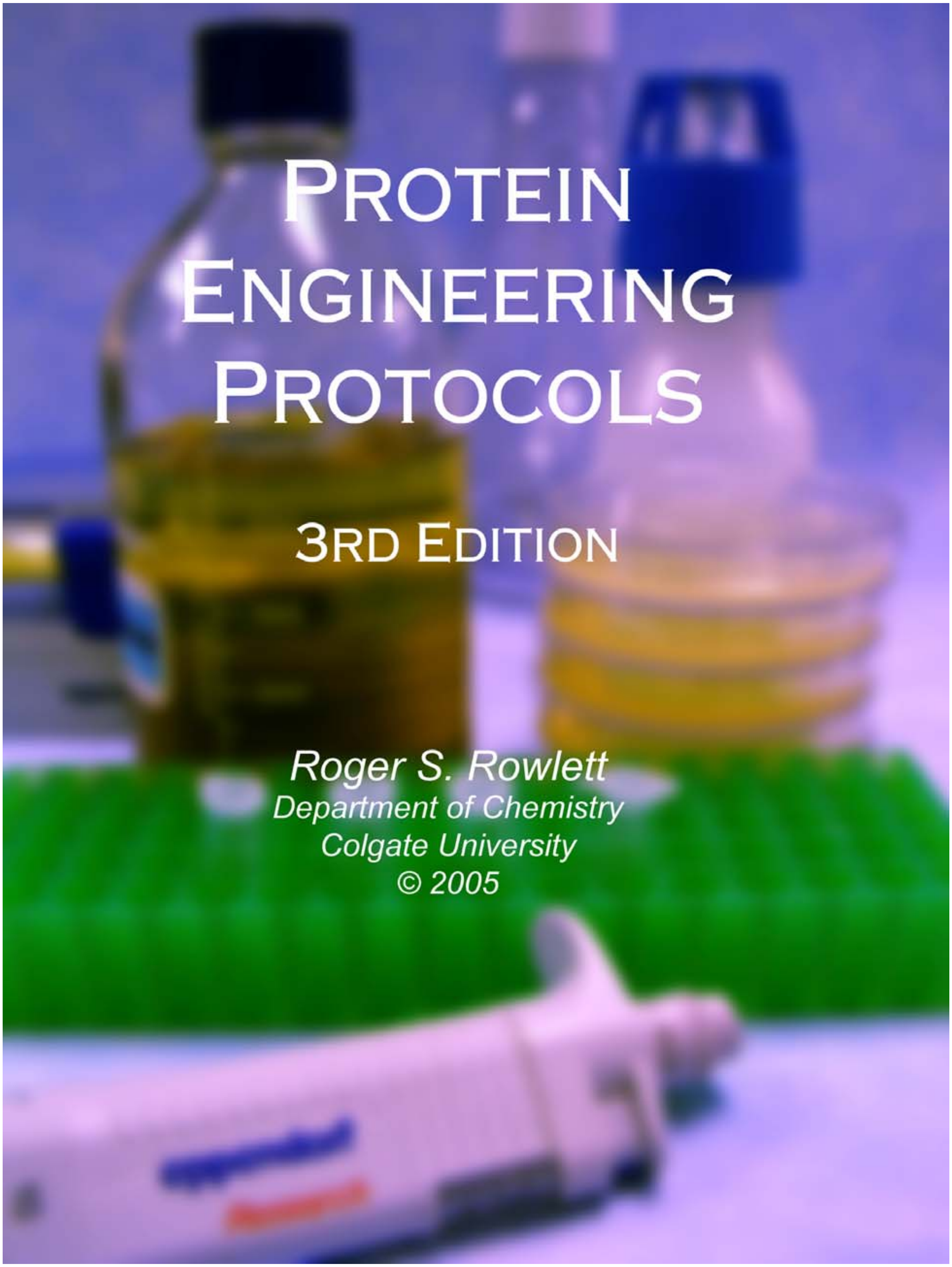

# Protein Engineering Protocols

## 3rd Edition


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## Introduction

This manual was originally written in 1999 to provide a compendium of up-to-date commonly used protocols which my research students use in my protein engineering laboratory at Colgate University. Much of the information contained in this manual was gleaned from my knowledgeable, kind, and very clever colleagues in the Laboratory of Dr. Edith Wilson Miles in the National Institute of Diabetes and Digestive and Kidney Diseases at the National Institutes of Health. Special thanks goes to Dr. Li-hong Yang, my very patient molecular biology and recombinant DNA mentor. I am forever in debt to his expert tutelage. Additional thanks go to the many Colgate undergraduates who have suggested and trialed new (to us) protein engineering methods in the research laboratory.

## Abbreviations Used

Protein engineering is a specialty which is unavoidably littered with jargon and abbreviations. Some of the more common acronyms and abbreviations used are listed here.

ATP – adenosine-5'-triphosphate
bp – base pair
BSA – bovine serum albumin
DMSO – dimethylsulfoxide
DNA – deoxyribonucleic acid
dNTP – deoxynucleotide tripshosphate
ds – double-stranded
DTT – DL-dithiothreitol
IPTG – isopropylthio-β-D-galactoside
LB medium – Luria-Bertani medium
nt – nucleotide
PAGE – polyacrylamide gel electrophoresis
PEG – polyethylene glycol
PCR – polymerase chain reaction
SDS – sodium dodecyl sulfate (*a.k.a.* sodium lauryl sulfate, sodium laureth sulfate)
ss – single-stranded
TB medium – "Terrific Broth" medium
TEMED – *N,N,N',N'*-tetramethylethylenediamine
X-Gal – 5-bromo-4-chloro-3-indolyl-β-D-galactoside

# Microbiology Protocols

## Preparation of Liquid Media

*LB medium*. This is a standard liquid medium used to grow cultures of *E. coli*. The recipe can be found in Appendix 1. All solid reagents should be added to the appropriate size Wheaton bottle (or other autoclavable container), and water added. When most of the solids have been dissolved (it is not necessary to completely dissolve all the solids) the solution should be neutralized by the addition of the indicated amount of NaOH. The bottle should be loosely capped—which should be secured from untwisting with tape—and autoclaved for 20 minutes at 250 °F. The solution should cool to 50 °C before any antibiotics, if needed, are added. (If you are able to hold the bottle in an ungloved hand without discomfort, it is cool enough for the addition of antibiotics.) After the addition of antibiotics, the solution should be swirled vigorously to mix thoroughly.

Media prepared without antibiotics can be stored at room temperature until opened and used the first time. Thereafter media should be stored in the refrigerator. Media prepared with antibiotics, especially ampicillin, should be stored in the refrigerator immediately.

*TB medium.* This is a standard "rich" medium used to grow *E. coli* cultures to very high cell density, and is thus useful for protein overexpression. The recipe can be found in Appendix 1. It is important to note that the nutrient media and the phosphate buffer solutions must be prepared and autoclaved separately. Once the solutions have cooled, they are combined using sterile technique, and antibiotics and any other additives added at that time. This medium should be swirled vigorously to mix and stored in the refrigerator if not used immediately.

## Preparation of agar plates

*LB agar plates.* Preparation is exactly as for LB medium, except that 15 g of agar is added to the mixture prior to autoclaving. After removal from the autoclave, the solution should be swirled to ensure even mixing of the agar, which will be concentrated at the bottom of the bottle. The solution must be cooled to 50 °C before adding antibiotics. Swirl to mix, but avoid introducing bubbles. Pour about 20 mL of the medium into 90 mm plates—just enough to cover the entire bottom of the plate about 2-3 mm deep—working quickly so that the agar medium does not harden in the bottle. Flame the neck of the bottle occasionally to maintain sterility. One liter of LB-agar should be sufficient to pour 40-50 plates. Plates should be appropriately marked to identify the antibiotic, if any, used in the medium. A suggested code is one black stripe on the edge of the lid to indicate an LB plate, two red stripes to indicate an LB-ampicillin plate, three green stripes to indicate an LB-chloramphenicol plate, *etc*.) Allow the plates to harden. If the plates "sweat" excessively, they may be placed in a 37 °C incubator overnight to remove condensastion. Plates should be stored inverted at 4 °C, and warmed to room temperature prior to use.

**Streaking out cultures from frozen glycerol stocks**

Remove an LB plate with the appropriate antibiotic, if necessary, and warm it to room temperature. If necessary, remove condensation by incubation in a 37 °C incubator. Label the bottom of the plate with your initials, date, and strain of *E. coli* used. For example the label

RSR
3-17-99
pARCANX/JM109

indicates that individual RSR streaked out a culture of *E. coli* strain JM109 harboring the pARCANX plasmid on March 17, 1999. If this plate is discovered in the incubator or refrigerator in June 1999 someone will realize that it needs to be discarded!

Sterilize an inoculating loop by heating in a flame until it glows cherry red over its entire length. Allow it to cool before using it to streak samples. Remove the cryovial containing the frozen glycerol stock from the –80 °C freezer, and *without letting the sample thaw*, take the sterile inoculating loop and scrape a little bit of ice from the surface of the frozen stock. *You do not need much material:* if you can see it on the loop, you have too much. Take the inoculating loop and touch it to the agar plate near one edge. In one continuous motion, drag the loop across the plate 20-25 times, as shown in Figure 1:

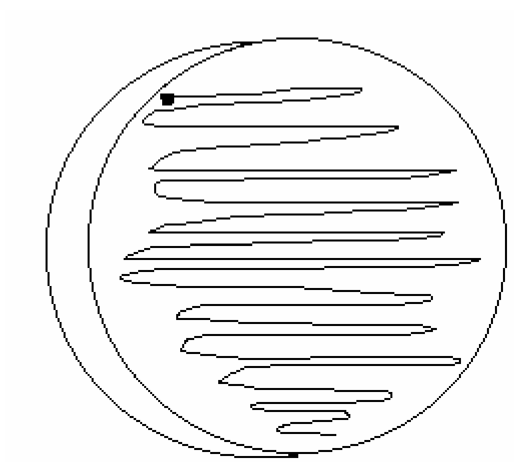

**Figure 1.** Streaking an agar plate to obtain single colonies (clones).

Replace the lid, invert the plate, and incubate overnight at 37 °C. If the plate has been streaked correctly, the bacterial growth will be nearly continuous and confluent near the beginning of the streak, but will thin out into single colonies somewhere farther down the plate. These single colonies represent growth from a single bacterial cell.

Streaked plates may be stored for a several weeks in the refrigerator. To keep plates from drying out, cut a strip of parafilm 1 × 10 cm and stretch it tightly around the edge of the plate, sealing the lid to the bottom. For longer term storage of bacterial cultures, frozen glycerol stocks should be prepared. Store plates inverted to prevent condensation from dripping on the agar surface.

## Preparation of liquid cultures of *E. coli*

Using sterile technique, pipet LB medium with the appropriate antibiotics, if needed, into a sterile culture tube. (For 2 mL cultures, use a 17 × 100 mm polypropylene or polycarbonate tube with a snap-top cap; for 5-10 mL culture, use a 50 mL conical polypropylene tube with a screw cap). Using a sterile pipettor tip scrape a *single* colony from the appropriate agar plate. Using sterile technique, tilt the culture tube until the medium is 1-2 cm from the top of the tube, and vigorously rub off the bacterial colony from the tip into the medium. For cultures in 17 × 100 mm tubes, replace the snap-top cap and adjust it so that it is in the "loose" position—this will allow in air for aerobic growth. For cultures in 50 mL conical tubes, replace the cap but leave it loosely screwed on; tape the cap to the tube to prevent it from unscrewing. Cultures are grown overnight at 37 °C with shaking on an orbital platform at ≈250 rpm. Overnight cultures should be used immediately, or within 24 hours if refrigerated.

## Preparation of frozen glycerol stocks of *E. coli*

An overnight culture of *E. coli* should be shaken by hand to resuspend cells thoroughly. Using sterile technique, pipet 0.5 mL of bacterial culture into a sterile 1.5 mL cryovial, and dilute with an equal volume of sterile 30% glycerol. Cap the vial tightly and mix the contents of the vial completely by inverting the vial repeatedly. Glycerol stocks may be stored for a day or two at –20 °C but should normally be immediately frozen at –80 °C. Frozen stocks at this temperature, and not subjected to repeated thawing and freezing, will remain viable nearly indefinitely.

## Preparation of competent cells[1]

The competent cells produced according to the following procedure are good for routine transformations, and typically yield ≈$10^6$-$10^7$ transformants per μg of DNA. High efficiency competent cells (>$10^8$ transformants per μg DNA) are generally required for transformations of ligation mixtures, and are best purchased commercially.

- Streak out *E. coli* JM109 (or other strain) on an LB plate. Incubate overnight at 37 °C.
- Transfer a colony into 5 mL of LB medium in a 50 mL conical tube. Shake overnight at 37 °C.
- Inoculate 5 mL of LB medium with 50 μL of overnight culture and incubate at 37 °C with shaking to an $OD_{600}$ of 0.3-0.4, about 2 hr. (The exact optical density is not that critical—even overnight cultures will produce reasonably competent cells.)
- Add an equal volume of ice-cold sterile 2× TSS buffer (20% PEG 8000-40 mM $MgSO_4$-10% DMSO) and incubate for 5-15 minutes.
- Quickly dispense 100 μL aliquots of the suspension into chilled, sterile, microcentrifuge tubes. Immediately snap-freeze cells by immersion of the tubes into liquid nitrogen.
- Store tubes at –80 °C until needed.

[1] Chung, C. T., & Miller, R. H. *Meth. Enzymol.* **1993**, *218,* [43]

## Transformation of competent cells

The following transformation protocol works well with home-made and many commercially obtained competent cells.

- Gently warm 50-100 µL of competent cells by hand until just thawed, then place in an ice bath.
- Add 1-10 µL (typically 5 µL) of ligation mixture, or 10-100 ng of DNA. Flick tube gently to mix.
- Place mixture on ice for 15-60 min. (Longer times will generate more transformants.)
- Add 500 µL of LB medium (*with no antibiotic*) supplemented with 5 uL of sterile 2.0 M glucose, mix gently, and incubate for 1 hr at 37 °C.
- Using a bent glass or stainless steel rod—sterilized by immersion in 95% ethanol flaming off three times— spread an aliquot of 100-400 µL (typically 400 µL) of the mixture on LB plates containing 50 µg/mL ampicillin or other appropriate antibiotic. (*Note:* if blue-white screening[2] is desired, add 35 µL 2% X-Gal and 15 µL 100 mM IPTG to the mixture before spreading.)
- Incubate plates overnight at 37 °C, but not more than 18 hr to prevent satellite colony formation.

## Transformation of Z-competent cells

Z-competent cells (Zymo research) are high efficiency (>$10^8$ cfu/µg DNA) commercial competent cells with an extremely simple transformation procedure, described below:

- Pre-warm an agar plate with the appropriate antibiotic selection factor to 37 ºC. *This step is critical to efficient transformation.*
- Take a single tube (100 µL) of Z-competent cells and thaw on ice.
- Add 1-5 µL of DNA to cells and mix gently.
- Incubate cells on ice for 10-60 minutes. (Longer times will generate more transformants.)

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[2] Many plasmid vectors, such as pUC18, contain a short segment of *E. coli* DNA which codes for the first 146 amino acids of the β-galactosidase gene, *lacZ*. In the middle of these gene there is a cloning site which inactivates this gene fragment if foreign DNA is inserted there. If such a plasmid is used in a host strain of *E. coli*, such as JM109, that contains a chromosomal copy of a gene which codes for the complementary C-terminus of β-galactosidase, the presence or absence of a DNA insert in a clone or bacterial colony can be evaluated by screening for β-galactosidase activity. If there is no gene insert in the vector of a clone or colony, the intact N-terminus of β-galactosidase can complement with the chromosomal C-terminus of the enzyme, yielding active protein. On the other hand, if a DNA insert fouls up the plasmid-encoded N-terminus of β-galactosidase, then the clone or colony will have no β-galactosidase activity. Clones (colonies) can be screened right on the agar plate if a chromogenic β-galactosidase substrate and inducing agent is included on the agar plate. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) is a colorless compound which turns blue when cleaved by the β-galactosidase. Thus, clones (colonies) of bacteria which have no DNA insert in the plasmid will appear blue, and those which have DNA insert will appear white. The blue color can be enhanced by leaving the plates out at room temperature for several hours after colonies have formed. Our laboratory rarely uses blue-white screening because the proportion of transformants with DNA insert should be in excess of 90% if the appropriate precautions are taken.

- Using a bent glass or stainless steel rod—sterilized by immersion in 95% ethanol and flaming off three times— spread an aliquot of 50-100 μL (typically 100 μL) of the mixture on LB plates containing 50 μg/mL ampicillin or other appropriate antibiotic.
- Incubate plates overnight at 37 °C, but not more than 18 hr to prevent satellite colony formation.

## Recombinant DNA Protocols

### Preparation of plasmid minipreps

Our laboratory relies upon commercial plasmid purification kits (Qiagen miniprep spin kit) which are highly reliable and produce very homogeneous plasmid preparations. Our protocol follows, and is adapted from the manufacturer's recommendations.

- Centrifuge 1.6-2.0 mL[3] of cell culture at 14 krpm for 3 min, save pellet
- Add 250 µL of Buffer P1 and resuspend pellet *completely* by vortexing[4]
- Add 250 µL of Buffer P2, mix *gently* by inversion, allow to lyse for *no more than* 5 min
- Add 350 µL of Buffer N3 to terminate lysis, mix *gently* by inversion
- Centrifuge at 14 krpm for 10 min, carefully remove supernatant to spin columns equipped with catch tubes
- Centrifuge spin columns at 6 krpm for 1 min, pulse at 14 krpm, and discard flow-through
- Wash with 500 µL of PB buffer, centrifuge at 6 krpm for 1 min, discard flow-through
- Wash with 750 µL of PE buffer, centrifuge at 6 krpm for 1 min, discard flow-through
- Centrifuge at 14 krpm for 1 min to remove last traces of PE buffer from spin column
- Place spin column in a 1.5 mL microcentrifuge tube[5]
- Add 100 µL of water, let stand 1 min, then centrifuge at 6 krpm for 1 min, pulse at 14 krpm.
- Use DNA immediately or store at –20 °C

### Quantifying DNA

The quantity of DNA can be determined from its absorbance at 260 nm. Its quality can be estimated by measuring the $A_{260}/A_{280}$ ratio.

- Pipet 5 µL of plasmid miniprep DNA (≈0.25 µg) into 195 µL of water in a 200 µL quartz silica cuvette. Measure the absorbance at 260 nm and at 280 nm[6]
- For ds-DNA, estimate the concentration using the formula $A_{260} \times 50$ = µg/mL ds-DNA.[7] Multiply by 40 (the dilution factor) to get the concentration of DNA in the original sample.
- The $A_{260}/A_{280}$ ratio should be greater than 1.8 for pure ds-DNA. For most purposes, however, ds-DNA less pure than this can be used successfully for most molecular biology purposes.

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[3] It is possible, especially for low copy-number plasmids to collect cells from as much as 10 mL of overnight culture for this protocol. This can be accomplished by successive centrifugation of 1.6-2.0 mL aliquots of culture in the same microcentrifuge tube. The extra quantity of cells usually presents no problem in the plasmid purification protocol.

[4] We have found that dragging the microcentrifuge tube rapidly back and forth across the holes in a microcentrifuge rack is highly effective in resuspending pelleted cells, and is usually superior to vortexing.

[5] It may be necessary to decapitate the lids from the tubes in order to fit them into the microcentrifuge.

[6] Most diode array instruments will do this simultaneously.

[7] For ss-DNA the formula is $A_{260} \times 33$ = µg/mL ss-DNA

### Preparation of restriction digests

One of the most common operations in recombinant DNA methodology is the digestion of DNA with restriction enzymes. The following protocol is satisfactory for most applications.

- Mix the following reagents in a 1.5 mL microcentrifuge tube, adding the enzyme(s) last:
  1.5 μL of appropriate 10× buffer[8]
  0.75 μL 20× acetylated BSA[9]
  5-10 μL of DNA[10]
  5-10 units of each restriction enzyme (< 1.5 μL total[11])
  water to 15 μL
- Flick the tube gently to mix, spin briefly in a microcentrifuge to collect the content of the tube in the bottom, and incubate for 1-3 hr at 37 °C
- Dilute samples with 3 μL of 6× agarose gel loading buffer (see Appendix 1 for recipe), mix, and centrifuge to collect the sample in the bottom of the tube.
- Run out samples immediately in agarose gel electrophoresis to separate products

### Dephosphorylating vectors

When vector DNA is linearized by digestion by one or more restriction enzymes, it is often desirable to prevent self-ligation of the vector in subsequent cloning steps. This is absolutely required if a vector is digested with a restriction enzyme that leave blunt ds-DNA ends. The simplest way to prevent self-ligation is to remove the 5'-phosphate groups which are required for ligation by T4 DNA ligase. The protocol is conveniently carried out on a 15 μL restriction digestion mixture described above.

- Immediately after completion of restriction enzyme digestion, incubate the mixture at 65 °C for 20 min to inactivate restriction enzymes.[12]
- Dilute the sample to 26 μL with water, add 3 μL 10× phosphatase buffer, and add 1 μL of calf intestinal alkaline phosphatase (1 unit/μL)
- Flick the tube gently to mix, spin briefly in a microcentrifuge to collect the content of the tube in the bottom, and incubate at 37 °C for 30 min
- Run out immediately in agarose gel electrophoresis to separate products

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[8] Consult the manufacturers recommendation for a buffer system that results in satisfactory activity for the restriction enzyme(s) used

[9] Diluted 5× from the 100× solution provided by the manufacturer. This additive is optional but enhances the activity of most restriction endonucleases.

[10] 10 μL of miniprep DNA (≈0.5 μg) or 5 μL of PCR products recovered from agarose gels is ideal. Generally 0.1-1.0 μg is required to visualize DNA fragments in agarose gels.

[11] If the restriction enzyme is added in a volume greater than 10% of the total volume of the digestion mixture, the glycerol concentration in the final mixture becomes very high and may promote non-specific cleavage of DNA

[12] Some restriction enzymes may require higher temperatures or longer inactivation times

### Phosphorylating oligonucleotides

When a DNA fragment is going to be ligated to a linearized, dephosphorylated vector with blunt ends, it is necessary for the DNA fragment to be 5'-phosphorylated for successful ligation with T4 DNA ligase. When the DNA fragment is a PCR product, this is most conveniently accomplished by phosphorylating the ss-DNA oligonucleotide primers used in PCR. A protocol for phosphorylating ss-DNA oligonucleotide primers is given below.

- Mix the following reagents in a 1.5 mL microcentrifuge tube, adding the enzyme(s) last:
  4 μL ss-DNA oligonucleotide (50 pmol/μL)
  5 μL 10× T4 ligase buffer with 10 mM ATP[13]
  1 μL T4 polynucleotide kinase (10 u/μL)
  water to 50 μL
- Flick the tube gently to mix, spin briefly in a microcentrifuge to collect the contents of the tube in the bottom, iand ncubate at 37 °C for 30 min
- Incubate at 70 °C for 10 min to inactivate enzymes
- Use solution immediately for PCR. Store unused portion at –20 °C

### Ligation protocol

Ligation is a critical step in recombinant DNA methodology which accomplishes the "stitching together" of two disparate fragments of DNA. This is a common point of failure in cloning attempts. A robust protocol for ligating a PCR product to a linearized vector follows.

- Mix the following reagents in a 1.5 mL microcentrifuge tube, adding the enzyme(s) last:
  2-3 μL of linearized vector (≈50 ng)
  4-5 μL of PCR product[14]
  1 μL 10× T4 ligase buffer with 10 mM ATP
  1-2 μL of T4 ligase (3-6 Weiss units per μL)[15]
  water to 10 μL
- Flick the tube gently to mix, spin briefly in a microcentrifuge to collect the contents of the tube in the bottom
- Incubate in the refrigerator (4 °C) overnight.[16] This solution may be used directly to transform cells. Store unused portion at –20 °C.

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[13] T4 ligase buffer is entirely satisfactory for carrying out phosphorylations using T4 polynucleotide kinase, and has the added bonus of usually containing the ATP required in the reaction. Alternatively, use 5 μL of the 10× polynucleotide kinase buffer supplied by the manufacturer. This buffer usually does not contain the ATP required for phosphorylation, so it will be necessary to also add 5 μL of 10 mM ATP to the reaction mixture. In this case, reduce the quantity of water used accordingly to maintain the total volume of solution at 50 μL.

[14] This would correspond to about half of the total PCR product obtained as described later

[15] For "sticky end" ligations, 3-6 Weiss units are typically adequate to obtain many recombinants; for blunt-end ligations, this amount of ligase is usually sufficient, but in difficult cases it may be desirable to use T4 ligase which is much more concentrated (typically 30 Weiss units per μL)

[16] Blunt-end ligations may be carried out at room temperature. One shortcut is to incubate blunt-end ligations for 3 hr at room temperature, and immediately use to transform cells. The remainder of the ligation mixture is placed in the refrigerator and allowed to ligate overnight. If no recombinants are obtained from the transformation, the transformation is repeated with an aliquot of the overnight ligation mixture. This is nearly always satisfactory.

### Quick Ligation™ Protocol

Our laboratory has experienced excellent ligation results using Quick T4 Ligase (New England Biolabs). In addition, this protocol takes substantially less time than traditional ligation, and is the preferred method whenever practical. The basic procedure follows.

- Mix the following reagents in a 1.5 mL microcentrifuge tube:
  1-3 µL of linearized vector (≈50 ng)
  4-5 µL of PCR product[17]
  water to 10 µL
- Add 10 µL of 2× Quick Ligation Buffer and mix gently
- Add 1 µL of Quick T4 Ligase and mix thoroughly
- Centrifuge briefly to collect the solution in the bottom of the microcentrifuge tube
- Incubate at room temperature for 5 minutes
- Chill on ice; use immediately for transformation or store at –20 ºC

### Basic PCR protocol

PCR is used to amplify a particular DNA fragment which is flanked by sequences complementary to two flanking ss-DNA oligonucleotide primers. A typical protocol follows.

- Mix the following reagents in a 0.5 mL PCR tube:[18]
  1 µL of plasmid template (≈50 ng)[19]
  0.5 µL of oligonucleotide primer #1 (50 pmol/µL)[20]
  0.5 µL of oligonucleotide primer #2 (50 pmol/µL)
  2 µL dNTP mix (2.5 mM each)
  2.5 µL 10× polymerase buffer[21]
  water to 24.5 µL
- Flick the tube gently to mix, spin briefly in a microcentrifuge to collect the contents of the tube in the bottom
- Using a thermal cycler or heating block, incubate the sample at 94 °C for 5 min, then cool to 4 °C or chill on ice for 2 min[22]
- Add 0.5 µL of *Pfu turbo* (2.5 units/µL, Stratagene) or *Vent* (2.5 units/µL, New England Biolabs) polymerase[23]
- Perform 20-30 cycles[24] of 94 °C (30 s) → 60 °C (1 min)[25] → 72 °C (1 min)[26]; at the end of cycling incubate for 7 min at 72 °C, and then chill to 4 °C or place on ice.

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[17] This would correspond to about half of the total PCR product obtained as described later
[18] It is possible to perform this basic PCR protocol at half-scale, if desired.
[19] 1 µL of a plasmid miniprep is typically about the right amount
[20] If using phosphorylated primers which have been kinased using the protocol decscribed previously, use 12.5 µL (50 pmol)
[21] Use the buffer solution provided by the manufacturer for the polymerase selected
[22] This step accomplishes what is called a “hot start” and may be in some cases critical for optimum PCR performance
[23] Both *Pfu* turbo and *Vent* are very high fidelity DNA polymerases, with very low error rates for dNTP incorporation.

- Dilute 20 µL of the PCR mix with 4 µL of 6× agarose gel loading buffer and run out on a 1% low-melt agarose gel
- Visualize bands on a UV transilluminator, cut out and purify desired PCR product (see electrophoresis protocols)

**Designing and storing ss-DNA oligonucleotide primers**

Commercially synthesized oligonucleotide primers are now quite inexpensive (typically 35¢ per nucleotide or less at scales appropriate for PCR) and can be ordered and received in a few days. We use primers from Integrated DNA Technologies, Coralville, IA. A number of design factors for successful PCR primers should be considered:

- PCR primers should be complementary to the DNA sequences flanking the 5' and 3' ends of the fragment to be copied; each primer should exactly match at least 18 nt of the fragment sequence
- Additional, non-complementary bases may be added to the 5'-end of either or both primers; this is commonly done to add unique restriction enzyme sequences to the PCR product to facilitate its cloning. There is generally no limit to how many "extra" bases can be added. For example, adding the sequence CCATGG to the 5' end of a nucleotide primer would add a *NcoI* restriction enzyme site in the final product. If restriction enzyme sites are introduced into PCR products in this fashion it is strongly recommended that some extra bases be added to the 5'-end to facilitate the direct digestion of PCR products by restriction enzymes.[27] The recommended additional sequence is "TGC." So, for example, to add an *NcoI* site to a PCR product, the sequence TGCCCATGG should be added to the 5'-end of the complementary DNA sequence.
- PCR primers should normally have $T_m$ values of 55-65 °C. $T_m$ values can be estimated by the formula $4 \times (G+C) + 2 \times (A+T)$, or any number of web-based $T_m$ calculators available on the internet can be utilized. Ideally, both PCR primers should have $T_m$ values that are matched closely, but this is not a rigorous requirement
- A typical PCR primer, with extra bases on the 5'-end for introducing unique restriction sites, is 24-28 nt long.
- Ideally, PCR primers should have approximately 50% GC content.
- Complementarity of the 3'-ends (especially the last three nt) for any pair of primers for PCR should be strictly avoided. Such self-complementary primers can anneal to each other and produce prodigious quantities of "primer-dimer" at the expense of the desired PCR product,

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[24] Generally, more product will be formed if a larger number of cycles are chosen; however, the chance of incorporation of an unintended point mutation increases with the number of cycles. Use the fewest number of cycles that give a satisfactory amount of product.

[25] The annealing temperature is normally chosen to be near or slightly below the predicted $T_m$ of the oligonucleoide primers. If no PCR product is formed, lower the annealing temperature 5 °C; if too many nonspecific products are formed, raise the annealing temperature 5 °C.

[26] A good rule of thumb is to allow 1 min of extension time for every 1000 bp of PCR product for high fidelity DNA polymerases like *Pfu* or *Vent*.

[27] Most restriction enzymes cannot digest a ds-DNA fragment if the restriction enzyme site is right at the end of the ds-DNA fragment. However, the addition of three additional bases to the 5'-end of the restriction site usually makes these sites accessible.

especially if there is high GC content at the 3' end of the primer. An example of PCR primers that can form "primer-dimers" is illustrated below:

5 '• • • • CTAGATCGATCG 3'
| | |
3' AGCTTCTGAT • • • • 5'

All PCR oligonucleotide pairs should be checked for possible primer-dimer formation prior to ordering or carrying out PCR.

- Reconstitute commercial primers in TE buffer at 500 pmol/µL and store at –80 deg C. Remove and dilute aliquots as required to 50 pmol/µL for routine PCR.

**Site-directed mutagenesis by megaprimer PCR**[28]

This two-step PCR-based method is certainly one of the simplest and most efficient methods of introducing specific point mutations into a DNA fragment coding for a protein. The method is summarized in Figure 2 below:

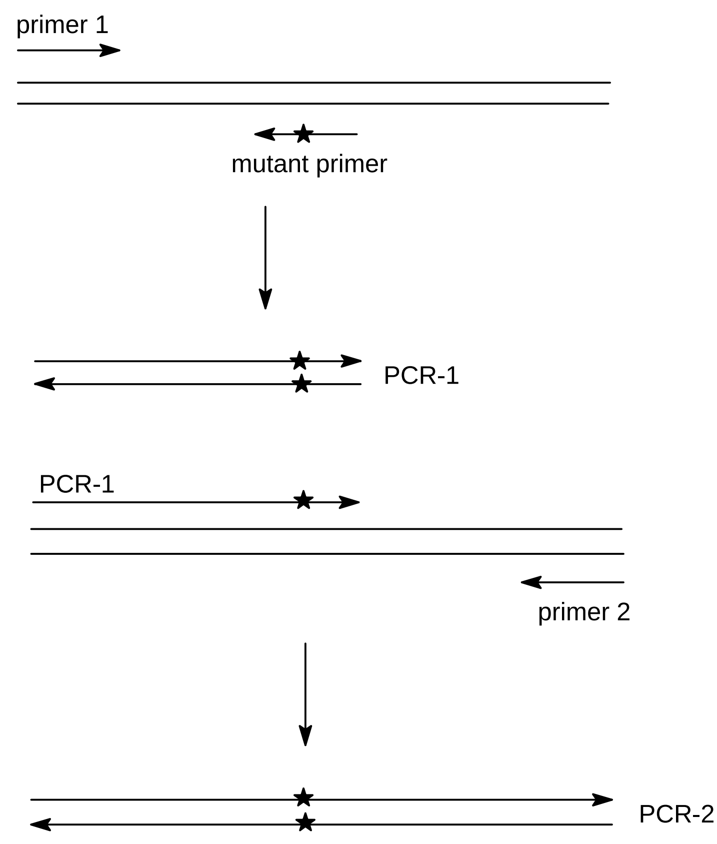


**Figure 2.** PCR-based site-directed mutagenesis. Location of the introduced mutation is indicated by the star.

[28] Sarkar, G. & Sommer, S. S. (1990) *Biotechniques* **8**, 404-407.

In the first round of PCR one flanking oligonucleotide primer (primer 1) is paired with an oligonucleotide primer (mutant primer) which is designed to introduce a point mutation in the desired location within the gene. The PCR product for this reaction (PCR-1) is gel purified and used as a primer in a second round of PCR with the other flanking primer (primer 2) to produce a DNA product corresponding to the entire gene, with the desired mutation included. The mutant primer should be designed in such a way as to have at least 9 exactly complementary nucleotides flanking the base mismatches required to introduce the desired mutation. Normally, the mutant codon should be checked against a usage table of codons[29] for *E. coli* to ensure that the codon is not rarely used for protein translation.

A typical protocol for site-directed mutagenesis follows:

- Mix the following PCR mix in a 0.5 mL PCR tube:[30]
  - 1 μL of plasmid template (≈50 ng)
  - 0.5 μL of oligonucleotide primer #1 (50 pmol/μL)[31]
  - 0.5 μL of mutant primer (50 pmol/μL)
  - 2 μL dNTP mix (2.5 mM each)
  - 2.5 μL 10× polymerase buffer
  - water to 24.5 μL
- Perform PCR according the protocol previously described
- Dilute 25 μL of the PCR mixture with 5 μL of 6× agarose gel loading buffer.
- Load 15 μL of the sample into each of two lanes of a 1½ % low-melt agarose gel and separate by electrophoresis; for PCR products, ϕX174/Hae III markers or a PCR ladder are necessary to locate the DNA fragment of the correct length, and should be loaded in a nearby lane
- Cut out the PCR product from the agarose gel, extract and purify using GeneClean[32]
- For the second PCR reaction, mix in a 0.5 mL PCR tube:
  - 1 μL of plasmid template (≈50 ng)
  - 0.5 μL of oligonucleotide primer #2 (50 pmol/μL)[33]
  - 10 μL of PCR product from first PCR[34]
  - 2 μL dNTP mix (2.5 mM each)
  - 2.5 μL 10× polymerase buffer
  - water to 24.5 μL
- Perform PCR according the protocol previously described
- Dilute 15 μL of the PCR mixture with 3 μL of 6× agarose gel loading buffer, and run out on a 1½ % low-melt agarose gel and separate by electrophoresis, using ϕX174/Hae III markers or a PCR ladder in an adjacent lane to help identify the DNA fragment of the correct length

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[29] One online source is http://www.clustalw.genome.ad.jp/kegg/codon_table/codon_eco.html
[30] It is possible to perform this and the subsequent PCR protocol at half-scale, if desired.
[31] Normally this primer contains extra nucleotides on the 5' end to add a restriction enzyme site and facilitate direct restriction enzyme digestion of the eventual PCR product
[32] The protocol will be described later; the contents of each lane are normally concentrated to 10 μL final volume
[33] Normally this primer contains extra nucleotides on the 5' end to add a restriction enzyme site and facilitate direct restriction enzyme digestion of the eventual PCR product
[34] Use the entire purified product from the first PCR reaction

- Cut out the PCR product from the agarose gel, extract and purify using GeneClean Turbo[35]
- Prepare a restriction digestion using 5 µL of the concentrated, purified PCR product
- Re-purify the digested DNA using the GeneClean, described later in this manual
- Perform a ligation of 4-5 µL of a solution of this DNA fragment to the desired vector digested with the same enzymes (and perhaps dephosphorylated as well)
- Transform competent *E. coli* cells with this ligation mixture, spread on LB plates with the appropriate antibiotic, and grow up overnight at 37 °C[36]
- Pick 2-3 colonies from this plate, grow up overnight in 5 mL of LB medium with the appropriate antibiotic, and prepare plasmid minipreps for each selected clone.
- Perform restriction digestions of miniprep plasmid DNA with the appropriate restriction endonucleases, and run out the products on a 1-1½% agarose gel to verify the plasmids contain the desired insert DNA.
- For clones showing evidence of the appropriate DNA insert in the vector, prepare frozen glycerol stocks from some of the overnight liquid culture. These cultures can be used to prepare samples of plasmid DNA for sequencing, or for conduction pilot overexpression trials (described later).

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[35] The final volume of this sample is typically 10 µL

[36] A successful transformation will normally result in >30 colonies, often hundreds. If fewer than 10 colonies are observed, it is likely most of these are background non-recombinants or the result of residual self-ligation of the vector. Under these circumstances, it is usually wise to check the condition of the competent cells, or try re-ligating with a higher concentration of T4 ligase.

## Electrophoresis Protocols

### Submarine agarose gel electrophoresis

Agarose gels are the preferred method of separating and purifying DNA fragments of 100-10,000 bp in length. For routine work, 6.5 × 9.0 × 1.0 cm minigels with 8-well combs which hold 20 µL samples per well are convenient. The exact method of pouring a gel will depend on the agarose gel apparatus used, but some general techniques are applicable to nearly all apparatus:

- Prepare agarose gel stock solutions by mixing agarose or low-melt agarose[37] with 1× TAE buffer (Appendix 2). This is conveniently done in a 500 mL Wheaton bottle. For a 1% gel, add 1 g of agarose for every 100 mL of TAE buffer. Typically no more than 200 mL are made at one time.[38]
- Loosen the cap and microwave agarose for ≈1 min for every 100 mL of agarose solution. Swirl to mix thoroughly but without introducing bubbles. The agarose should be fully melted before proceeding. If necessary microwave for additional time to fully melt agarose.
- If the solution is being used for the first time, add 50 µL of ethidium bromide solution (10 mg/mL) to the agarose solution.
- Allow the agarose solution to cool enough so that it can be held in the bare hand, and pour the gel and introduce the comb. Allow the gel to harden. Once the gel takes a partial set, it can be placed in a –20 °C freezer for 5-10 minutes to speed complete hardening.
- Gently remove the comb,[39] place the gel in the apparatus, and fill the apparatus with 1× TAE buffer deep enough to cover the gel completely 2-3 mm deep.
- Load samples with a pipettor by placing the tip just below the top of the well. The high density of the loading buffer will ensure the samples sink to the bottom of the well.
- Run the samples out at 120 V constant[40] for approximately 30-40 min as desired to achieve the necessary separation,
- Disconnect the power, remove the gel tray, and place it on a UV transilluminator. *Don a UV face shield before engaging the UV lamp.* DNA will appear as orange bands within the gel.
- Gels are conveniently photodocumented with a camera equipped with Polaroid type 67 film and a special light-blocking hood. An exposure of ½ second at *f*8 is usually adequate for most gels.

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[37] Agarose is much cheaper than low-melt agarose. The latter is used for preparative purposes, when bands are sliced out of the gel and the DNA must be recovered for further use. The former can be used for analytical purposes.
[38] Additional agarose and TAE buffer can be added directly to partially used bottles of agarose as needed; it is not necessary to remove remaining agarose and buffer prior to making additional solution.
[39] Rips and tears in the wells can be minimized by pouring a little 1× TAE buffer on the top of the gel around the comb to lubricate its removal.
[40] The literature usually recommends much lower voltages for agarose gel separations, with consequently longer separation times. While lower voltages result in better resolution, it is not necessary for routine recombinant DNA work.

**Recovering DNA fragments from agarose gels**

One of the most convenient methods for recovering purified DNA bands visualized in ethidium bromide stained agarose gels involves cutting out gel slices and purifying/recovering on silica gel using a commercial product (*GeneClean*, Bio101, Inc.)

- Select the desired DNA band and, using a knife,[41] make vertical slices in front of, behind, and to each side of the band to free it from the rest of the gel.
- Slipping the knife into the cut previously made below the gel band, gently tip the knife and lift the gel slice from the rest of the gel, and place in a tared microcentrifuge tube. A typical gel slice should weigh 100-250 mg.
- If necessary, slices may be frozen at this stage for later processing.

Purify/recover the DNA using the reagents provided in the GeneClean Turbo kit, as described below:

- To each gel slice, add an equal volume of GeneClean Turbo salt solution and mix well[42]
- Heat at 50-55 °C for 5 min or until gel slice has completely melted
- Mix solution well and transfer (<600 μL) to a GeneClean Turbo cartridge
- Place cartridge into a catch tube and spin at 6,000 rpm for 1 min, then pulse at 14,000 rpm; empty catch tube as needed. The cartridge should be free of liquid before proceeding.
- Add 500 μL of GeneClean Turbo Wash solution to the cartridge; spin at 6,000 rpm for 1 min, then pulse at 14,000 rpm; empty the catch tube as needed.
- Repeat the previous wash step
- Empty catch tube and spin at 14,000 rpm for 2 min to completely remove wash solution from the spin filter
- Transfer the cartridge to a clean 1.5 mL microcentrifuge tube
- Add 30 μL of water to the cartridge filter and allow to stand for 5 min
- Spin at 14,000 rpm for 2 min to collect the DNA
- For most uses, the DNA solution should be evaporated to dryness in a vacuum evaporator, and stored at –20 ºC until needed.
- To reconstitute DNA solution, add 10 μL of water to the bottom of the microcentrfuge tube, flick or vortex gently to mix, and centrifuge briefly to collect solution at the bottom of the tube. Use immediately, or store at –20 ºC.

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[41] A satisfactory knife can be constructed by taking a standard thin, flat spatula, grinding each end flat, and sharpening, if necessary. Such a knife is about the same width as a gel lane in an 8-well agarose gel apparatus.

[42] If purifying DNA from solution, e.g., restriction digests of PCR products, add at least 5× volume of GeneClean Turbo solution and mix well. Omit the heating step and then follow the GeneClean Turbo protocol othwerwise as described.

**SDS-polyacrylamide gel electrophoresis**

*Preparing gels.* For routine work, precast 10 cm × 10 cm × 1 mm minigels are convenient. However, the following protocol is appropriate for preparing four (4) homemade disposable polystyrene gel cassettes for an X-Cell II Mini-Cell gel electrophoresis apparatus (Novex), but could easily be adapted for other systems. *Caution: wear gloves and protective clothing when preparing acrylamide gels! Acrylamide is a potent, cumulative neurotoxin.*

- Tape bottom of gel cassettes, if necessary, and arrange upright in a rack
- Prepare a 12% resolving gel mixture in a small Erlenmeyer flask according to the recipe described in Appendix 2.
- Add the TEMED and ammonium persulfate in that order, and mix thoroughly by swirling.
- Take up the mixture in a syringe, attach a narrow cannula or needle, and fill the gel cassettes with the gel mixture to within 2 cm of the top. Place 1-2 mL of remaining gel mixture in a microcentrifuge tube and cap tightly.
- Working quickly, gently layer 2-3 mm of distilled water on the top of the gel mixture in each cassette, using a syringe and needle.
- Allow the gel to polymerize for about 1 hr.[43] You may use the gel mixture in the microcentrifuge tube as a guide as to when polymerization is complete.
- Prepare a 4% stacking gel mixture in a small Erlenmeyer flask according to the recipe described in Appendix 2.
- Add the TEMED and ammonium persulfate in that order, and mix thoroughly by swirling.
- Take up the mixture in a syringe, attach a narrow cannula or needle, and fill the gel cassettes with the gel mixture all the way to the top. Place 1-2 mL of remaining gel mixture in a microcentrifuge tube and cap tightly.
- Insert well-forming combs into each gel cassette, taking care not to trap bubbles under the comb.
- Allow the gel to polymerize for about 1 hr. You may use the gel mixture in the microcentrifuge tube as a guide as to when polymerization is complete.
- Gels may be used immediately, or can be stored in a zip-lock plastic bag with a damp paper towel. Gels can be stored for about 2-3 weeks before drying out.

*Preparing samples for SDS-polyacrylamide gel electrophoresis.* The following protocol describes how to prepare samples for SDS-PAGE:

- For clarified liquid samples, mix equal volumes of protein-containing solution and 2× SDS-PAGE loading buffer, prepared as described in Appendix 2. Suggested final protein concentrations are about 0.1-1.0 μg/μL for homogeneous proteins or 1.0-10.0 μg/μL for protein mixtures. Heat sample to 95 °C for 5 min prior to loading.
- For whole cells (*E. coli*), pellet 1 mL of overnight culture in a microcentrifuge tube, and wash with 1.0 mL of 0.9% NaCl. To the washed pellet, add 25 μL of water and 25 μL of 2×

[43] If the gel is insufficiently gelled after 1 hr, it will be necessary to begin again. To increase the rate of polymerization, add 10-20% more TEMED or ammonium persulfate to the gel mixture. If the mixture instead gels too quickly, decrease the TEMED or ammonium persulfate by 10-20%.

SDS-PAGE loading buffer. Heat sample to 95 °C prior to loading, and centrifuge at 14000 ×g for 3 min to pellet cell debris before loading.

*Running and visualizing gels.* The following protocol is typical for running an SDS-PAGE minigel. Wear gloves and protective clothing when initially handling and washing gels:

- Prepare 800 mL of 1× SDS-PAGE running buffer from 10× SDS-PAGE running buffer prepared as described in Appendix 2.
- Remove the comb from a previously prepared gel cassette, and rinse the wells thoroughly with 1× running buffer to remove any unpolymerized acrylamide.
- Remove the tape from the bottom of the gel cassette, and place it into the gel appraratus.
- Fill the middle chamber with 1× running buffer above the top of the wells in the gel. Check for leaks into the outer compartment.
- Load up to 20 µL of sample into wells, as desired, using either a microsyringe or a micropipettor equipped with a gel-loading tip.
- Fill the outer compartment with the remainder of the 1× running buffer, and affix the top of the gel apparatus.
- Attach the leads to a power supply and run gel(s) at a constant 250 V.[44] The gel run should be complete (as indicated by tracking dye migration to the bottom of the gel) in about 40-45 min.
- When the gel run is completed, switch off the power supply and disconnect the power leads.
- Remove the gel cassette from the apparatus, and using a knife, separate the halves of the gel cassette. Cut off the "foot" of the gel (which protrudes into the slot at the bottom) and the stacking gel.
- Place the remaining portion of the gel in a container and complete cover with about 200-250 mL of staining solution prepared as described in Appendix 2.
- Cover *loosely*, and microwave at full power for 2 min. Place on a shaker and slowly agitate for 10-15 min. Alternatively, the gel can be safely stained overnight at room temperature, if desired.
- When staining is complete, pour off the stain (it can be recycled and used many times) and rise the gel and its container with distilled water to remove excess stain solution.
- Add 200-250 mL of destaining solution prepared as described in Appendix 2, cover *loosely*, and microwave at full power for 2 min. Place a few crumpled laboratory tissues into the container with the gel.[45] Place on a shaker and slowly agitate until destaining is adequate to visualize bands. Typically only 30-60 min is required.

*Drying gels.* Gels are conveniently air-dried between membranes (Novex) for permanent storage or imaging:

- Soak two drying membranes in distilled water.

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[44] The literature suggests using a lower voltage, but this results in unnecessarily long run times. No degradation in performance is noted for standard tris-glycine SDS-PAGE gels at 250 V.
[45] The tissues help absorb the staining dye, and speed the destaining procedure.

- Pour off destain solution from gel, and place gel between drying membranes, using plenty of distilled water to remove all bubbles.
- Assemble drying rack to hold gel sandwich. Dry overnight at room temperature.
- Dried gels can conveniently imaged using a color flatbed scanner, and images analyzed using Scion-Image[46] software.

**Non-denaturing gel electrophoresis**

Non-denaturing gel electrophoresis can be carried out in a manner directly analogous to SDS-PAGE, by simply omitting the denaturants and reducing agents from the gels, running buffer, and loading buffer. The required changes in the SDS-PAGE protocol are noted below:

- When preparing separating or stacking gel solutions, omit SDS from the recipe given in Appendix 2.
- Running buffer should be prepared without SDS, but otherwise as given by Appendix 2.
- 2× Loading buffer should be prepared without SDS or β-mercaptoethanol, but otherwise as in Appendix 2
- Samples should not be heated prior to loading on the gel.[47]
- Exact run times may vary from 30 min to 2 hr depending on the sizes and charges of proteins separated.

**DNA sequencing sample preparation**

Plasmids DNA can be conveniently sequenced using a dye-terminator-based cycle sequencing method. We are currently using the *ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit* supplied by PE Biosystems, in conjunction with an ABI 310 capillary electrophoresis sequencing system. Sequencing reactions can be carried out in any heated-top thermal cycler with adjustable temperature ramp times.

*Cycle sequencing protocol.* The basic protocol for generating sequencing fragments has been slightly modified to conserve reagents:[48]

- For each sequence, pipet into a 0.5 mL PCR tube:
  4 μL terminator reaction ready mix[49]
  4 μL dilution buffer[50]
  2 μL oligonucleotide primer (1.6 pmol/μL)
  10 μL miniprep plasmid template (≈500 ng)
- Mix and centrifuge

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[46] Scion-Image software for Windows is available free from www.scioncorp.com
[47] For whole-cell samples, an alternative method (to heating) of releasing cell contents should be used, such as sonication or freeze-thawing.
[48] Thanks go to Dr. James Leebens-Mack for supplying the modified protocol.
[49] *Store at –20 °C. Do not heat to melt; thaw on ice.* This soution includes, dye terminators, dNTPs, AmpliTaq DNA polymerase, pyrophosphatase, $MgCl_2$, and Tris buffer, *p*H 9.0. *Store at –20 °C. Do not heat to melt; thaw on ice.*
[50] 200 m*M* Tris-Cl (*p*H 9.0) -1 m*M* $MgCl_2$, made by mixing 200 μL 1 *M* Tris, *p*H 9.0, 5 μL 1 *M* $MgCl_2$, and 795 μL of water.

- Perform 25 cycles as follows[51]:
  96 °C for 30 s
  50 °C for 15 s
  60 °C for 4 min
- At end of cycling hold at 4 °C
- Spin down sample prior to purifying extension products

*Purifying extension products.* The dye terminators are removed from the cycle-sequencing extension products as follows:

- Hydrate a Centri-Sep (Princeton Separations P/N CS-901) spin column by adding 0.8 mL water; allow to hydrate for 2 hr at room temperature.[52]
- Tap the column to settle gel and remove bubbles. Add a little more water if necessary.
- Remove upper, then lower end caps; allow column to drain completely by gravity.
- Insert column into wash tube, spin at 350-750 ×*g* for 2 min to remove interstitial fluid.
- Insert column into a 1.5 uL microcentrifuge tube.
- Carefully pipet extension reaction mixture on to *center* of gel material.
- Spin at 350-750 ×*g* for 2 min.
- Dry the collected sample in a vacuum centrifuge for 10-15 min, or until dry.[53]
- Resuspend dried sample in 10-20 µL of TSR buffer; vortex and spin.
- Denature at 95 °C for 2 min, vortex, spin, and chill on ice until loaded into the ABI 310.

[51] Ramp time should be set at 1°C/s for optimal results
[52] Hydrated columns can be stored in the refrigerator for 1-2 days; warm to room temperature before use. Columns can also be re-used. Immediately after use, discard Sephadex from column. Rinse thoroughly with water and centrifuge 700 µL of water through the column. After columns have air-dried, fill with 1 mL hydrated Sephadex G-50 (10 g per 150 mL water), let drain, and centrifuge as you would for new Centri-Sep columns.
[53] Do not over-dry! Dried samples may be stored in the freezer until ready for electrophoresis.

## Protein Overexpression Protocols

### Pilot scale overexpression

It is frequently advisable to assess protein overexpression levels obtained with a plasmid/host combination before committing to large scale overexpression trials. The following protocol is convenient for preliminary assessment of overall protein overexpression in *E. coli* harboring vectors with *trc* or *lac* promoters and an ampicillin resistance gene.

- Using sterile technique, transfer 2 mL of LB medium containing 100 μg/mL ampicillin into a sterile 10 mL (17 ×100 mm) culture tube.
- Inoculate the medium with 100 uL of a fresh, overnight *E. coli* culture harboring the appropriate overexpression plasmid.
- Incubate with shaking (250 rpm) for 2½-3 hr.[54]
- Using sterile technique, add IPTG to a final concentration of 0.2-1.0 m*M*, to induce overexpression.
- Incubate with shaking (250 rpm) for 6 hr to overnight.
- Perform SDS-PAGE analysis of whole cells as described previously.

In order to determine what, if any, fraction of the overexpressed protein is produced in soluble form, it is necessary to grow a slightly larger culture in order to produce cell-free extracts. This is conveniently done immediately following the preliminary pilot overexpression.

- Using sterile technique, place 50 mL of LB medium containing 100 μg/mL ampicillin into a sterile 250 mL culture flask.
- Using sterile technique, pipet 1.0 mL of fresh, overnight *E. coli* culture into each of two (2) sterile 1.5 mL microcentrifuge tubes.
- Centrifuge at 6000 ×g for 3 minutes, remove the supernatant, and resuspend in 1.0 mL of sterile growth medium.
- Centrifuge at 6000 ×g for 3 minutes, remove the supernatant, and wash once again with 1.0 mL of sterile growth medium.
- Resuspend cells in each tube with 1.0 mL of sterile growth medium, and use the contents of both tubes (2.0 mL) to inoculate the 50 mL of growth medium previously prepared.
- Incubate with shaking (250 rpm) for 2½-3 hr.
- Using sterile technique, add IPTG to a final concentration of 0.2-1.0 m*M*, to induce overexpression.
- Incubate with shaking (250 rpm) for 6 hr to overnight.
- Harvest cells by centrifugation at 8000 ×g for 5 min in a 50 mL centrifuge tube.
- Wash cells twice by resuspension and centrifugation in cold 0.9% NaCl.
- Take up wet cell pellet (typically 0.50-1.0 g) in 5 mL of the appropriate extraction buffer.[55]

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[54] For maximum yield of overexpresed protein, cell cultures should be growyn until the turbidity as measured by $A_{600}$ is between 0.6-1.0.

[55] A suggested buffer is 50 mM MOPS-1 mM DTT-1 mM EDTA, *p*H 7.0. Typically, protease inhibitors are added to limit proteolysis of the desired protein. The exact composition of the buffer will depend on the optimum conditions required for the protein of interest.

- Break open cells using an appropriate technique and perform SDS-PAGE analysis

**Production-scale overexpression**

The large-scale overexpression of target proteins typically requires 1-4 L of cell culture. Such cultures cannot be started directly from single colonies on agar plates, but must be scaled up gradually. The following protocol is typical for most overexpression systems, and should observe proper sterile technique:

- The desired strain of *E. coli* harboring the desired overexpression plasmid should be streaked out on an agar plate containing the appropriate antibiotic[56] and grown overnight.
- A single colony should be used to inoculate 10 mL of LB medium containing the appropriate antibiotic, and the culture shaken overnight at 37 °C. Alternatively, cells can be grown until $A_{650}$ reaches 0.6-1.0, and then stored at 4 ºC for up to 24 hr.
- For each 1 L of overexpression culture, place 10 mL of overnight scale-up culture in a 50 mL centrifuge tube.
- Pellet cells at 6000 ×*g* for 5 min, and wash with 10 mL fresh LB medium with antibiotic as described above.
- Resuspend washed and pelleted cells in 10 mL of fresh LB medium.
- Prepare overexpression cultures by adding 1 L of TB medium containing the appropriate antibiotic to 2.5-3.0 L culture flasks.[57]
- Inoculate each 1 L of overexpression medium with the entire contents (10 mL) of one 50 mL centrifuge tube.
- Incubate at 37 °C with vigorous shaking for 2½-3 hr, or until $A_{600}$ reaches 0.6-1.0, then induce by adding IPTG to a final concentration of 0.2-1.0 m*M*.[58]
- Grow cells 6 hr to overnight with vigorous shaking at 25-37 °C.[59]
- Harvest cells immediately, or store at 4 °C for no more than a few hours before processing. The typical yield of wet pelleted cells is 20-30 g per liter of culture for cultures grown at 37 ºC.

**Harvesting overexpression cultures**

The extraction of overexpressed soluble proteins from *E. coli* cultures requires removal of the culture medium, breakage of cells, and clarification of the extract. All operations should be carried out at 4 °C or with samples placed on ice to minimize protein denaturation and proteoloysis. For cell breakage we are currently using a BeadBeater™ (Biospec) which efficiently disrupts cells by agitation with small glass beads. A typical protocol follows:

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[56] For expression vectors expressing ampicillin resistance, 50 μg/mL of ampicillin is recommended.
[57] The use of Tune-Air culture flasks is highly recommended for final overexpression. These uniquely shaped and baffled flasks generate cell densities several times higher than standard or baffled Erlenmeyer flasks.
[58] IPTG is hideously expensive; if growing cultures overnight, lower concentrations of IPTG can be used without significant effect on the overexpression yield.
[59] The appropriate post-induction growth temperature should be determined by experiment. Inclusion body formation is often reduced by overexpression at lower temperatures.

- In tared 500 mL centrifuge bottles, pellet cells by centrifugation at 8000 ×*g* for10 min, discarding the supernatant
- Resuspend each bottle of pelleted cells in 250 mL of 0.9% NaCl by vortexing, scraping and/or shaking.
- Centrifuge at 8000 ×*g* for10 min, and discard the supernatant.
- Weigh the bottles to determine the mass of wet, pelleted cells.
- Resuspend cells in 2 mL/g of extraction buffer[60] by scraping, vortexing, and/or shaking vigorously.[61]
- Combine resuspended cells in one container, and add any protease inhibitors desired.
- Fill the pre-chilled homogenzation chamber of the BeadBeater™ half-full with 0.1 mm glass beads, and add resuspended cells. *Top off the chamber with additional extraction buffer so that there will be no air space left when the cap is attached*.[62]
- Disrupt cells 15 seconds, followed by a 45 second rest period to re-chill the homogenization chamber contents. Repeat this disruption/rest cycle a total of 8 times for complete homogenization.[63]
- Load the crude cell extract into the required number of appropriately balanced 50 mL centrifuge tubes.
- Centrifuge at 48000 ×*g* for 40-60 min to clarify the cell extract.
- Filter the supernatant to remove the last remnants of glass beads before subjecting to standard protein purification techniques.

### Thrombin cleavage of purification tags

Many proteins are conveniently expressed with purification tags, e.g., 6× His-tags, maltose binding protein, glutathione-*S*-transferase, *etc*. to simplify purification to homogeneity and/or to improve expression levels of soluble protein. These tags must often be removed before the protein of interest can be studied. One of the most common removal methods involves proteolytic cleavage of the purification tag utilizing a cloned thrombin recognition site. A typical protocol for thrombin cleavage of a tagged protein using a Novex Thrombin Cleavage Capture Kit follows:

- In a 2.0 mL microcentrifuge tube add:
  - ≈2 mg tagged protein (up to 1600 μL)
  - 200 μL 10× thrombin cleavage buffer
  - water to a total volume of 1800 μL
- Add 200 μL diluted biotinylated thrombin[64] and mix gently by inversion

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[60] See footnote 44 for suggested extraction buffer composition.

[61] It is most convenient to resuspend wet packed cells from each liter of culture in 40 mL extraction buffer. This solution will conveniently fit into the small homogenization chamber of the BeadBeater™ when properly filled with glass beads.

[62] It is critical that there is no air in the homogenization chamber. Air in the chamber will reduce disruption efficiency and may denature extracted protein.

[63] These conditions are appropriate for the small (15 or 50 mL) homogenzation chambers. The large (350 mL) chamber will require cycles of 1 minute disruption and 3 minutes rest.

- Incubate at room temperature for 2-24 hours[65]
- Remove 2-5 µL for later SDS-PAGE analysis
- Thoroughly resuspend streptavidin agarose slurry by inversion and pipet 32 µL into reaction mixture with a wide-bore pipet tip[66]
- Incubate for 30 min at room temperature. Mix gently by inversion every few minutes to keep streptavidin agarose resuspended
- Transfer no more than 350 µL at a time of the reaction mixture to the sample cup of a spin filter
- Centrifuge at 500 ×*g* for 30 s. Remove filtrate with a transfer pipet to a clean 2.0 mL microcentrifuge tube
- Repeat the previous two steps until all of the reaction mixture is processed, taking care to save the filtrate solution, which is your cleaved protein
- Store protein at 4 ºC and purify protein as soon as practical using gel exclusion chromatography

[64] The exact dilution of thrombin should be determined by prior cleavage trials, and could vary from 1:20 to 1:400 dilution. To minimize non-specific cleavage, the greatest dilution of thrombin should be used that gives complete cleavage in the desired time interval.
[65] The exact incubation time should be determined by prior cleavage trials. Utilize the shortest cleavage time that results in complete cleavage using the desired thrombin dilution.
[66] In the absence of wide-bore pipet tips, cut off a portion of the end of a standard pipet tip to accommodate passage of streptavidin agarose particles

# Appendix 1—Molecular Biology Reagents

## MEDIA

**LB medium**

10 g tryptone
5 g yeast extract
10 g NaCl
*Add 950 mL water, dissolve solids, add 0.2 mL 5 M NaOH, make to 1000 mL with water. Autoclave and store at room temperature. Refrigerate after opening.*

- *For agar plates, add 15 g agar prior to autoclaving*
- *For ampicillin plates, add 1 mL sterile 50 mg/mL ampicillin after cooling to 50°C*

**TB Overexpression Medium**

12 g tryptone
24 g yeast extract
8 mL 50% glycerol
*Make to 900 mL and autoclave.*

2.31 g $KH_2PO_4$
12.54 g $K_2HPO_4$
*Make to 100 mL and autoclave.*

*Cool to 55 °C or less, combine, and add any antibiotics desired.*

## BUFFERS

**1 *M* Tris**

121.1g Tris base
*Dissolve in 800 mL water, adjust pH to desired value (pH 7.4, 7.8, or 8.0) with HCl, make to 1000 mL with water. Sterilize by autoclaving.*

**0.5 *M* EDTA (*p*H 8.0)**

186.1 g disodium EDTA
*Dissolve in 800 mL of water, adjust pH to 8.0 with NaOH, make to 1000 mL. Sterilize by autoclaving.*

**TE Buffer**

10 mL 1 *M* Tris (desired *p*H)
2 mL 0.5 *M* EDTA (*p*H 8.0)
*Make to 1000 mL with water.*

**TAE Buffer (50 ×)**

242 g Tris
57.1 mL acetic acid
100 mL 0.5 *M* EDTA (*p*H 8.0)
*Make to 1000 mL with water.*

- *1 × composition is: 40 mM Tris-acetate; 1 mM EDTA*

**TBE Buffer (5 ×)**

54 g Tris
27.5 g boric acid
20 mL 0.5 *M* EDTA (*p*H 8.0)
*Make to 1000 mL with water.*

- *1 × composition is: 90 mM Tris-borate; 2 mM EDTA*

**Agarose Gel Loading Buffer (6 ×)**

0.25% (w/v) bromophenol blue
40% (w/v) sucrose
*Make to volume with water. Store at 4 °C.*

**Transformation Buffer (TSS, 2 ×)**

5 g PEG 8000
0.25 g $MgSO_4 \cdot 7\ H_2O$
*Add 22.5 mL LB, adjust pH to 6.5-6.8 if necessary. Sterilize by filtration. Add 2.5 mL DMSO. Composition is 20% PEG, 40 mM $Mg^{2+}$, 10% DMSO. Store at 4 °C.*

## REAGENTS

**10% Ammonium Persulfate**

1 g ammonium persulfate
*Make to 10 mL with water. Store in aliquots at –20 °C.*

**5% Ampicillin**

500 mg ampicillin, sodium salt
*Dissolve in 10 mL of water. Sterile filter and store at 4 °C.*

**100 m*M* IPTG**

238 mg IPTG
*Dissolve in 10 mL of water. Sterile filter in 1 mL aliquots. Store at –20 °C.*

**2% X-Gal**

20 mg X-Gal
*Dissolve in 1 mL of DMF. Store in dark bottle at –20 °C.*

**1% Ethidium Bromide**

100 mg ethidium bromide
*Dissolve in 10 mL of water. Store in dark bottle.*

**Phenol**

*Add 0.1 g of 8-hydroxyquinoline to 100 mL of liquid phenol. Add 100 mL of 500 mM Tris base, stir 10 min at low speed with a stir bar, decant aqueous layer. Check phenol layer with pH paper to verify pH is ≥ 8.0, reequilibrate if necessary. Equilibrate similarly 2× with 100 mL of 50 mM Tris • Cl ( pH 8.0). Store the equilibrated mixture under an equal volume of 50 mM Tris • Cl (pH 8.0) at 4 °C in a dark bottle.*

**Chloroform**

24 parts chloroform
1 part isoamyl alcohol
*Store in a dark bottle.*

**5 *M* Sodium Hydroxide**

263 mL concentrated (19.1 *M*) sodium hydroxide

*Dilute to 1000 mL with water. Store in a plastic bottle.*

**2 *M* NaOH–1 m*M* EDTA**

40 mL 5 *M* sodium hydroxide

0.2 mL 0.5 *M* EDTA

*Dilute to 100 mL with water. Store in a plastic bottle.*

**3 *M* Sodium Acetate (*p*H 4.9)**

408.1 g sodium acetate trihydrate

*Dissolve in a minimum volume of water, titrate to pH 4.9 with glacial acetic acid. Make to 1000 mL with water. Sterilize by autoclaving.*

# Appendix 2–Electrophoresis Reagents

## SDS-POLYACRYLAMIDE

**50% Acrylamide/Bis (29:1) Solution**

48.3 g acrylamide
1.7 g bis-acrylamide
*Make to 100 mL with water. Store in a dark bottle.*

**Separating Gel Buffer (1 *M* Tris-HCl, *p*H 8.8)**

30.3 g Tris
*Add 150 mL water and adjust to pH 8.8 with HCl. Make to 250 mL with water.*

**10% SDS**

10.0 g SDS
Make to 100 mL with water.

**Stacking Gel Buffer (0.375 *M* Tris-HCl, *p*H 6.8)**

11.4 g Tris
*Add 150 mL water and adjust to pH 6.8 with HCl. Make to 250 mL with water.*

**10% Ammonium Persulfate**

1 g ammonium persulfate
*Dissolve in 10 mL water. Aliquot and store at –20 °C.*

**Recipe for 12% separating gel (25 mL, enough for four 1.0 mm minigels):**

| | |
|---|---|
| 50% Acrylamide/Bis (29:1) Solution | 6.0 mL |
| Separating Gel Buffer (1 *M* Tris-HCl, *p*H 8.8) | 9.4 mL |
| 10% SDS | 250 μL |
| water | 9.0 mL |
| TEMED* | 20 μL |
| 10% Ammonium Persulfate* | 300 μL |

*Add just prior to polymerization—open time is 5-10 min, full polymerization in 1 hr; layer with deionized water to prevent inhibition of polymerization by oxygen in air.

**Recipe for 4% stacking gel (12.5 mL, enough for four 1.0 mm minigels):**

| | |
|---|---|
| 50% Acrylamide/Bis (29:1) Solution | 1.0 mL |
| Stacking Gel Buffer (0.375 *M* Tris-HCl, *p*H 6.8) | 4.2 mL |
| 10% SDS | 125 μL |
| water | 6.7 mL |
| TEMED* | 20 μL |
| 10% Ammonium Persulfate* | 500 μL |

*Add just prior to polymerization—open time is 5-10 min, full polymerization in 1 hr; insert comb immediately after pouring.

**Running Buffer (10 ×)**

29.0 g Tris
144.0 g glycine
10.0 g SDS
*Make to 1000 mL with water. Do not adjust pH.*

**Loading Buffer (2 ×)**

2.5 mL 0.5 M Tris • Cl, *p*H 6.8
2.0 mL glycerol
4.0 mL 10% SDS
0.5 mL 0.1 % bromophenol blue
0.5 mL β-mercaptoethanol
*Make to 10 mL with water. Aliquot and store at –20 deg C.*

**Running Conditions:**

*250 V constant, expect 60-80 mA per gel at start, 15-25 mA per gel at end. Total run time is approximately 45 min.*

**Low Toxicity Staining Solution**

0.25 g Coomassie Blue R-250
100 mL ethanol
100 mL water
*Stir until dye is dissolved. Add 25 mL acetic acid and make to 250 mL with water. Store at room temperature in a dark bottle.*

**Low Toxicity Destaining Solution**

400 mL ethanol
100 mL acetic acid
*Make to 1000 mL with water. Store at room temperature*