product and vector. Add to a 1.5-ml micro-centrifuge tube:

$10 \times$ T4 DNA ligase buffer	2 µl
Digested vector DNA	50–100 ng
Digested PCR products	100–300 ng
T4 DNA ligase	1 µl
Water	up to 20 µl

Incubate at room temperature for 1–2 h or overnight at 16 °C.

- **4.6** Transform competent *E. coli* and plate cells on selective plates. Grow overnight at 37 °C.
- **4.7** Pick single colonies into 5 ml LB containing antibiotic and grow overnight at 37 °C, with shaking.
- **4.8** Isolate plasmid DNA using a Plasmid miniprep kit and sequence the inserts to determine whether they contain mutations.

8.3. Tip

Digests of the purified PCR product and vector DNA should be set up in separate tubes.

8.4. Tip

BSA is required by some restriction enzymes. Consult the manufacturer's product material to determine whether it is necessary. BSA does not exhibit an inhibitory effect on restriction enzymes and may be routinely included in digests if so desired.

8.5. Tip

The incubation time and temperature may need to be adjusted depending on the enzyme(s) to be used. Consult the manufacturer's product material for the optimal conditions for a specific enzyme.

8.6. Tip

If you can screen genetically for a phenotype or have a functional assay, use this to prescreen for potentially interesting mutants prior to DNA sequencing.

8.7. Tip

If possible, transform E. coli via electroporation since this will give the greatest number of transformants, increasing the chances of obtaining interesting mutations.

See Fig. 20.3 for the flowchart of Step 4.

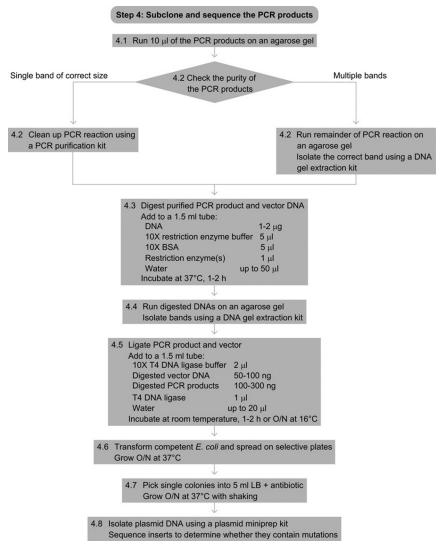


Figure 20.3 Flowchart of Step 4.

REFERENCES

Referenced Literature

Cadwell, R. C., & Joyce, G. F. (1992). Randomization of genes by PCR mutagenesis. PCR Methods and Applications, 2, 28–33.

Referenced Protocols in Methods Navigator

Explanatory chapter: PCR -Primer design Molecular Cloning Transformation of Chemically Competent *E. coli* Transformation of *E. coli* via electroporation Isolation of plasmid DNA from bacteria Agarose Gel Electrophoresis



Megaprimer Method for Mutagenesis of DNA

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Abstract

To create mutations in a gene of interest, including point mutations, short insertions, or deletions.

1. THEORY

Alteration of a gene of interest is a fundamental tool to study the structure and function of DNA and proteins in biomedical research. Point mutations are often produced to study the role of specific amino acid residues in protein activity and interactions. Insertions and deletions can also be incorporated to study protein function.

One commonly used method for producing these alterations is the megaprimer method (Sarkar and Sommer, 1990), which utilizes a two-step PCR-based protocol to incorporate changes followed by incorporation into a target plasmid. This is a flexible and robust technique allowing a wide range of alterations to be incorporated into a gene of interest.

Another commonly used method involves replication of the complete plasmid and gene of interest while incorporating the mutation (e.g., Quikchange mutagenesis, Stratagene).

2. EQUIPMENT

PCR Thermocycler Microcentrifuge Micropipettors Agarose gel electrophoresis equipment UV light box 0.2-ml thin-walled PCR tubes 1.5-ml microcentrifuge tubes Scalpel Micropipettor tips

3. MATERIALS

High-fidelity DNA polymerase [e.g., Phusion (New England Biolabs)] dNTP mix Agarose Tris base Glacial acetic acid EDTA Ethidium bromide DNA gel extraction kit

3.1. Solutions & Buffers

Steps 3 & 5 50× TAE agarose gel running buffer

Component	Stock	Amount	
Tris base		242 g	
Glacial acetic acid		57.1 ml	
EDTA, pH 8	0.5 M	100 ml	

Add water to 1 l

Ethidium bromide stock solution (10 mg ml⁻¹)

Dissolve 10 mg ethidium bromide in 1 ml water

Use an amber colored 1.5-ml tube or wrap tube with aluminum foil

4. PROTOCOL

4.1. Duration

Preparation	About 1 h
Protocol	About 8 h

4.2. Preparation

Obtain or purify DNA containing the gene of interest.

Design and order standard (outside) forward and reverse primers for amplification of the gene with appropriate restriction endonuclease

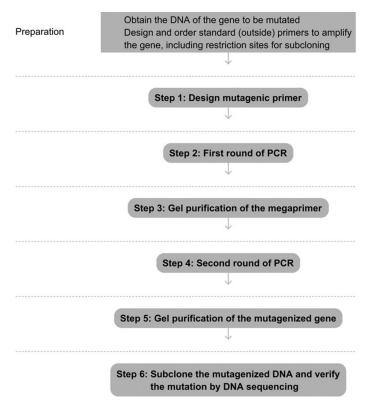


Figure 21.1 Flowchart of the complete protocol, including preparation.

recognition sequences and overhangs for subsequent subcloning (see Explanatory chapter: PCR -Primer design).

See Fig. 21.1 for the flowchart of the complete protocol.

5. STEP 1 DESIGN MUTAGENIC PRIMER

5.1. Overview

Design a primer that incorporates the mutation of interest along with flanking regions overlapping the gene of interest.

5.2. Duration

1 h

1.1 Determine the nucleic acid sequence for the desired mutation.

- **1.2** Select regions of DNA upstream and downstream of the mutation site that each have a $T_{\rm m} \sim 45 \,^{\circ}{\rm C}$.
- **1.3** If the mutation lies within the first half of the gene, order the reverse complement of the above sequence.
- 1.4 If the mutation lies within the second half of the gene, order the designed sequence.

5.3. Tip

The mutagenic primer can be purified as needed (see Purification of DNA Oligos by Denaturing Polyacrylamide Gel Electrophoresis (PAGE)).

6. STEP 2 FIRST ROUND OF PCR

6.1. Overview

Generate the megaprimer by PCR, in this example using Phusion DNA polymerase (New England Biolabs).

6.2. Duration

2 h

- 2.1 If the mutation is in the first half of the gene, use the standard (outside) forward primer and reverse mutagenic primer. If the mutation is in the second half of the gene, use the forward mutagenic primer and the standard (outside) reverse primer. Dilute all primers to a working stock concentration of 25 µM.
 - $5 \times$ Phusion HF buffer 4 µl 10 mM dNTP mix 0.4 µl Template DNA (200 ng ml⁻¹) 0.5 µl Primer A 0.4 µl Primer B 0.4 µl Phusion DNA polymerase 0.2 µl Water to 20 µl
- **2.2** Add to a 0.2-ml thin-walled PCR tube:

5–10 s for 10–30 s
for 10-30 s
101 10 50 3
for 15–30 s/1 kb
for 5–10 min
hold

2.3 Run PCR with the following cycling conditions:

6.3. Tip

Cast agarose gel while PCR is running. See Fig. 21.2 for the flowchart of Steps 2 and 4.

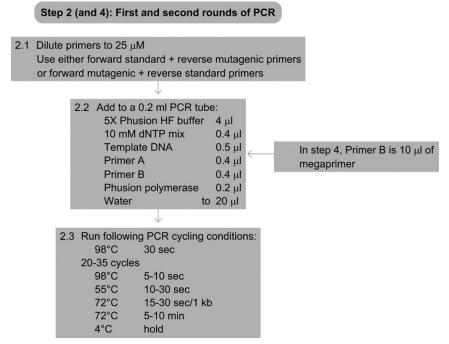


Figure 21.2 Flowchart of Step 2 (and 4).

7. STEP 3 GEL PURIFICATION OF THE MEGAPRIMER7.1. Overview

Run an agarose gel (see Agarose Gel Electrophoresis) to purify the megaprimer to use in subsequent PCR.

7.2. Duration

1.5h

- **3.1** Prepare a 2% (w/v) agarose gel. Add 2 g agarose to $100 \text{ ml} 1 \times \text{TAE}$.
- **3.2** Microwave in glass flask or beaker to dissolve agarose. Swirl flask occasionally to prevent boiling over. Cool on bench for 10 min.
- **3.3** Add 2 μ l of ethidium bromide and swirl to mix. Pour the gel into gel casting box and allow gel to fully harden.
- **3.4** Load the PCR sample from Step 2 and run the agarose gel at 100 V for \sim 30 min.
- **3.5** Examine gel under a UV light box, excise the megaprimer using a scalpel, and put it in a 1.5-ml microcentrifuge tube.
- **3.6** Dissolve the gel fragment and isolate the megaprimer using a gel extraction kit.

7.3. Tip

Work quickly when excising gel band to minimize UV exposure. Alternatively, use a handheld UV light source.

7.4. Caution

Ethidium bromide is a mutagen. Wear gloves and handle with care. Dispose of gel and buffer appropriately according to local regulations.

See Fig. 21.3 for the flowchart of Steps 3 and 5.

8. STEP 4 SECOND ROUND OF PCR

8.1. Overview

Utilize the megaprimer to produce the mutated gene by PCR.

8.2. Duration

2 h

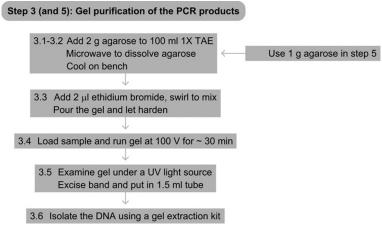


Figure 21.3 Flowchart of Step 3 (and 5).

4.1 If the mutation is in the first half of the gene, use the (forward) megaprimer (10 μ l) and the standard (outside) reverse primer (0.4 μ l). If the mutation is in the second half of the gene, use the standard

(outside) forward primer (0.4 μ l) and (reverse) megaprimer (10 μ l).

4.2 Add to a 0.2-ml thin-walled PCR tube:

$5 \times$ Phusion HF buffer	4 µl
10 mM dNTP mix	0.4 µl
Template DNA (200 ng ml ^{-1})	0.5 µl
Primer A	0.4 µl
Primer B (megaprimer)	10 µl
Phusion DNA polymerase	0.2 µl
Water	to 20 µl

4.3 Run PCR with the following cycling conditions:

98 °C	30 s
20-35 cycles:	
At 98 °C for	5–10 s
At 55 °C for	10–30 s
At 72 °C for	15–30 s/1 kb
At 72 °C for	5–10 min
At 4 °C	hold

8.3. Tip

The protocol is designed to utilize excess megaprimer for the second round of PCR, which significantly improves the efficiency and allowable megaprimer size (Smith and Klugman, 1997). As needed, the amount of megaprimer and template can be varied to maximize production of the desired product.

8.4. Tip

Cast agarose gel while PCR is running. See Fig. 21.2 for the flowchart of Steps 2 and 4.

9. STEP 5 GEL PURIFICATION OF THE MUTAGENIZED GENE

9.1. Overview

Run an agarose gel to purify the mutagenized gene (see Agarose Gel Electrophoresis).

9.2. Duration

1.5 hours

- **5.1** Prepare a 1% (w/v) agarose gel. Add 1 g agarose to $100 \text{ ml } 1 \times \text{TAE}$.
- **5.2** Microwave in glass flask or beaker to dissolve agarose. Swirl flask occasionally to prevent boiling over. Cool on bench for 10 min.
- 5.3 Add 2 μ l of ethidium bromide and swirl to mix. Pour the gel into gel casting box and allow gel to fully harden.
- **5.4** Load the PCR sample from Step 4 and run the agarose gel at 100 V for \sim 30 min.
- **5.5** Examine gel under a UV light box, excise the DNA (gene with incorporated mutation) using a scalpel, and put it in a 1.5-ml microcentrifuge tube.
- **5.6** Dissolve the gel fragment and isolate the DNA using a gel extraction kit.

9.3. Tip

Work quickly when excising gel band to minimize UV exposure.

See Fig. 21.3 for the flowchart of Steps 3 and 5.

> 10. STEP 6 SUBCLONE THE MUTAGENIZED DNA AND VERIFY THE MUTATION BY DNA SEQUENCING 10.1. Overview

Use standard protocols to digest the vector DNA and the PCR product with the appropriate restriction enzymes and gel-purify the DNA. Ligate the insert into the digested vector (see Molecular Cloning), transform *E. coli* (see Transformation of Chemically Competent *E. coli* or Transformation of *E. coli* via electroporation), screen colonies for the presence of the insert (see Colony PCR), purify (see Isolation of plasmid DNA from bacteria) and sequence the plasmids to verify the mutation.

10.2. Duration

4-7 days

- 6.1 Digest the PCR product and 1 μ g of the desired vector DNA with the chosen restriction enzymes.
- **6.2** Prepare a 1% (w/v) agarose gel (as above). Run digested DNAs on gel at 100 V for \sim 30 min and gel-purify the DNA (as earlier).

$10 \times$ T4 DNA ligase buffer	2 µl
Prepared vector	50–100 ng
Annealed insert	100–300 ng
T4 DNA ligase	1 µl
Water	up to 20 µl

6.3 Add to a 1.5-ml microcentrifuge tube:

Incubate at room temperature for 1-2 h or overnight at 16 °C (a PCR theyrmocycler can be used).

- **6.4** Transform competent *E. coli* of choice. Plate cells on selective media and incubate overnight.
- **6.5** Pick colonies and screen for the presence of the insert by colony PCR or by restriction enzyme digestion.
- **6.6** Purify the insert-containing plasmids and subject them to DNA sequencing to verify the presence of the mutation.

See Fig. 21.4 for the flowchart of Step 6.

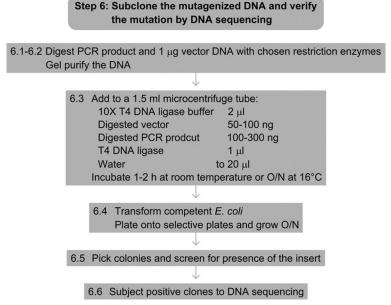


Figure 21.4 Flowchart of Step 6.

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Explanatory Chapter: Troubleshooting PCR

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Abstract

The purpose of this chapter is to assist with any problems you are having with the polymerase chain reaction (PCR) protocol from General PCR.

1. THEORY

The simple theory behind PCR is to use custom-designed primers, template DNA, a thermostable DNA polymerase, and nucleotides (dNTPs) to amplify a particular region of DNA so that it can be easily detected or manipulated. There are many applications for PCR including gene cloning, manipulation, mutagenesis, genotyping, detection and diagnosis of pathogens, DNA sequencing, and analyzing expression levels of a gene.

PCR comprises three main steps:

- *Denaturation*: this separates the double-stranded DNA into single-stranded DNA.
- *Annealing*: the primers bind to the complementary target sequence in the template DNA.
- *Extension*: the thermostable DNA polymerase synthesizes the complementary DNA strand, starting from the 3'-end of the primer.

All PCR should be evaluated to confirm that a PCR product of the expected size was made. This is most commonly done by agarose gel electrophoresis (see Agarose Gel Electrophoresis). This chapter assists in the troubleshooting of PCR in the event that there was no product produced or a product of the wrong size was produced.

2. EQUIPMENT

PCR thermal cycler Micropipettors Aerosol barrier pipet tips Sterile microcentrifuge tubes 0.2-ml thin-walled PCR reaction tubes or plates Surface decontamination wipe (e.g., DNA AWAY[®], Molecular Bioproducts) Agarose gel electrophoresis equipment

3. MATERIALS

Template DNA Primers dNTPs Taq DNA polymerase (or other thermostable DNA polymerase) $10 \times$ PCR buffer Magnesium chloride (MgCl₂, 25 mM) Sterile ultra pure water Ethidium bromide Normal melting agarose TAE buffer DNA ladder

4. PROTOCOL

Rather than being a standard protocol, this chapter lists common problems with PCR, the reasons why they might have occurred, and steps that can be taken to prevent these problems in the future. Most often, after performing PCR, an agarose gel is run with the PCR product alongside a DNA ladder to verify the size of the product. Two problems can be immediately apparent: first, there is no PCR product, and second, there is a PCR

problem	Possible reasons	Fixes	
No product formed	• Problem with reagents	 Repeat the experiment, as a reagent may have been unintentionally left out Check that the reagents had been fully thawed and mixed thoroughly Try a new vial of dNTPs as they can be damaged by repeated freeze-thaw cycles Use a different polymerase Try using an additive such as DMSO or glycerol 	
	• Quality of the template DNA is poor	• Use a NanoDrop spectrophotometer to check the quantity and quality of template DNA	
	• Too much or too little starting template	 Remake template DNA. Older stocks can degrade, particularly for genomic DNA Set up a series of reactions with varying amounts of template (between 10 and 200 ng DNA) 	
	Primers are not working	 Check that the primers have been diluted to the correct concentration Make sure that the sequence of the primer is what you expected it to be synthesized as. Typos are common! Increase primer concentration Redesign the primers 	
	• PCR inhibitors are present in the template DNA	• Demonstrate that a control gene or another DNA sequence can be effec- tively amplified using the same tem- plate DNA. If it can, there is another problem with the PCR reaction	
	• Problem with the set- tings on thermal cycler	 Check the cycler – Are the temperatures and times as you expect? Change the annealing temperature. Find the optimum temperature by using a gradient cycler and test a range from the lowest primer T_m to 10 °C below the T_m 	

Table 22.1 Troubleshooting the lack of a PCR product seen on an agarose gel PCR

problem	Possible reasons		Fixes	
		•	Try the reaction in another cycler – the calibration of the one you are using may be off	
	• The template DNA is GC-rich	•	Use a polymerase buffer intended for GC-rich templates. Test a full range of the additive (e.g., GC melt)	

Table 22.1	Troubleshooting the lack of a PCR product seen on an agarose gel—cont'd
PCR	

Table 22.2 Troubleshooting PCR products which are either too long or too shortPCR problemPossible reasonsFixes

PCR problem	Possible reasons	Fixes
Wrong sized product formed	Cross-contamination	• Use the following order when setting up the reac- tions: actual samples, posi- tive controls, and then negative controls
	• One or both primers anneal to an off-target site on the template	 Use aerosol barrier pipettor tips Pre- and postamplification samples should be manipulated in physically separated areas. Set up a separate lab bench area and a set of pipettors for running gels
Long nonspecific products	 One or both primers anneal to an off-target site on the template 	 Decrease the annealing and/or extension time Decrease the extension temperature to 62–68 °C Increase the annealing temperature. Increase the MgCl₂ concentration to 3–4.5 mM, while keeping the dNTP concentration constant Use less primers, DNA template, and/ or polymerase If none of the above works. BLAST the primer for repetitive sequences and try designing new primer(s)

PCR problem	Possible reasons	Fixes	
Short nonspecific products or 'primer dimers'	• Small PCR bands about the size of one primer or both together, which are formed by the annealing of a primer to itself or to the other primer	 Increase the annealing temperature and/or time. Try to find the optimal annealing temperature using a gradient PCR machine 	
	 One or both primers anneal to an off-target site on the template, resulting in a very short PCR product 	 Increase the extension time and/or temperature to 74–78 °C Titrate the MgCl₂ concentration to 3–4.5 mM, while keeping the dNTP concentration constant Use less primer or Taq polymerase Try a 'hot start' polymerase Increase the amount of the DNA template If none of the above works, BLAST the primer for repetitive sequences and try designing new primer(s) 	

 Table 22.2 Troubleshooting PCR products which are either too long or too short—cont'd

product(s) but it is the wrong size. Table 22.1 gives some reasons and possible fixes for when there is no PCR product produced. Table 22.2 gives some reasons and possible fixes for when there is a PCR product, but it is the wrong size.

Since there are five main reagents in a PCR, you can alter any of them in order to optimize the reaction for your particular gene of interest and product. It is preferred to only change one aspect of the reaction at a time. The following gives examples of how each reagent, MgCl₂, Taq DNA polymerase, the primers, dNTPs, and template DNA, can cause problems in PCR and acceptable ranges of concentrations for each reagent.

 $MgCl_2$: Magnesium plays several roles in PCR: it is an essential cofactor for thermostable DNA polymerases, it stabilizes double-stranded DNA, and it elevates the T_m . A deficient or low Mg^{2+} concentration requires stricter base pairing when the primers and DNA anneal, and can cause a small or no yield of PCR product. If Mg^{2+} ions are in excess, there can be an increased yield of nonspecific products, and the reliability of DNA polymerases can be reduced, promoting the misincorporation of nucleotides and the samples appearing as a smear on the gel. Since dNTPs sequester Mg^{2+} ions, any alteration in dNTP concentrations will require additional changes in the concentration of MgCl₂. Normally, the MgCl₂ concentration is best between 1 and 4 mM.

Taq DNA polymerase: Low levels of Taq polymerase can cause incomplete primer elongation or untimely termination of the PCR product synthesis during the extension step. Too much polymerase will result in an excessive background of unwanted DNA fragments, which will look like a smear on the gel. A markedly overabundant amount of polymerase can cause the reaction to completely fail with no product formed at all. A polymerase concentration of 1 unit per 25 μ l of reaction mix is sufficient to generate a clean PCR product.

Primers: Low primer concentration generally results in a cleaner product, yet raising the primer concentration does not lead to a corresponding increase in the amount of product formed. Using elevated concentrations of primers can result in the creation of primer-dimers, nonspecific primer binding, and the generation of undesirable PCR products. Conversely, to amplify short target sequences, such as 100 base pairs, a higher number of molecules of PCR product are required to provide a specified amount of amplified DNA (in nanograms). In this case, a higher primer concentration could be beneficial. The suggested primer concentration is between 0.1 and 1 μ M of each primer. Primer base composition and priming sites can also dramatically affect the PCR performance, see chapter Explanatory chapter: PCR -Primer design.

dNTPs: A suboptimal concentration of nucleotides can lead to incomplete primer elongation or untimely termination of synthesis at some point in the extension step. Excessive dNTP concentrations can inhibit PCR, preventing product formation. The usual dNTP concentration is between 40 and 200 μ M for each of the four dNTPs. To amplify longer DNA fragments, a higher dNTP concentration may be required.

DNA template: The concentration of the template DNA should be balanced with the number of cycles in the reaction. When the total amount of DNA is extremely small, there is increased likelihood of loss, contaminating DNA from impurities, and/or degradation. Using too much DNA can lead to off-target annealing of the primers as well as poor DNA synthesis due to the obstructed diffusion of polymerase. However, reducing the number of cycles may help to resolve these problems. Contamination can come from unlikely sources such as the dust floating in the air or particles of skin or hair from your body, which can carry both DNA and DNA-degrading nucleases. To prevent this, autoclave the tubes, wear gloves, and clean the working space using an oxidizing substance (e.g., 6% H_2O_2), 100% ethanol, or a surface decontamination wipe (e.g., DNA AWAY[®], Molecular Bioproducts).

Weak amplification of your target can be improved by:

- Increasing the amount of primers, DNA template, and/or polymerase.
- Checking primer sequences for mismatches and/or increasing the primer length by five nucleotides (see also Explanatory chapter: PCR –Primer design).
- Increasing the annealing time or decreasing the annealing temperature.
- Increasing the number of cycles.
- Trying an additive of 5% (v/v, final concentration) DMSO or glycerol.
- Extracting the weak PCR product from an agarose gel using a DNA gel purification kit and using this as the template in a new reaction.

Nonspecific band amplification can be fixed by:

- Repeating the reaction with a negative control, such as water. The nonspecific bands are most likely from contaminating foreign DNA. If this is the problem, use new stocks for all reagents. Also, remember to always use autoclaved PCR tubes and wear gloves.
- Using less DNA template.
- Increasing the annealing time if the nonspecific products are shorter than your target. If they are longer than your target, reduce the annealing time.
- Increasing the annealing temperature.
- Redesigning the primer(s) to increase the length at its 3'-end as the extra bands may be from similar sequences to your target. Increasing the 3' primer length by adding further matching base pairs will make the primer more specific for your target because correct sequence binding will be enhanced while the extension of the nonspecific sequences will be blocked. When trying to eliminate aberrant bands, it is usually not a good idea to increase the length of a primer at its 5'-end, because this will increase its ability to anneal to the off-target sequences. In some cases shorter primers will work better because they do not base pair as well to the off-target sequences.

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General PCR Agarose Gel Electrophoresis Explanatory chapter: PCR -Primer design



Explanatory Chapter: Quantitative PCR

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Abstract

Quantitative PCR (qPCR), also called real-time PCR or quantitative real-time PCR, is a PCRbased technique that couples amplification of a target DNA sequence with quantification of the concentration of that DNA species in the reaction. This method enables calculation of the starting template concentration and is therefore a frequently used analytical tool in evaluating DNA copy number, viral load, SNP detection, and allelic discrimination. When preceded by reverse-transcription PCR, qPCR is a powerful tool to measure mRNA expression and is the gold standard for microarray gene expression data confirmation. Given the broad applications of qPCR and the many technical variations that have been developed, a brief survey of qPCR, including technical background, available chemistries, and data analysis techniques will provide a framework for both experimental design and evaluation.

1. THEORY

The defining feature of qPCR is 'real-time' measurement of the concentration of a DNA species of interest during a PCR, rather than end-point measurement at the completion of the amplification series. Although endpoint analysis is extremely valuable in determining the presence or absence of template DNA in a reaction, the results can be very misleading. Much like other enzyme-catalyzed reactions, product formation in a PCR reaction follows a saturation curve (Fig. 23.1). Early amplification cycles generate product in an exponential fashion; the PCR is working at top efficiency. As the PCR progresses, the efficiency decreases because of lowered concentrations of consumables such as dNTPs and primers, and enzyme degradation, and product formation enters a linear phase. Finally, the reaction progresses into a plateau phase where product formation is greatly decreased because of the depletion of consumables. The PCR cycle at which each phase transition occurs is unique to each reaction and is largely dependent on the concentration of template DNA. PCRs containing a large template concentration will generally plateau in earlier cycles than PCRs containing a lesser amount of template. By end-

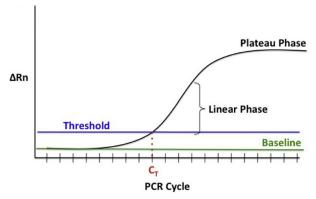


Figure 23.1 Sample qPCR plot. Important values are indicated. Baseline: background fluorescence prior to product accumulation. Threshold: 10 times the standard deviation of baseline fluorescence for the first 15 cycles. C_T : Amplification cycle at which fluorescence exceeds the threshold. $\Delta Rn =$ normalized fluorescence (subtracted background). Linear phase: The most efficient amplification cycles when the rate of product accumulation is greatest. Plateau phase: Product accumulation slows then ceases as the reaction is exhausted.

point analysis, however, these two conditions are indistinguishable if both reactions have reached the plateau phase by the last amplification cycle performed. For this reason, measurement of PCR product accumulation during the PCR is a much more sensitive assay than end-point analysis.

Analysis of product accumulation in qPCR is accomplished through use of fluorescent dyes that specifically label dsDNA or a specific DNA species of interest; the amount of fluorescence is proportional to the quantity of DNA present. Although many models of qPCR thermocyclers are in production, all share common features: a standard thermocycler platform (96- or 384well) is coupled with an excitation source (usually a laser or tungsten lamp), a camera for fluorescence detection, and computer and software for data processing. Depending on the available excitation source and detection filters, a variety of fluorescent dyes may be used in qPCR.

2. TERMINOLOGY

Pioneering studies by Heid et al. (1996); Gibson et al. (1996) defined many of the important parameters in qPCR (Fig. 23.1).

Baseline: During cycles 1–15 of the qPCR, fluorescence is below the limits of detection, but it is increasing.

Rn_B: The fluorescence level at the baseline.

Rn_F: The fluorescence level at a given point in the qPCR.

 Δ Rn: The normalized fluorescence level at a given point in the qPCR; Rn_F/Rn_B.

Threshold: A Δ Rn value defined as 10 times the standard deviation of baseline fluorescence during the first 15 amplification cycles. The threshold may be adjusted for a group of different reactions so that it is in the linear amplification range for each, but should be held constant across samples to be compared.

 $C_{\rm T}$: The amplification cycle at which the Δ Rn exceeds the threshold. The $C_{\rm T}$ is a quantitative description of the starting concentration of template.

3. EQUIPMENT

qPCR thermocycler qPCR plates (depends on thermocycler used) Micropipettors Multichannel pipettor Micropipettor tips

4. MATERIALS

Primers Fluorescent probe TaqMan probe

5. PROTOCOL 5.1. Preparation

Several factors must be considered when designing a qPCR experiment. Once a target of interest has been identified, PCR primers must be designed to amplify that sequence, a detection method must be chosen, control reactions must be planned, and the method of data analysis established. Each of these points is addressed individually in the subsequent sections.

5.2. Duration

Preparation	1–2 days
Protocol	About 1 day

6. STEP 1 PRIMER DESIGN

Primer quality (see Explanatory chapter: PCR -Primer design) plays an essential role in qPCR: well-designed primers with minimal selfcomplementarity and high specificity give rise to reproducible results; primers with a tendency toward dimerization or mishybridization will greatly reduce PCR efficiency and confound results. PCR amplicons should be 50–150 bp. In mRNA expression studies, it is desirable for the PCR product to cross an exon–exon border to reduce background amplification from contaminating genomic DNA and/or splice variants. Primer T_m should be 58–60 °C, length should be 18–30 bases, and G-C content at 40–60%. To increase specificity, an adenine or thymine at the 3' position should be avoided. The use of software to design primers is preferable as it optimizes these parameters and matches primers very efficiently. Primer3 is an excellent open-source program that is available over the internet (Rozen and Skaletsky, 2000). Commercially available software for qPCR primer design exists as well. Prior to using newly designed primers in a qPCR reaction, their specificity can be tested by performing a standard PCR; if spurious amplification is observed, the primers should be rejected and the design process restarted.

7. STEP 2 CONTROL GENE

qPCR is an extremely sensitive technique. Small differences in reaction composition may be magnified over the course of the PCR. In order to correct for this eventuality, normalization against an internal control is critical. Performing additional qPCR reactions using the same template and identical setup but instead measuring the expression (or copy number) of a housekeeping gene will provide a 'loading control' against which experimental data may be normalized. These control reactions should be performed simultaneously alongside experimental reactions. The most commonly used control genes include β -actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and ribosomal RNA. These genes have become well established as controls, since they are ubiquitously expressed and do not appear to vary dramatically under many experimental conditions. Care should be taken when selecting a control gene, however, as there have been reports of unexpected changes in expression of control genes, particularly GAPDH (reviewed in Suzuki et al., 2000). A thorough literature search with planned experimental conditions in mind is necessary for the best choice of control gene. Once a control has been decided upon, amplification primers should be designed in a fashion identical to that for the experimental genes.

8. STEP 3 AMPLIFICATION EFFICIENCY

In order to compare expression or copy number data between two samples and/or genes, they must first be normalized against a control gene. One assumption of normalization of experimental data against a control gene is that they have relatively equal amplification efficiencies (AE). PCR efficiency may vary because of primer hybridization, GC-content of the amplicon, and disparities in amplicon size. If the amplification efficiencies are equal, varying the starting template concentration will have an equal effect on amplification of both genes and the control gene remains an appropriate control. If, however, AEs differ, an increase in template concentration may manifest as variable increases in product accumulation between the experimental and control genes, and the results are no longer directly comparable. Therefore, AEs must be matched as closely as possible.

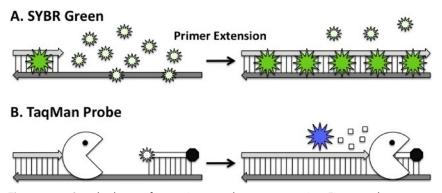


Figure 23.2 Standard curve for starting template concentration. Five template concentrations falling in the linear range are shown. The trend line and corresponding equation are also shown. Each point represents the average of three replicates. In this case, the efficiency is calculated as 0.99.

If necessary, however, corrections that consider mismatched AEs are available (Pfaffl, 2001; Yuan et al., 2001).

The standard method to determine the AE is to generate a standard curve. qPCR is performed with a series of dilutions of the template; the $C_{\rm T}$ for each reaction is then plotted against the log template concentration, and the slope of the resulting line is calculated (Fig. 23.2). The AE is defined as

$$AE = 10^{(-1/slope)} - 1$$

(Rasmussen, 2001). An AE of 1.0, corresponding to an efficiency of 100% and a standard curve slope of -3.33, is optimal. A template concentration in the middle of those falling within the -3.33 slope should be chosen for future experiments. Efficiencies of 0.9–1.1 (corresponding to 90–110%) are acceptable. In order to compare two AEs, a plot of template dilution versus $\Delta C_{\rm T}$ where

$$\Delta C_{T} = C_{T, \text{ target gene}} - C_{T, \text{ reference gene}}$$

should be constructed; a slope of approximately zero indicates equivalent AEs.

9. STEP 4 PROBE CHOICE

Multiple technical variations exist to detect the DNA species of interest in a qPCR reaction, but all rely upon fluorescent labeling of the DNA of interest and subsequent measurement of fluorescence during each cycle of the PCR. The most commonly used methods, DNA intercalating agents (specifically SYBR Green) and TaqMan probes, are described here. A more comprehensive review has been compiled by Mackay and Landt (2007).

9.1. SYBR green I

DNA intercalating agents have long been used to label double-stranded (ds) DNA. Although ethidium bromide has been the most common labeling agent in the past, SYBR Green I (SG), which does not carry the same exposure risks for humans and exhibits brighter fluorescence, has largely supplanted ethidium bromide in qPCR applications. SG is a nonspecific minor groove-binding molecule, and exhibits a large increase in fluorescence when in the dsDNA-bound form (Zipper et al., 2004; Fig. 23.3(a)). One major advantage of SG is its nonspecific nature, which allows it to be used for multiple samples with little or no preparation. This

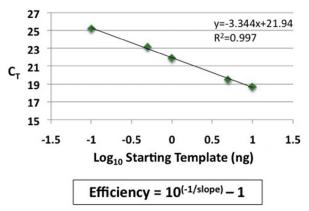


Figure 23.3 Popular qPCR detection methods. Gray arrows: single-stranded DNA (ssDNA); arrowheads: 3' ends; vertical bars: hydrogen bonds. (a) SYBR Green I (SG) is a DNA intercalating agent that binds dsDNA with high affinity. Free or ssDNA-bound SG (light green star) has a low level of intrinsic fluorescence; however, when bound to dsDNA, SG fluorescence increases greatly (bright green star). Generation of dsDNA over the course of a PCR results in a higher proportion of dsDNA-bound SG and an increase in fluorescence. (b) TaqMan probes are short primers (white) that are dual-labeled with a 5' fluorophore (white and blue stars) and a 3' quencher (black octagon). The probe hybridizes to complementary ssDNA, holding the fluorophore and quencher in close proximity. Due to FRET between the two dyes, there is little fluorescence (white star). As primer extension occurs, Taq polymerase (chomper) encounters the TaqMan probe, and the enzyme's 5' exonuclease activity cleaves the probe, liberating the 5' fluorophore from the 3' quencher. Fluorescence increases as a result (blue star).

same feature, however, may be a disadvantage if primers are not carefully designed; SG will bind any dsDNA, including primer dimers and non-specific PCR products. As a result, the fluorescence due to formation of the product of interest may be masked, making analysis extremely difficult. Use of primer-design software typically ameliorates these complications.

9.2. TaqMan probes

Taq polymerase, frequently used for standard PCR applications, exhibits 5'exonuclease activity: if the enzyme encounters a double-stranded region during extension, the polymerase will nick the nontemplate strand, freeing its constituent nucleotides. This property is exploited by the TaqMan probe (Lee et al., 1993; Fig. 23.3(b)). A pair of PCR primers is designed, and an additional primer is designed within the amplicon. This internal primer is the foundation of the TaqMan probe and is labeled at the 5' end with one fluorescent reporter dye, typically FAM, TET, VIC, or HEX, and the 3' end is labeled with a quencher dye, often TAMRA, DABCYL, or a black hole quencher (BHQ). As this technology relies upon FRET (fluorescence resonance energy transfer) between the reporter and quencher dyes, the choice of reporter and quencher dye is not arbitrary; the two dyes must have overlapping excitation/emission spectra. When the reporter and quencher are in close proximity (labeling the intact primer), FRET occurs and there is no fluorescence observable. As Taq polymerase extends through this region and encounters the probe, the enzyme's exonuclease activity nicks the 5' end of the probe, liberating the reporter dye from the primer anchoring the quencher. As the reporter and quencher are no longer in close proximity, FRET does not occur and fluorescence is observed. A major advantage of TaqMan probes is the added specificity conferred by the probe's hybridization between the two PCR primers. One drawback is that a separate probe must be designed for each gene of interest; however, the multiple available combinations of reporters and quenchers in concert with sequence specificity conferred by the probe itself enable multiplexing, that is, the combination of several probes in one reaction (Gibson et al., 1996).

When designing TaqMan probes, several guidelines must be kept in mind: the probe should be 15–30 nucleotides long, be \sim 30–80% GC, have a $T_{\rm m}$ 10 °C higher than the amplification primers, and be free of nucleotide runs. It is also desirable to have fewer than 2 GCs at the 3' end to introduce a small degree of instability to prevent mispriming. Additionally, a G in the 5' position should be avoided as it may quench fluorescence (Arya et al., 2005).

If maximal specificity is desired, such as for allelic discrimination, a non-fluorescent quencher (such as a BHQ) may be coupled with a minor groove binder (MGB). MGBs increase $T_{\rm m}$, allowing shorter probes, and show higher specificity for single base mismatches (Kutyavin et al., 2000).

10. STEP 5 DATA ANALYSIS

Two methods are widely used to analyze qPCR data: absolute and relative quantification. Regardless of the method of analysis to be employed, all qPCR reactions should be performed in triplicate simultaneously to further protect against error.

10.1. Normalization

Small variations in the composition of qPCR reactions may be magnified by PCR amplification and increase variability both within and between samples. For this reason, normalization against an internal control is necessary. qPCR on control genes should be performed on the same samples and at the same time as experimental runs.

10.2. Absolute quantification

To determine the exact concentration of starting template, a standard curve may be generated from reference DNA (or cDNA) and used to quantify experimental samples. Ideally, the reference DNA source will be the gene of interest on a plasmid so that its concentration can be accurately calculated by spectrophotometry and copy number can be extrapolated. Copy number versus $C_{\rm T}$ is plotted to generate a standard curve; alternatively, the log starting template concentration may be used. Experimental data may then be compared with this curve to calculate copy number. In both constructing a standard curve and defining experimental samples, the raw $C_{\rm T}$ average should be normalized against an internal control gene.

10.3. Relative quantification

A relative quantification, or 'fold-change' description, is typically sufficient for most applications. The most commonly used method of relative quantification is the $\Delta\Delta C_{\rm T}$ analysis: The $C_{\rm T}$ of the target gene in a sample is normalized against the reference gene, yielding a $\Delta C_{\rm T}$ value for that gene and sample. The experimental strain is then compared with the reference strain,

Ratio =
$$\frac{(AE_{target})^{\Delta C_{T,target}(control-sample)}}{(AE_{reference})^{\Delta C_{T,reference}(control-sample)}}$$

Figure 23.4 $\Delta\Delta C_T$ analysis for the relative quantification of qPCR data. The efficiency can be considered in calculating the ratio of gene expression and/or copy number.

generating a $\Delta\Delta C_{\rm T}$. Finally, the fold-change is calculated by raising 2 to $-\Delta\Delta C_{\rm T}$ (Livak and Schmittgen, 2001).

$$\Delta C_{T} = C_{T, \text{ target gene}} - C_{T, \text{ reference gene}}$$
$$\Delta \Delta C_{T} = [\Delta C_{T}]_{\text{experimental}} - [\Delta C_{T}]_{\text{reference}}$$
$$2^{-\Delta\Delta CT} = \text{fold change}$$

This method, however, assumes equal AEs, which is not always the case. Because of the exponential nature of PCR, differences in AE can be dramatically magnified after repeated rounds of amplification, resulting in an inability to directly compare experimental and control data. If necessary, the efficiency can be considered in calculating the ratio of gene expression and/or copy number (see Fig. 23.4):

10.4. Summary

Quantitative PCR is a powerful tool for determining copy number and relative concentration of nucleic acids. Proper choice and use of a detection method and data analysis will ensure accuracy and reproducibility. Although qPCR technologies are rapidly evolving, the basic concepts covered here provide a foundation upon which to evaluate and make appropriate decisions regarding qPCR experimental design and execution.

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Explanatory chapter: PCR -Primer design



General PCR

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Abstract

The primary purpose of polymerase chain reaction (PCR) is to rapidly make many copies of a specific region of DNA or RNA so that it can be adequately detected, often by agarose gel electrophoresis. PCR is routinely used to amplify, modify, and clone genes for expression studies. There are many other applications for PCR, including paternity testing, biological relationships, mouse genotyping, diagnosing genetic diseases, forensics, and finding bacteria and viruses.

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1. THEORY

There are four main components of a PCR reaction: primers, the DNA template, thermostable DNA polymerase, and nucleotides (dNTPs). Primers are custom designed, short oligonucleotides that are complementary to the ends of the piece of DNA you are interested in amplifying. The template DNA provides the starting material for the reaction and contains the region of DNA you want to amplify. In the case of RT-PCR, the template is cDNA made from the RNA. The thermostable DNA polymerase copies the DNA using the oligonucleotides as primers in a cycle that is repeated many times, resulting in the exponential amplification of the DNA between the primers. This can continue until the primers and/or the free nucleotides are used up or the double-stranded DNA reaches a reaction-inhibiting concentration.

There are three main steps in a PCR reaction: denaturation, annealing, and extension. These are repeated using an automated thermal cycler until sufficient amplification has taken place (often > 25 cycles). Denaturation occurs at 94 °C and it separates the double-stranded DNA template into single-stranded DNA. Annealing occurs at a lower temperature, usually between 45 and 60 °C. Annealing allows the primers to base pair with their complementary sites on the template DNA. This formation of double-stranded DNA provides a place for the polymerase to attach and begin the process of copying the template. This last phase, known as extension, usually takes place at 72 °C. Because both strands of DNA are copied during the PCR process, there is an exponential growth in the number of gene copies.

The PCR reaction must be analyzed to confirm that the desired product was made. This is most commonly done using an agarose gel (see Agarose Gel Electrophoresis). For some applications, it is important to know the sequence as well. PCR products can be sequenced directly or cloned into a plasmid vector (see Molecular Cloning) and individual clones sequenced (see sequencing methods on Sanger Dideoxy Sequencing of DNA and Explanatory Chapter: Next Generation Sequencing). If there are problems with the PCR, you can troubleshoot the protocol (see Explanatory Chapter: Troubleshooting PCR).

2. EQUIPMENT

PCR thermal cycler Agarose gel electrophoresis equipment Micropipettors Aerosol barrier micropipettor tips Sterile 1.5-ml microcentrifuge tubes 0.2-ml thin-walled PCR tubes (or plates)

3. MATERIALS

Template DNA Primers dNTPs (100 mM each dATP, dCTP, dGTP, dTTP) Thermostable DNA polymerase (e.g., Taq polymerase or a high fidelity enzyme) $10 \times PCR$ buffer Tris base Hydrochloric acid (HCl) Ammonium sulfate $[(NH_4)_2SO_4]$ Tween-20 Magnesium chloride (MgCl₂) Agarose $50 \times$ TAE buffer Ethidium bromide $6 \times$ DNA gel loading dye Sterile ultra pure water

3.1. Solutions & buffers

Component	Final concentration	Stock	Amount
Tris–HCl, pH 8.8	750 mM	1 M	7.5 ml
$(NH_4)_2SO_4$	200 mM	1 M	2.0 ml
Tween-20	0.1% (v/v)	10%	100 µl

Step 1 10× PCR buffer

Add purified water to 10 ml

Final concentration	Stock	Amount
2.5 mM	100 mM	25 µl
2.5 mM	100 mM	25 µl
2.5 mM	100 mM	25 µl
2.5 mM	100 mM	25 µl
	2.5 mM 2.5 mM 2.5 mM	2.5 mM 100 mM 2.5 mM 100 mM 2.5 mM 100 mM

dNTP mix

Add purified water to 1 ml

25 mM MgCl₂

Dilute 25 µl 1 M MgCl₂ in 975 µl sterile ultra pure water

4. PROTOCOL

4.1. Preparation

Isolate DNA from the source of choice, which can be cells, human or lab animal tissue, or another origin. Kits can be purchased to isolate DNA from a variety of sources or standard protocols can be used. If you wish to isolate genomic DNA, see Preparation of Genomic DNA from Bacteria, Preparation of genomic DNA from *Saccharomyces cerevisiae*, or Isolation of Genomic DNA from Mammalian cells.

Design and order PCR primers (see Explanatory chapter: PCR - Primer design).

Dilute PCR primers in purified water to a working concentration of $10 \ \mu\text{M}$.

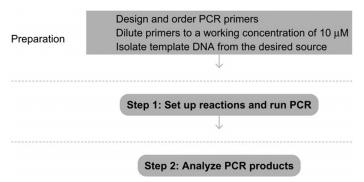
4.2. Duration

Preparation	Variable, 1–2 days
Protocol	\sim 4 h

4.3. Caution

If using a phenol or phenol-chloroform extraction technique, make sure to wear the appropriate gloves, eye protection, and use a hood ventilation system.

See Fig. 24.1 for the flowchart of the complete protocol.





5. STEP 1 POLYMERASE CHAIN REACTION

5.1. Overview

Set up the reactions and run the PCR in a thermal cycler.

5.2. Duration

3–4 h

1.1 Prepare a PCR Master Mix. On ice, combine the following reagents in a sterile 1.5-ml microcentrifuge tube. Note that these amounts are for a single PCR reaction; the volumes should be multiplied by the total number of reactions you are performing.

$10 \times$ PCR buffer	2.5 μl
Primer 1 (10 μM)	1.25 µl
Primer 2 (10 μM)	1.25 µl
dNTP mix (2.5 mM each)	2.5 μl
MgCl ₂ (25 mM)	1.6 µl
Taq DNA polymerase	0.1 µl
Sterile ultra pure water	10.8 µl

- 1.2 Dispense 20 μ l of the reaction mixture into 0.2-ml thin-walled PCR tubes. Add 5 μ l of the template DNA (50–200 ng) for a total reaction volume of 25 μ l.
- **1.3** Quickly vortex samples and centrifuge for a short time to ensure there are no air bubbles in the reaction mix. Alternatively, mix by gently pipetting up and down.

Temperature	Time	Purpose	# Cycles
94 °C	1 min	1	Initial denaturing
94 °C	1 min	25-35	(Denaturing)
55 °C	1 min		(Annealing)
72 °C	1 min (per kilobase PCR product)		(Extension)
72 °C	4 min	1	Final extension
4 °C	Hold	1	Stop/hold

1.4 Place tubes in a thermal cycler with the following cycling conditions:

5.3. Tip

Use a DNA contamination control product (e.g., DNA AwayTM wipes, Molecular BioProducts) to clean your bench top and pipettors to prevent contamination of your PCR. These are particularly useful when performing nested PCR and degenerate PCR.

5.4. Tip

Thaw samples and reagents on ice!

5.5. Tip

Use aerosol barrier pipette tips to reduce contamination. Always use a new tip when pipetting samples or reagents.

5.6. Tip

Run a negative and positive control reaction with each PCR.

5.7. Tip

Some $10 \times PCR$ buffers supplied with the enzyme may already contain MgCl₂. Check the recipe of your buffer.

See Fig. 24.2 for the flowchart of Step 1.

Step 1: Set up reactions and run PCR					
	-				
1.1		· · · · · · · · · · · · · · · · · · ·	a PCR Ma R buffer	ster Mix	x. For each reaction, add to a 1.5 ml tube:
			(10 μM)		2.5 μl 1.25 μl
			2 (10 μM)		1.25 μl
			ix (2.5 mM	l each)	
			25 mM)	,	1.6 μl
	Та	q poly	/merase		0.1 μl
	Ste	erile u	ltra pure w	/ater	10.8 μl
					\downarrow
	1.2		•		Mix to 0.2 ml thin-walled PCR tubes
	Add 5 µl template DNA (50-200 ng) \downarrow			\downarrow	
		1.3	Run PCF	R using t	he following cycling conditions:
			94°C	1 mii	n
			25-35 cy		
			94°C	1 mii	
			55°C		
	72°C 1 min per kilobase of PCR product				
			72°C 4°C	4 mii Hold	
			4.0		

Figure 24.2 Flowchart of Step 1.

6. STEP 2 ANALYZE PCR PRODUCTS

6.1. Overview

Run a portion of the PCR reaction on an agarose gel to assess the quantity and quality of the PCR product (see Agarose Gel Electrophoresis).

6.2. Duration

About 2 h

- **2.1** Prepare a 1% agarose gel in $1 \times$ TAE buffer plus ethidium bromide.
- 2.2 Mix 5 µl PCR reaction and 1 µl.
- 2.3 Load samples and a DNA ladder on the gel. Run gel at 100 V for 30–45 min (depending on the expected size of the PCR product).
- 2.4 Visualize bands using a UV gel documentation system.
- **2.5** If appropriate, clone the PCR product into a plasmid vector and/or directly sequence the PCR product.

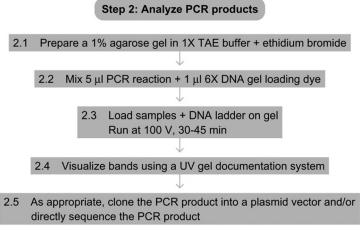


Figure 24.3 Flowchart of Step 2.

6.3. Tip

If there is little or no PCR product, the conditions may have to be optimized (see *Explanatory Chapter: Troubleshooting PCR*).

See Fig. 24.3 for the flowchart of Step 2.

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Agarose Gel Electrophoresis Molecular Cloning Sanger Dideoxy Sequencing of DNA Explanatory Chapter: Next Generation Sequencing Explanatory Chapter: Troubleshooting PCR Preparation of Genomic DNA from Bacteria Preparation of Genomic DNA from *Saccharomyces cerevisiae* Isolation of Genomic DNA from Mammalian cells Explanatory chapter: PCR -Primer design



Colony PCR

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Abstract

Colony PCR is a method for rapidly screening colonies of yeast or bacteria that have grown up on selective media following a transformation step, to verify that the desired genetic construct is present, or to amplify a portion of the construct.

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1. THEORY

Strategies for genetic manipulation of yeast and bacteria usually require linking a desired modification with a selectable marker on a construct that is transformed into the organism. The construct can be either a linear piece of DNA that must then undergo homologous recombination in order to be stably integrated into the genome, or a circular plasmid that can be maintained under selection. Following transformation, the organism is plated onto selective media. A successful transformation will result in the growth of one to a few hundred colonies on the plate. However, there are many possible scenarios that can lead to retention of the selectable marker and subsequent growth of a colony in the absence of the desired genetic construct. Therefore, a screening method is required to distinguish colonies carrying the correct construct from colonies carrying the selective marker in some other context. Colony PCR involves designing PCR primers that will yield a specific product of known size only if the desired construct is present, and ideally, a product of a different size if the site of the desired manipulation remains unaltered. This technique takes advantage of the high sensitivity of PCR - the small amount of template DNA that is required to give an easily visualized band on an agarose gel following PCR amplification can be recovered from a very crude preparation of cells. Colony PCR is thus a powerful tool for rapidly and easily screening through potentially large numbers of colonies to distinguish true positives from false positives. In most cases it is a superior alternative to the older strategy of growing small cultures from several colonies, preparing microgram quantities of DNA from each culture, and performing restriction digests to verify that the desired construct is present. Additionally, in the case of a cloning strategy that utilizes a step in one organism to generate a construct that is then recovered, amplified by PCR, and transformed into a different organism, colony PCR can be used directly to generate the product for the second transformation.

2. EQUIPMENT

Benchtop microcentrifuge Vortex mixer PCR Thermocycler Microwave oven Gel electrophoresis equipment Transilluminator, or other UV light source Petri dishes, containing colonies to be tested Micropipettors Micropipettor tips 0.2-ml thin-walled PCR tubes

3. MATERIALS

Selective culture media, on which colonies are growing Sodium hydroxide (NaOH) Purified water (can be distilled and autoclaved, or filtered by Milli-Q or similar filtration system) PCR primers Taq polymerase (or similar thermostable DNA polymerase) dNTP mix (may be supplied with Taq enzyme, or contains: dATP dGTP dTTP dCTP) PCR buffer (may be supplied with Taq enzyme, or contains: Tris-HCl, pH 8.8 $(NH_4)_2SO_4$ Tween 20 $MgCl_2$) Agarose Tris Base Boric Acid (H₃BO₃) EDTA Ethidium Bromide Ficoll 400 Bromophenol blue Xylene Cyanol DNA ladder

3.1. Solutions & buffers

Step 1 Sodium Hydroxide (20 mM)

Component	Stock	Amount/10 ml
NaOH	10 N	20 µl

Add purified water to 10 ml

Component	Final concentration	Stock	Amount/1 ml
dATP	2.5 mM	100 mM	25 µl
dCTP	2.5 mM	100 mM	25 µl
dGTP	2.5 mM	100 mM	25 µl
dTTP	2.5 mM	100 mM	25 µl

Step 2 dNTP mix

Add purified water to 1 ml

$10 \times$ PCR buffer

Component	Final concentration	Stock	Amount/10 ml
Tris–HCl (pH 8.8)	750 mM	1 M	7.5 ml
$(NH_4)_2SO_4$	200 mM	1 M	2.0 ml
Tween 20	0.1% (v/v)	10%	100 µl
MgCl ₂	15 mM	1 M	150 µl

Add purified water to 10 ml

PCR primers

Amounts given for PCR primers assume a working stock concentration of 10 µM.

•				
Component	Final concentration	Stock	Amount/liter	
Tris base	445 mM	N/A	54 g	
Boric Acid	445 mM	N/A	27.5 g	
EDTA	10 mM	0.5 M	20 ml	

Step 3 5× TBE buffer

Add purified water to 11

1% Agarose Gel Mix

Component	Final concentration	Stock	Amount/100 ml
Agarose	1% w/v	N/A	1 g
TBE buffer	$1 \times$	$5 \times$	20 ml
Ethidium Bromide	$0.5 \ \mu g \ ml^{-1}$	10 mg ml^{-1}	5 µl

Add water to 100 ml and heat to boiling in microwave

Component	Final concentration	Stock	Amount/10 ml
Ficoll	15% w/v	N/A	1.5 ml
Bromophenol Blue	0.25% w/v	N/A	25 mg
Xylene Cyanol FF	0.25% w/v	N/A	25 mg

б×	DNA	loading	dye

Add water to 10 ml

4. PROTOCOL 4.1. Duration	
Preparation	variable
Protocol	3–5 h

4.2. Preparation

PCR primers must be designed and synthesized prior to starting this protocol. Commercial primer synthesis has become very economical and widely available, and the design of appropriate primers is critical to the success of colony PCR. It is usually worthwhile to design primers specifically for this purpose rather than use suboptimal primers that may have been designed for other purposes. Ideally, primers designed to verify the integration of a construct by homologous recombination should include one primer against the construct and one against the genome adjacent to the desired site of integration. Colony PCR is most effective for products less than 1 kb in length, especially when the organism is S. cerevisiae. Larger products have been successfully obtained, especially from E. coli colonies, but if the goal is simply to verify an integration event, smaller products are usually better. It is best to design primers such that a product is obtained whether the desired construct is present or not, but the product sizes are unique in each case. Sometimes it is necessary to design more than one primer set to accomplish this. At a minimum, primers that give a product only if the desired construct is present can be used, but will not distinguish between a failure to obtain the desired construct and a failure of the PCR reaction in the case that no product is obtained. See Fig. 25.1 for an example.

See Fig. 25.2 for the flowchart of the complete protocol.

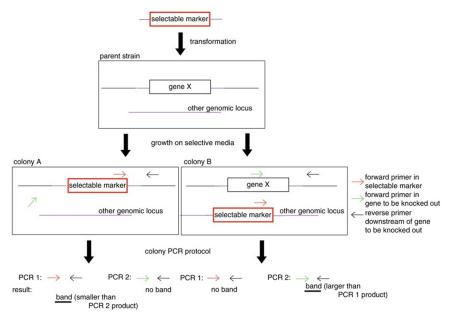


Figure 25.1 An example of primer locations for a colony PCR to validate a gene deletion. A construct consisting of a selectable marker flanked by sequences upstream and downstream of a gene to be deleted is transformed into the organism. Colonies that grow on selective media presumably express the selectable marker, but some colonies may have integrated the marker into a random genomic locus (as in colony B) rather than into the locus of the gene to be deleted (as in colony A). A forward primer against sequence within the selectable marker combined with a reverse primer against sequence just downstream of the gene to be deleted (PCR 1) will yield a product only if the marker has integrated in the appropriate locus. A forward primer against sequence within the gene to be deleted, combined with a reverse primer against sequence just downstream of that gene (PCR 2) will yield a product only if the correct integration has not occurred. Thus, a colony with the correct genotype should yield a band for PCR 1 but not PCR 2. Each colony should yield a band for either PCR 1 or PCR 2, however - failure of both reactions would indicate a failure of the PCR protocol, rather than a negative result for the presence of the desired construct. All the three PCR primers should have a similar melting temperature, preferably above 60 °C, and should not be complementary to each other. Product sizes should be \sim 200 bp-1 kb.

5. STEP 1 EXTRACTION OF DNA FROM A COLONY

5.1. Overview

Although PCR requires only a very small amount of template DNA, it is more successful, especially for *S. cerevisiae*, if cells are treated to break down the cell wall and allow the DNA to escape.

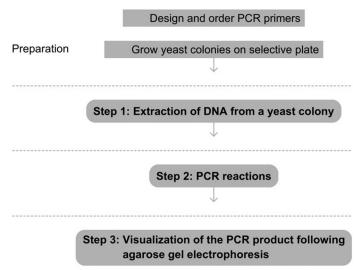


Figure 25.2 Flowchart of the complete protocol, including preparation.

5.2. Duration

15 min

- 1.1 Start with large ($\sim 2 \text{ mm}$) colonies. Colonies can be restreaked into a larger patch on a second plate of selective media if they are very slow-growing, or if the first plate is crowded with colonies.
- 1.2 For each colony to be screened, pipette $20 \ \mu l \ 20 \ mM$ sodium hydroxide into a 0.2-ml thin-walled PCR tube. Using a pipettor tip, scrape up about half of a colony and pipette up and down in the $20 \ \mu l$ sodium hydroxide to disperse the colony in the liquid. Label the colony on the plate so that positive colonies can later be identified.
- 1.3 Incubate tubes for 8 min at 100 $^{\circ}$ C in a PCR machine, with a heated lid.
- **1.4** Briefly spin the tubes in a microfuge to collect liquid at the bottoms of the tubes. Mix each tube vigorously using a vortex mixer at top speed for about 15 s.
- **1.5** Spin tubes in a microfuge for about 30 s at top speed to pellet the cell debris.
- **1.6** Use the supernatant as the template DNA in a PCR reaction, as described in Step 2.

5.3. Tip

Because PCR is so sensitive in detecting very small amounts of DNA, it is possible for DNA from dead cells that have failed to grow on the selective media to be amplified in

colony PCR. Depending on how the primers for the colony PCR have been designed, this can lead to a false band in a negative control reaction. Usually these false bands are faint. This can be minimized by restreaking colonies onto a fresh plate of selective media and using these cells for colony PCR, but adding this step costs 1-2 days.

5.4. Tip

This step is probably not necessary if the organism is E. coli. Good results have been observed when a small amount of an E. coli colony is scraped up with a pipetor tip and dispersed directly into a PCR reaction mix. This also has been effective with some other yeast species and may even sometimes be effective for S. cerevisiae. This step will not decrease the efficacy of the PCR, however, and probably helps the DNA escape the cell wall.

5.5. Tip

A single E.coli colony can be used for simultaneous PCR amplification and overnight growth. First, disperse some of the scraped colony into the PCR master mix. Disperse the remainder into a tube containing 1-2 ml of LB medium plus the appropriate antibiotic and grow overnight at 37 °C with shaking.

See Fig. 25.3 for the flowchart of Step 1.

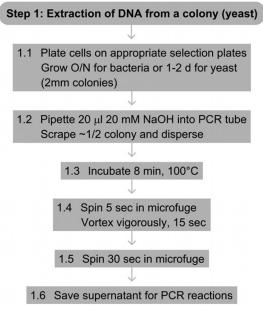


Figure 25.3 Flowchart for Step 1.

6. STEP 2 PCR 6.1. Overview

A standard PCR reaction is used to amplify the product of interest from the DNA that has been extracted from the colony in Step 1. A typical set of PCR conditions is described, but any commercially available thermostable polymerase and its supplied buffer should work (see General PCR).

6.2. Duration

2–2.5 h

2.1 Make a PCR master mix, and keep on ice:

per sample:

16.25 μ l purified water

2.5 μ l 10× PCR buffer

 $2.5 \ \mu l \ dNTP \ mix$

1.25 µl forward primer

1.25 µl reverse primer

0.25 µl 100× Taq

- **2.2** Pipette 24 μl of the PCR master mix into 0.2-ml thin-walled PCR tubes. Keep tubes on ice.
- **2.3** Pipette 0.5–1 μl of the supernatant from each sample into a PCR tube containing PCR master mix.
- **2.4** Use a thermocycler to carry out PCR. Typical reaction conditions are as follows, for a product up to 1 kb in length:

5 min at 95 °C repeat 30 times: 15 s at 95 °C 15 s at 57 °C 1 min at 72 °C finish with: 5 min at 72 °C

6.3. Tip

If skipping Step 1, increase the amount of water per reaction by 1 μ l, and pipette 25 μ l PCR master mix into a 0.2-ml thin-walled PCR tube for each colony to be tested. Then disperse the colonies directly into this liquid. Be sure to label the colonies on the plate so positives can later be identified.

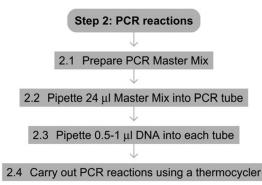


Figure 25.4 Flowchart for Step 2.

6.4. Tip

If using a HotStart Taq polymerase, it is not necessary to place the tubes on ice while setting up the reactions.

6.5. Tip

When preparing a PCR master mix, make enough for the number of reactions plus 0.5-1 extra.

6.6. Tip

If the goal of the colony PCR is to produce a sufficient quantity of a construct for a subsequent transformation step into a different organism, a larger volume PCR can be carried out. It may also be advisable to use a higher fidelity thermostable polymerase, or mixture of polymerases, since any mutations acquired during this PCR step could be deleterious in future cloning steps. Check the supplier's manual if you use a high-fidelity thermostable polymerase. The extension is often carried out at a lower temperature.

See Fig. 25.4 for the flowchart of Step 2.

7. STEP 3 VISUALIZATION OF PRODUCT ON AN AGAROSE GEL

7.1. Overview

The PCR products that have been generated are now separated by size on an agarose gel containing a DNA-intercalating dye such as ethidium bromide (see Agarose Gel Electrophoresis).

7.2. Duration

1.5 h

3.1 Cast the agarose gel mix into a gel tray after boiling in a microwave oven to completely dissolve the agarose. Take care while heating the

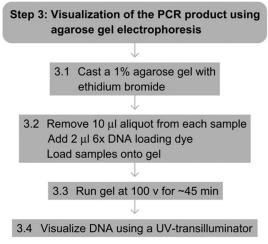


Figure 25.5 Flowchart for Step 3.

gel mix not to allow it to boil over. This can be done while the PCR is running. Fill the gel tank with $1 \times$ TBE, the buffer in which the gel will be run. Note that ethidium bromide is toxic. Wear gloves when handling the gel and take care to dispose of it properly.

- **3.2** Add 2 μl DNA loading dye to 10 μl of each sample. Load onto the gel. Load DNA ladder (as commercially supplied, or about 500 ng DNA in a volume of about 10 μl, containing loading dye) in one lane.
- **3.3** Run the gel at ~ 100 V for 45 min, or until xylene cyanolhas run about three quarters the length of the gel.
- 3.4 Use a UV light box to visualize the DNA bands on the gel.

7.3. Tip

If the goal of the colony PCR is to produce a sufficient quantity of a construct for a future transformation step, run only a small fraction of the PCR product on a gel to verify that the product is the correct size. If a single band is obtained in high enough amounts, the PCR mixture can be used directly for a transformation of some organisms, including S. cerevisiae.

See Fig. 25.5 for the flowchart of Step 3.

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Chemical Transformation of Yeast

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Abstract

Transformation of chemically competent yeast cells is a method for introducing exogenous DNA into living cells. Typically, the DNA is either a plasmid carrying an autonomous replication sequence that allows for propagation or a linear piece of DNA to be integrated into the genome. The DNA usually also carries a marker that allows for selection of successfully transformed cells by plating on the appropriate selective media.

1. THEORY

Since the phenomenon was first reported in 1983, transformation of yeast cells by treatment with alkali cations has become the most commonly used method for yeast transformations. Cells are incubated with a mixture of lithium acetate, polyethylene glycol (PEG), single-stranded carrier DNA, and DNA to be transformed. Next, cells are briefly heat-shocked and plated on selective media. The precise mechanism by which treatment with alkali cations allows for exogenous DNA to enter a yeast cell remains unclear, but it is thought that interactions among the negatively charged PEG, the monovalent cations, and the yeast cell surface result in an increase in permeability of the cell wall and plasma membrane, and that membrane potential then helps drive the exogenous DNA across.

2. EQUIPMENT

Roller drum (30 °C) Rotary shaker (30 °C) Magnetic stirrer Magnetic stir bars Spectrophotometer Bench top or clinical centrifuge Microcentrifuge Vortex mixer Bunsen burner Micropipettors Micropipettor tips Pipet-aid Inoculating loop (optional) 250-ml Erlenmeyer flask, autoclaved Sterile 17×100 -mm glass culture tubes Sterile glass or plastic serological pipettes, 5–25 ml Disposable spectrophotometer cuvettes 50-ml polypropylene conical tubes 1.5-ml polypropylene microcentrifuge tubes, autoclaved 5-mm glass beads, autoclaved (optional) Sterile 100×15 mm disposable Petri plates

3. MATERIALS

YPAD medium (YPD medium supplemented with 400 μM adenine hemisulfate) Bacto agar PEG 3350 Lithium acetate (LiOAc·2H₂O) Salmon sperm DNA Tris base Hydrochloric acid (HCl) EDTA Distilled water

3.1. Solutions & buffers

Prepare $1 \times$ YPAD liquid and agar plates, and $2 \times$ YPAD liquid medium. Prepare selective medium agar plates, either containing an antibiotic (if an antibiotic resistance marker is used) or lacking an essential nutrient (if a nutritional marker is used). See the yeast media recipes on *Saccharomyces cerevisiae* Growth Media

Step 2 50% PEG 3350

Add 50 g PEG 3350 to about 30 ml distilled water in a 150-ml beaker and stir until completely dissolved, heating gently if necessary. Bring the volume up to 100 ml with water and sterilize by autoclaving. This solution can be stored at room temperature, but is best prepared fresh every few months

1 M Lithium Acetate

Dissolve 10.2 g lithium acetate dihydrate in 100 ml distilled water and sterilize by autoclaving

2 mg ml^{-1} single-stranded carrier DNA

Prepare 100 ml sterile TE by adding 1 ml of 1 M Tris–HCl, pH 8.0 and 200 μ l of 0.5 M EDTA to 99 ml sterile water. Dissolve 200 mg of salmon sperm DNA in the 100 ml TE on a magnetic stir plate at 4 °C. This will take several hours. Dispense in 1–5 ml aliquots. Denature carrier DNA by boiling for 5 min and then immediately chilling on ice before use. Small aliquots can be denatured and then stored at -20 °C and used again without reboiling; however, repeated boiling up to 4 times will not adversely affect it. All aliquots should be stored at -20 °C

Component	Final concentration	Stock	Amount per transformation
PEG 3350	33%	50%	240 µl
Lithium Acetate	100 mM	1 M	36 µl
Single-stranded carrier DNA	$280~\mu g~ml^{-1}$	2.0 mg ml^{-1}	50 µl
DNA to be transformed	1 μg/ transformation	About 40 ng μl^{-1}	24 µl

Transformation Mix

4. PROTOCOL

4.1. Preparation

Prior to the start of this protocol, the DNA to be transformed into yeast must be prepared (see Molecular Cloning). Depending on the goals of the transformation, the DNA can be of different forms and sources. Often, a plasmid carrying a selectable marker and an autonomous replication sequence is transformed; the plasmid can be prepared by miniprep isolation of plasmid DNA from a bacterial stock carrying the plasmid, if a stock of the plasmid DNA is not already available. Transformation with plasmid DNA is usually highly efficient. Transformation with a linear piece of DNA that is intended to integrate into the genome by homologous recombination is also very common. There are a couple sources of the linear piece of DNA. It is usually PCR amplified from a cassette carrying a selectable marker using primers that carry sequences to direct homologous recombination. Alternatively, a plasmid carrying a selectable marker and other sequences of interest can be digested with restriction endonucleases. PCR product can be used directly for transformation if the reaction yields a single product in sufficient quantity. If multiple PCR products are present, or in the case of a fragment that has been cut out of a plasmid, the desired linear piece of DNA should be gel-purified away from the other pieces. Because homologous recombination is itself a somewhat low-frequency event, and is required for successful maintenance of the selectable marker, transformation by a linear piece of DNA that must undergo homologous recombination is usually much less efficient than transformation by plasmid DNA. Thus, it is recommended that the higher efficiency version of the protocol is followed for linear pieces of DNA, and that a full 1 µg of DNA is used per transformation (i.e., follow

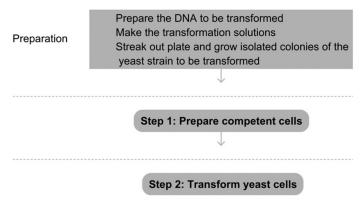


Figure 26.1 Flowchart of the complete protocol, including preparation.

all of Step 1). For plasmid transformations, the lower efficiency protocol and less DNA can usually be used successfully (see Tip after Step 1).

Make solutions (dissolving salmon sperm DNA can take quite a while).

Grow the yeast strain that is to be transformed as patches on an YPAD agar plate.

4.2. Duration

Preparation	Variable
Protocol	About 1 day $+3-4$ days for transformed yeast colonies to grow

See Fig. 26.1 for the flowchart of the complete protocol.

5. STEP 1 PREPARATION OF COMPETENT CELLS 5.1. Overview

The efficiency of transformation correlates with the growth status of the yeast cells used for the transformation – cells in log phase growth transform most efficiently. The protocol described below gives the highest transformation efficiency. If high efficiency is not required (for a transformation using plasmid DNA, for example), see the tip below for a faster version of this step.

5.2. Duration

1 day

1.1 Set up a 5 ml starter culture by scraping up a colony of the yeast strain to be transformed and dispersing it into 5 ml of YPAD medium in a

 17×100 -mm glass culture tube. Work near a Bunsen burner flame with aseptic technique.

- **1.2** Allow starter to grow to saturation overnight at 30 °C (or room temperature if the strain is temperature sensitive) on a roller drum.
- **1.3** The next morning, measure the OD at 600 nm of the starter culture, using a spectrophotometer. Dilute the culture 1:20 with YPAD medium in a plastic cuvette before making a measurement, in order to bring the OD within the linear range of the spectrophotometer.
- **1.4** Use the starter culture to inoculate a 50 ml culture in $2 \times$ YPAD in a sterile 250-ml Erlenmeyer flask at an OD₆₀₀ of 0.2.
- **1.5** Allow the culture to grow at 30 $^{\circ}$ C on a rotary shaker for 4–5 h, or until the OD₆₀₀ reaches ~1.0–1.5.
- **1.6** Transfer the culture to a 50-ml conical centrifuge tube. Harvest the yeast cells by spinning at 3000 rpm at room temperature for 5 min in a benchtop centrifuge.
- **1.7** Discard the supernatant and resuspend the pellet in 20 ml of sterile, room temperature water.
- 1.8 Pellet the cells by spinning again at 3000 rpm for 5 min.
- 1.9 Resuspend the pellet in 1 ml sterile water and transfer it to a 1.5-ml microcentrifuge tube. Use $100 \ \mu l$ of this cell suspension per transformation.

5.3. Tip

Remember to take into account the 1:20 dilution of the overnight starter culture. For example, if the OD_{600} reading is 0.4, then the actual OD_{600} of the starter culture is 8. Usually this will require adding $\sim 1-2$ ml of the starter culture to 48–49 ml of 2× YPAD media, although the exact amount needed will vary greatly with the growth rate of the strain and the amount of time it had been left to grow.

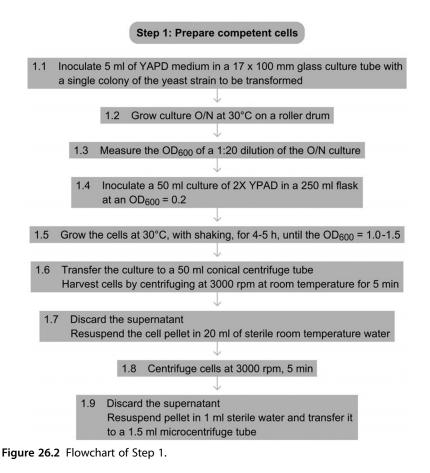
5.4. Tip

If a very high efficiency of transformation is not required, cells can be used directly from a fresh patch on an agar plate. The patch should be no more than a few days old. Scrape approximately a $50-\mu$ l volume of cells off of the plate and disperse them in $100 \ \mu$ l of sterile water in a microcentrifuge tube. Follow the rest of the protocol as written.

5.5. Tip

Cells harvested by this protocol can be stored at $4 \,^{\circ}$ C for up to a week with only minor losses in transformation efficiency. If planning to store cells, pellet them out of the sterile water suspension and resuspend them in 100 mM lithium acetate instead.

See Fig. 26.2 for the flowchart of Step 1.



6. STEP 2 TRANSFORMATION OF YEAST CELLS 6.1. Overview

An exogenous piece of DNA is introduced to the cells in the presence of lithium acetate and polyethylene glycol. The addition of single-stranded salmon sperm DNA as a carrier dramatically increases the efficiency of transformation.

6.2. Duration

- 1.5–2 h
- **2.1** Transfer 100 μ l of the cell suspension into a 1.5-ml microcentrifuge tube. Centrifuge the cells at 10 000 rpm for 1 min in a microcentrifuge. Discard the supernatant.

- 2.2 Add 360 µl Transformation Mix and resuspend the cells by mixing vigorously using a vortex mixer.
- **2.3** Incubate 15–30 min at 30 $^{\circ}$ C. Ensure mixing by taping the tubes to a roller drum.
- 2.4 Heat-shock cells by incubating for 15–40 min in a 42 °C water bath.
- 2.5 Collect cells by spinning at 10 000 rpm for 1 min in a microcentrifuge.
- **2.6** Discard supernatant and gently resuspend the cell pellet in 500 μ l of sterile water.
- 2.7 Pipette 20–200 µl cell suspension onto the surface of an agar plate, depending on expected transformation efficiency. For plasmid transformations, generally less is required, while for transformations with linear DNA more cells should be plated.
- **2.8** Add 5–20 autoclaved 5 mm glass beads to each plate by shaking them out of their bottle. Replace the lids on the plates and shake back and forth briefly to disperse the cells over the surface of the plate. A whole stack of plates can be shaken simultaneously. After the transformation reaction has been spread over the surface of the plate, dump the glass beads into a receptacle containing 70% ethanol. They can be washed, autoclaved, and reused.
- **2.9** Incubate plates at 30 °C, or at room temperature for temperaturesensitive strains. For antibiotic resistance markers, replica plate to antibiotic-containing plates the day after plating to YPAD. It should take 3–4 days for sizeable yeast colonies to grow up following transformation.

6.3. Tip

The optimal heat shock time varies from strain to strain. Generally, longer heat shock times improve transformation efficiency, but may lead to increased cell death, especially for temperature-sensitive strains. Extremely temperature-sensitive strains can be transformed by incubating overnight at room temperature rather than at 30 and 42 °C as described above.

6.4. Tip

Some commonly used antibiotics and their concentrations to use for selection include Geneticin (G418, 0.2 mg ml⁻¹), nourseothricin (NAT, 0.2 mg ml⁻¹), and hygromicin B (HPH, 0.3 mg ml⁻¹).

6.5. Tip

If the yeast strain being transformed is unfamiliar, it is advisable to perform a mock transformation with water being added to the transformation mix instead of DNA, to use as a negative control.

6.6. Tip

If unsure what efficiency is expected, plate 20 μ l on one plate and 200 μ l on a second plate. For plating 20 μ l, add 100–200 μ l of sterile water directly to the plate. If the transformation efficiency is anticipated to be very low, plate the entire transformation reaction, splitting it between at least two plates. Increased plating density lowers the transformation efficiency. If using a nutritional marker for selection, plate directly onto

Step 2: Transform yeast cells	
2.1 Transfer 100 µl of the competent cell suspension into a 1.5 ml tube Centrifuge at 10,000 rpm for 1 min Discard the supernatant	
2.2 Add 360 µl Transformation Mix: 33% PEG 3350 100 mM LiOAc 280 µg/ml ssDNA 1 µg DNA (to be transformed) Resuspend cells by vortexing ↓ 2.3 Incubate cells at 30°C for 15-30 min Tape tube to a roller drum to mix ↓ 2.4 Heat shock cells in a 42°C water bath for 15-40 min ↓ 2.5 Centrifuge cells at 10,000 rpm for 1 min ↓ 2.6 Discard supernatant Resuspend pellet in 500 µl sterile water ↓ 2.7 Pipette 20-200 µl of the transformation mix onto an agar plate (lower amount for transformations with plasmid DNA, higher amounts for transformations with linear DNA)	
2.8 Add 5-20 autoclaved 5 mm glass beads onto each plate	
Close the lid and shake back and forth to distribute the cells over the plate	Remove the glass beads into a receptacle containing 70% ethanol Wash and autoclave beads for reuse
2.9 Incubate plates at 30°C for 3-4 days until sizable yeast colonies grow	For antibiotic resistance markers, initially plate cells on YPAD plates After 1 day replica plate onto YPAD + antibiotic plates

Figure 26.3 Flowchart of Step 2.

selective media. If using an antibiotic resistance marker, plate onto an YPAD agar plate.

6.7. Tip

Alternatively, a bent glass rod that has been sterilized by dipping in ethanol and flaming can be used to spread the transformation reaction over the surface of the plate (see how to flame-bend a glass Pasteur pipette and spread the yeast on Pouring Agar Plates and Streaking or Spreading to Isolate Individual Colonies).

See Fig. 26.3 for the flowchart of Step 2.

REFERENCES

Related Literature

Gietz, R. D., & Schiestl, R. H. (2007). High-efficiency yeast transformation using the LiAc/ SS carrier DNA/PEG method. *Nature Protocols*, 2(1), 31–34.

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Referenced Protocols in Methods Navigator

Saccharomyces cerevisiae Growth Media Molecular Cloning Pouring Agar Plates and Streaking or Spreading to Isolate Individual Colonies



Transformation of *E. coli* Via Electroporation

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Abstract

To create electro-competent *E. coli* and transform them with a plasmid of choice via electroporation.

1. THEORY

Electroporation of *E. coli* is a popular alternative to traditional heatshock transformation of chemically competent cells. A high-voltage current is applied to the cells, which temporarily permeabilizes the plasma membrane and allows DNA or other small molecules to enter. The main advantages of electroporation over heat-shock transformation are the higher efficiency in the uptake of plasmid DNA and a faster, less involved production of competent cells.

2. EQUIPMENT

Electroporator for bacteria (e.g., Gen Pulser Xcell Electroporation System, Bio-Rad) Refrigerated centrifuge Refrigerated microcentrifuge Shaking incubator (37 °C) Incubator (37 °C) 15-ml sterile polypropylene snap-cap tubes Electroporation cuvette, 0.1 cm gap 15-ml glass centrifuge tube Rubber adaptors (to fit glass centrifuge tube into floor centrifuge rotor) Pipettes Micropipettors Micropipettor tips 1.5-ml microcentrifuge tubes Kimwipes

3. MATERIALS

Plasmid DNA (to be transformed) LB agar plates (selective plates containing appropriate antibiotic) Lysogeny broth (LB) Bacto agar Sterile ddH₂O Glycerol (optional)

4. PROTOCOL

4.1. Preparation

Inoculate 5 ml of LB with E. coli. Grow overnight at 37 °C with shaking.

Chill the 15-ml glass centrifuge tube, a 1.5-ml microcentrifuge tube, the electroporation cuvette, and the ddH_2O on ice. Run the floor centrifuge for a few minutes to cool the chamber to 4 °C.

4.2. Duration

Preparation	15 min $+$ overnight	
Protocol	About 2 h	

4.3. Tip

For most simple plasmid transformations, it is not necessary to harvest bacteria at early to mid log phase. However, if the transformation efficiency is low, dilute 1 ml of the overnight culture in 20 ml of LB and grow at 37 °C with shaking until the OD₆₀₀ measures between 0.4 and 0.6 (1–1.5 h). Use 10 ml to make 50 μ l of competent cells as described subsequently.

4.4. Tip

For all steps, keep reagents, tubes, and bacteria on ice at all times to ensure the production of high-quality electro-competent cells. Keeping the bacteria at a low temperature during the electric pulse also helps prevent electrically induced heating and thus increases cell survival.

See Fig. 27.1 for the flowchart of the complete protocol.

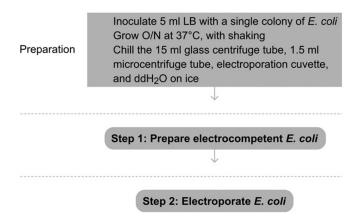


Figure 27.1 Flowchart of the complete protocol, including preparation.

5. STEP 1 CREATE ELECTRO-COMPETENT E. COLI

5.1. Overview

E. coli are washed several times with ice-cold water to prepare them for the uptake of plasmid DNA during electroporation.

5.2. Duration

20-30 min

- **1.1** Pour the bacterial culture into the prechilled 15-ml glass centrifuge tube on ice.
- **1.2** Centrifuge at 7000 rpm at 4 °C, for 5 min.
- **1.3** Discard the supernatant.
- **1.4** Resuspend the pellet in 1 ml of sterile, ice-cold ddH₂O and transfer into a chilled 1.5-ml microcentrifuge tube.
- 1.5 Spin in a microcentrifuge at 7000 rpm at 4 °C, for 5 min.
- 1.6 Discard the supernatant.
- **1.7** Resuspend the pellet in 1 ml of ice-cold ddH_2O .
- 1.8 Repeat steps 1.5–1.7 two more times for a total of three washes.
- **1.9** Spin a final time at 7000 rpm at 4 °C, for 5 min, discard the supernatant, resuspend the pellet in 50 µl of ice-cold ddH₂O, and put the cells on ice.

5.3. Tip

If you want to store the electro-competent cells at $-80^{\circ}C$ for later use, substitute 10% glycerol for ddH₂O in Step 1.4 and in all subsequent steps.

5.4. Tip

This protocol can be scaled up to make larger quantities of competent cells. See Fig. 27.2 for the flowchart of Step 1.

6. STEP 2 ELECTROPORATION OF *E. COLI*

6.1. Overview

Plasmid DNA is added to electro-competent *E. coli*. An electric pulse mediates the uptake of the DNA by the bacteria.

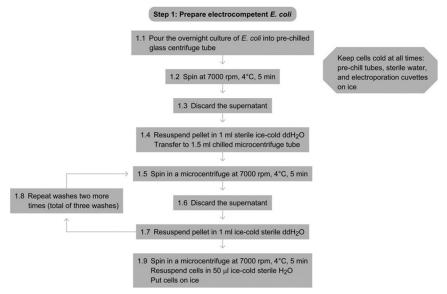


Figure 27.2 Flowchart of Step 1.

6.2. Duration

1 h and 10 min

- **2.1** Add 50–100 ng of supercoiled plasmid to the electro-competent *E. coli* and mix gently (do not pipette up and down).
- **2.2** Transfer the bacteria to a chilled electroporation cuvette. Be careful to pipette straight in between the metal plates and avoid introducing any bubbles.
- **2.3** Cap the cuvette and tap it lightly on the bench to settle the bacteria/ DNA mix.
- 2.4 Put the cuvette back on ice and carry it to the electroporator.
- **2.5** Turn on the electroporator and set it to 1.8 kV, 25 μ F, 200 Ω . This is a standard setting for most *E. coli* strains. Other bacterial strains may require an adjustment of the electroporation conditions.
- **2.6** Wipe the cuvette briefly with a Kimwipe to remove any residual water or ice, and then place it in the electroporation chamber.
- **2.7** Push the pulse button. The time constant displayed should be around 4 ms.
- **2.8** *Immediately* after the pulse has been delivered, add 1 ml of LB (or other growth medium, e.g., SOC) to the cuvette and pipette quickly but gently up and down. Be aware that the transformation efficiency

decreases proportionally to the lag time between the electric pulse and the addition of media.

- 2.9 Transfer the mixture to a fresh 1.5-ml microcentrifuge tube.
- 2.10 Incubate at 37 °C for 1 h with shaking.
- **2.11** Evenly spread 100 μ l of your transformation onto a selective plate. Electroporation is highly efficient and often yields a very large number of colonies. To ensure that individual, medium-sized colonies can be picked the next day, mix 10 μ l of the transformation with 90 μ l of LB and spread this 1:10 dilution evenly onto another

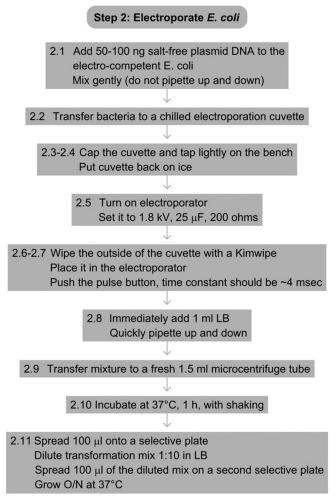


Figure 27.3 Flowchart of Step 2.

selective plate. Incubate both plates upside down overnight at 37 $^{\circ}\mathrm{C}.$

6.3. Tip

Using low amounts of well-purified DNA is key to a successful electroporation. Even small amounts of residual salt from DNA preparations may interfere with the electric current and cause arcing, resulting in excessive cell death.

6.4. Tip

Warm up the selective plates at $37 \,^{\circ}$ C while the bacteria are recovering. Cold plates lead to lower transformation efficiency.

See Fig. 27.3 for the flowchart of Step 2.

REFERENCES

Related Literature "Gene Pulser Xcell Electroporation System" Instruction Manual, BioRad



Transformation of Chemically Competent *E. coli*

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Abstract

To introduce DNA into E. coli cells.

1. THEORY

Transformation of E. *coli* is an important step that allows the introduction of heterologous DNA using plasmid vectors or introducing mutations via homologous recombination events.

2. EQUIPMENT

Shaking incubator (37 °C) UV/Vis spectrophotometer Refrigerated low-speed centrifuge (4 °C) Water bath (42 °C) Incubator (37 °C) Erlenmeyer flask, 500 ml (sterile) 10-cm Petri plates 0.2-µm filters 14-ml sterile polypropylene snap-cap tubes Disposable cuvettes 50-ml sterile polypropylene tubes (e.g., Corning 430829) 2-ml sterile screw-capped conical tubes with no skirt (e.g., Phenix SCS-02 S) 5–10-ml glass vials or autoclavable screw-capped tubes Glass spreader

3. MATERIALS

Antibiotic of choice Bacto agar Bacto tryptone Bacto yeast extract Calcium chloride (CaCl₂) Magnesium chloride (MgCl₂) Magnesium sulfate (MgSO₄) Manganese chloride (MnCl₂) 3-(*N*-Morpholino)-propanesulfonic acid (MOPS) Potassium acetate (KOAc) Potassium chloride (KCl) Rubidium chloride (RbCl) Sodium chloride (NaCl) Glucose Glycerol Acetic acid Sodium hydroxide (NaOH) Potassium hydroxide (KOH) Dry ice Ethanol Liquid nitrogen

3.1. Solutions & buffers

Step 1 Psi Media

Component	Amount
Tryptone	20 g
Yeast extract	5 g
MgCl ₂	5 g

Dissolve in 900 ml deionzed water. Adjust the pH to 7.6 with KOH. Add water to 1 l and autoclave to sterilize

Note: You can also use LB medium (low salt) supplemented with 4 mM $MgSO_4$ and 10 mM KCl or SOB (also commercially available)

Component	Final concentration	Amount
Potassium acetate	30 mM	1.18 g
RbCl ₂	100 mM	4.84 g
CaCl ₂ ·2H ₂ O	10 mM	0.59 g
MnCl ₂ ·4H ₂ O	50 mM	3.96 g
Glycerol	15% (v/v)	60 ml

Tfb I (Transformation Buffer I)

Dissolve in 300 ml water. Adjust the pH to 5.8 with dilute acetic acid. Add water to 400 ml and pass through a 0.2- μ m filter to sterilize

Component	Final concentration	Amount
MOPS	10 mM	0.21 g
$CaCl_2 \cdot 2H_2O$	75 mM	1.1 g
RbCl ₂	10 mM	0.12 g
Glycerol	15% (v/v)	15 ml

Tfb II (Transformation Buffer II)

Dissolve in 75 ml water. Adjust the pH to 6.5 with dilute NaOH. Add water to 100 ml and pass through a 0.2-µm filter to sterilize

Component	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g

Step 2 LB Agar (Miller's high salt)

Dissolve in 900 ml water. Adjust the pH to 7.2 with \sim 0.2 ml 5 N NaOH. Add water to 1 l and autoclave to sterilize. Cool to \sim 55 °C, add appropriate antibiotic and pour plates

Note: LB Agar is available commercially as a premixed powder

SOC media

Component	Final concentration	Stock	Amount
Yeast extract	0.5%	_	0.5 g
Tryptone	2%	_	2.0 g
NaCl	10 mM	3 M	0.33 ml
KCl	2.5 mM	1 M	0.25 ml
MgCl ₂	10 mM	1 M	1 ml
MgSO ₄	10 mM	1 M	1 ml
Glucose	20 mM	1.1 M	1.82 ml

Dissolve in 90 ml of deionized water and bring the volume to 100 ml. Dispense into 5–10 ml aliquots and autoclave to sterilize

4. PROTOCOL

4.1. Preparation

Pour selective agar plates and let harden.

Pick a single colony from a freshly streaked plate (without antibiotics!) and inoculate a small culture (2-5 ml) of Psi media in a sterile 14-ml snap-cap tube. Grow overnight at 37 °C with shaking at 250 rpm.

Store TfbI and TfbII at 4 °C to make sure they are chilled.

4.2. Duration

Preparation	About 15 min		
Protocol	About 4–6 h		

See Fig. 28.1 for the flowchart of the complete protocol.

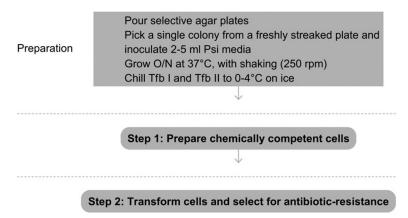


Figure 28.1 Flowchart of the complete protocol, including preparation.

5. STEP 1 PREPARE COMPETENT CELLS

5.1. Overview

Grow cells to mid-log phase and make competent by chemical treatment.

5.2. Duration

3–5 h

- **1.1** Inoculate 100 ml of Psi media with 0.5 ml of the overnight culture and incubate at 37 °C with vigorous shaking.
- **1.2** When A_{600} reaches 0.4–0.5, place on ice and chill for 5–10 min.
- **1.3** Transfer the cells to 50-ml sterile chilled polypropylene centrifuge tubes. Pellet cells at $5000 \times g$, 4 °C for 5 min.
- **1.4** Discard supernatant carefully and gently resuspend the cell pellet in 0.4 volume of ice-cold Tfb I (20 ml for each 50 ml tube). Do not vortex; keep on ice while resuspending.
- **1.5** Incubate the cells on ice for 15 min.
- **1.6** Pellet the cells at $2000 \times g$, 4 °C for 10 min.
- **1.7** Discard the supernatant carefully and gently resuspend in 0.02 volume of ice-cold TfbI II (1 ml for 50 ml of culture). Keep the tube on ice.
- 1.8 Aliquot 50 μ l into 2-ml prechilled sterile screw-capped tubes with conical bottoms.
- **1.9** Flash-freeze in a dry ice-ethanol bath or liquid nitrogen and store at -80 °C.

5.3. Tip

Use sterile technique at all times; the bacteria are not antibiotic-resistant at this point.

5.4. Tip

If reusable tubes are used to pellet the cells, they must be very clean and free of soap residue.

5.5. Tip

Cells and transformation buffers should be kept cold at all times. It is also preferable to use chilled pipettes and do everything in the cold room if possible.

5.6. Tip

The incubation in Tfb I can vary from 5 min to 1-2 h without any harm. The cells must be kept on ice.

5.7. Tip

Do not use standard 1.5-ml conical microcentrifuge tubes – they do not work well in the heat shock step of the transformation.

See Fig. 28.2 for the flowchart of Step 1.

6. STEP 2 TRANSFORM COMPETENT CELLS

6.1. Overview

Introduce DNA into competent cells and select for antibiotic-resistant bacteria.

6.2. Duration

2 h

- **2.1** Equilibrate a water bath to 42 °C. A dry heating block will work if the tube fits snugly, but it is not as good as a water bath.
- **2.2** Thaw one vial of competent cells on ice for each transformation. Handle gently since cells are sensitive to temperature changes and mechanical lysis.
- **2.3** Add 1–5 μl of DNA (10 pg to 100 ng) to a vial of thawed competent cells. DO NOT VORTEX OR PIPETTE UP AND DOWN.
- **2.4** Incubate on ice for 30 min.
- **2.5** Heat-shock cells for 30 s at 42 $^{\circ}$ C. Do not go any longer or shake the tube.
- **2.6** Remove the tube from the water bath and place on ice for 2 min.
- 2.7 Add 250 µl of SOC media to each vial.
- **2.8** Make sure that the cap is tight and incubate the tube on its side in a $37 \degree$ C shaking incubator (200–250 rpm) for 1 h.

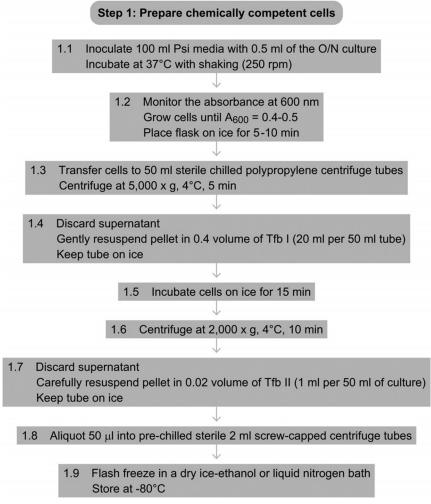


Figure 28.2 Flowchart of Step 1.

2.9 Spread from 20 to 200 μ l on an appropriate selective plate. The plates should be at room temperature or prewarmed to 37 °C. Incubate overnight at 37 °C.

6.3. Tip

Validate the chemically competent cells by plating untransformed cells on LB plates (without antibiotic – should have a lawn of cells) and LB plates containing antibiotic (should not have any colonies growing on them).

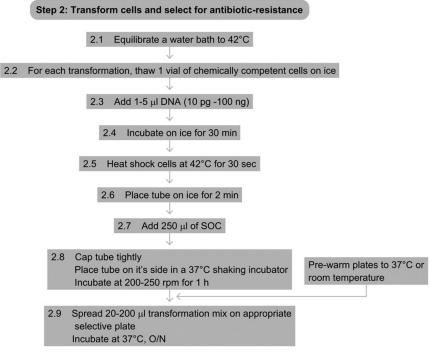


Figure 28.3 Flowchart of Step 2.

6.4. Tip

Transform the cells with 10, 30, and 100 pg of supercoiled plasmid DNA and determine the transformation efficiency (# colonies per microgram DNA).

6.5. Tip

Bacteria are transformed more efficiently using supercoiled DNA than ligated DNA.

6.6. Tip

Transformed cells can be stored at $4 \degree C$ for 24-48 h with minimal loss of viability. Transformation efficiency varies depending on DNA.

See Fig. 28.3 for the flowchart of Step 2.

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