Prather Lab
Molecular Cloning Protocols

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Safety

- When working with live cells, always use proper aseptic technique and work in a biosafety hood or near an open flame. When working with volatile or dangerous chemicals (other than EtBr), always work in the chemical fume hood.
- When working with cells (dead or alive), DNA, or chemicals, always wear latex gloves. Wearing a lab coat and safety glasses is also recommended. Some form of eye protection is especially recommended if you do not wear glasses or if you wear contacts.
- Do not wear contacts when working with volatile chemicals (acetone, ethanol, etc.) outside of the chemical hood. Contact lenses, being made of organic polymers and plastics themselves, readily absorb organic chemicals from the air. Chemical-laced contacts are very painful to wear.
- Certain chemical solvents (like chloroform) will rapidly degrade latex gloves. When using these chemicals, wear the purple nitrile gloves found in the chemical hood, and change gloves frequently.
- You should wash your hands thoroughly with soap and water whenever you take your gloves off (if you have been working with cells/chemicals).

Cell/DNA Storage

- Cells that you are going to kill do not have to be kept sterile. For instance, you can miniprep live cells outside of the biosafety hood.
- Cells in liquid culture are viable for up to 24 hours after entering stationary phase. Cells on a plate stored at 4^{o}C are viable for 30-45 days. Cells frozen in the -80^{o}C freezer are good for several years.
- DNA usually does not need to be kept sterile, but try to keep DNA that will be used for transformations sterile.
- DNA can be frozen in buffered solution in the -20^{o}C freezer for several years. Digested DNA (with sticky ends) can also be stored in the -20^{o}C freezer for up to a year. DNA can also be stored in buffered solution in the 4^{o}C refrigerator for several weeks.
- DNA that you wish to preserve (i.e. new plasmids that you create or obtain from other labs) should always be stored in transformed cells in the -80°C freezer.

Waste Disposal

- *Non-hazardous materials* (packaging, weigh boats, non contaminated used gloves etc.) can be thrown in the trash.
- *Biological and large sharps* (broken glass, serological pipets, etc.) should be disposed of in a biological sharps bin. There are three of these bins in the lab, one near each biosafety hood and one in the main aisle near the chemical hood. For your convenience, a smaller red biosharps container may be used for your bench or in the biosafety hoods for small pipet tips and loops. However, it is your responsibility to empty these containers into the three larger containers in the aisles. Please retain and reuse the smaller containers.
- *Solid biological waste* (used tubes, old plates, etc.) should be disposed of either in the white biohazard trash bins or one of the smaller clear biohazard trash bags on the

benches. The small clear biobags should be placed in the white biohazardous bins when full.

- *Liquid biological waste* (liquid cultures) should be treated with a 10 % (v/v) bleach solution (final concentration) for several minutes before being sewered. The empty culture tubes, cuvettes, etc. may then be disposed of as solid biological waste. Do not throw full culture tubes in the solid biological waste as the media will most likely leak during later autoclaving.
- *Miniprep, Gel Extraction and PCR Cleanup flow through waste* may be disposed of in the plastic jugs located by the benchtop microfuge. However, Buffer QG waste (& ADB buffer) must be separated from other effluent. Note the labels on the containers before adding new waste.
- *Chemicals* (acetone, methanol, etc.) should be disposed in the waste jugs in the chemical fume hood in a compatible waste container. Due to the low volume of chemical wastes in the lab, chemicals are pooled into one of 4 waste streams: acids, bases, oxidizers and organic waste. Ensure that you dispose of waste in the appropriate stream. If a container is missing, start a new one. Similarly, if you fill a container, arrange for its pickup by contacting EHS. Should you have a large volume of waste to dispose of (eg. from HPLC use) feel free to create a separate container. However, in all cases, ensure a red tag is on the waste container and remember to update the tag as you add to the containers.
- *Solid chemical (EtBr) waste* should be disposed of in the bucket by the gel imager. This includes contaminated waste such as stained gels and gloves
- *Chemical sharps* (EtBr contaminated tips, other non-biologically contaminated tips) should be placed in the blue wide mouth containers by the gel staining area or in the chemical fume hood. When full, empty these containers into the large white drum on the floor by the solid chemical waste disposal bucket.
- *Liquid EtBr* waste is transferred to the large jug in the gel staining area after expiration. When full, the container is decontaminated through a carbon filter. Refer to the "Prather Lab Chores" document for decontamination protocols.

Making Sterile Liquid LB Media

1. In an autoclavable container, dissolve *LB Miller* (not LB Lennox) powder in deionized water to a final concentration consistent with what is recommended on the container label. Make sure all of the powder is dissolved. The resulting solution should be clear and yellow.

2. Label the container with your initials, the identity of the media (LB), and the current date.

3. Loosely cap the solution (you should be able to easily twist the cap but not pull it straight up off of the jar), place a small piece of autoclave tape on the cap, and autoclave your LB solution at 121^{o}C for 20 to 40 minutes (longer times may be needed for solutions with agar). Immediately after autoclaving, tighten the cap. After autoclaving, the media should be clear and yellow to yellowish-brown in color.

4. The LB media is now sterilized and ready to use (once cooled). The media should only be opened inside a sterile biosafety hood. When the media becomes cloudy, it has become contaminated and should be discarded. When not in use, the media should be tightly capped and stored at room temperature on your bench.

   Note that if you need to make antibiotic-containing liquid media, you should first measure out the amount of LB you will need for your cell culture and then add antibiotics to that portion of LB only. Most antibiotics do not keep well at room temperature for long periods of time, thus you should never add antibiotics directly into your bottle of LB.

Making LB Plates

1. In an autoclavable jar, prepare a solution of LB Agar (Miller) powder in deionized water (use the amount of powder recommended on the label). 500 mL of solution makes about 20-25 plates (1 sleeve). Alternatively, LB Miller and Bacto Agar can be combined per the recommended concentration on the containers and used in place of LB Agar powder. Note that while the LB in this mixture will dissolve (forming a yellow-tinted solution), the agar will not. However, completely suspend the agar before autoclaving. Clumps will burn and not dissolve.

2. Label the media jar with your initials, the identity of the media (LB/Agar) and the current date.

3. Loosely cap the solution (you should be able to easily twist the cap but not pull it straight up off of the jar), place a small piece of autoclave tape on the cap, and autoclave the solution at 121^{o}C for 40 minutes. Immediately after autoclaving, tighten the cap. After autoclaving the media will be clear and yellow to yellowish-brown in color.

4. Place the sealed jar of autoclaved LB/agar in a biosafety hood to cool. While the autoclaved media cools, set out 20-25 empty plates per 500 mL solution made. Label each plate with your initials, the current date, and the identity of the media in the plate (LB, LB/Amp, etc.). A 500 mL autoclaved solution should take roughly 45-75 minutes to cool.

5. Once the solution has cooled to where you can keep your hand on the jar continuously (T < 50^{o}C), add antibiotics to the media, if desired.

Plate type	**Volume to add to 500 mL LB/agar solution**	**Final [antibiotic]**
LB/Amp100	500 µL of 100 mg/mL (stock) ampicillin	100 µg/mL
LB/Cm25	368 µL of 34 mg/mL (stock) chloramphenicol	25 µg/mL

6. While the solution is still warm, pour or pipet roughly 20 mL of solution into each plate. Be careful not to get any bubbles in the plates. If you observe any bubbles, simply pipet them up out of the plate.

7. Let the plates cool for about an hour. During this time the plates should solidify.

8. Place the plates in the 4^{o}C refrigerator upside down (agar side up), making sure they are capped. Plates can also be stored on the left hand side of the cold room (56-459). LB plates are generally good for months if refrigerated. Plates with antibiotics expire after 30-45 days. Despite the low temperature, antibiotics degrade leading to lower effective concentrations with time. You should not use plates that are have expired or plates that have visibly been contaminated or damaged.

Growing *E. coli* Cells in Liquid Culture

1. Determine what volume of culture you would like to grow. Tasks like miniprepping and cell transfer or amplification (i.e. growing up cells from a plate) generally require few cells, so a 3-5 mL culture in a culture tube will suffice.

2. In the biosafety hood, put the appropriate volume of sterile media (3-5 mL for a culture tube, 30-50 mL for a sterile culture flask) in the appropriate growth container (either a culture tube or a sterile culture flask).

3. In the biosafety hood, add any antibiotics to your media as appropriate. Ranges of effective concentrations can be found in Sambrook et. al, Molecular Cloning Manual, *Book 3*, Appendix 2, Table A2.1

4. In the biosafety hood, inoculate your culture using appropriate cells from another source. Sources of cells include:

    - Cells from another liquid culture (pipet them in).
    - Cells from another plate (use a loop or pipet tip to gently brush them off the plate and dip the loop into your media).
    - Cells from -80^{o}C frozen stock (without thawing out the cells, use a loop or pipet tip to scrape some of the cell-containing ice out of the vial and put this ice into your culture).

5. Cap the culture tube (only to the first stop) or flask and incubate it in a 37^{o}C shaker. Generally, for the culture to reach stationary phase, it must be incubated overnight (>12 hrs).

6. You can monitor how many cells are in your culture at any time by measuring the absorbance of the culture at 600 nm using the lab spectrophotometer.

Plating/Streaking Cells

1. Take the appropriate media plates out of the 4°C refrigerator and set them lid-side down in a biosafety hood to help remove condensation. Let them sit in the biosafety hood for 20-30 minutes.

2. Add appropriate reagents, inducer and/or other chemicals to your plate and spread (e.g. add 1 mg of X-gal and 4 μM (final) IPTG for blue-white selection).

3. Add cells onto your plate. You can do this in two ways:

    - From liquid culture, add no more than 100 μL of liquid culture onto your plate and spread evenly using sterile glass beads. Remember that you can always dilute your cultures with sterile media or concentrate them by pelleting in a centrifuge followed by decanting and resuspension.

    - From another plate, use a sterile loop to gently pick up a colony from one plate. Streak the loop across the new plate to deposit the cells from the loop. This technique is called streaking.

4. For *E. coli* cells, incubate the plates overnight at the appropriate temperature (typically 37°C). After incubation, the cells may cover the entire plate to form a lawn, or if you diluted them enough, you will see small, 0.5-2 mm diameter circles of cells called colonies. A colony forms from a single cell, so all cells in a colony are clones of each other. In most instances, you want to obtain plates where you get single colonies. If you get a lawn, streak a tiny bit of the lawn across a fresh plate and grow that plate up overnight.

5. Once satisfactory growth has been achieved, wrap the edges of your capped plate tightly with a strip of parafilm. Store the plate on your shelf in the 4°C refrigerator for up to 30-45 days.

DNA Electrophoresis

DNA electrophoresis is a method used to separate and visualize DNA. An electric field can be used to pull DNA through an agarose gel matrix because the phosphate backbone in DNA is negatively charged. Smaller pieces of DNA will move faster through the gel than larger pieces. A molecular weight marker (DNA ladder) containing DNA fragments of known length can be used to determine the size (in base pairs) of the DNA samples run on the gel.

The migration rate of the DNA fragments will depend on the density of agarose in your gel. However, the migration rate is not linearly related to the fragment size so different gel densities yield different resolutions. Thick gels (>1.5 % (w/v) agarose) give low resolution of large fragments, but high resolution of smaller fragments (<500 bp). The opposite is true for thinner gels – smaller fragments move quickly, stick together and never resolve while thicker bands

separate and resolve. Typical gels run in our lab contain 0.7 or 0.8% (w/v) agarose. The chart below, from Biorad, can be used to select the best gel concentration for your application.

Table 1: Gel concentration required for DNA separation

% (w/v) Agarose	DNA fragment size (kb)
0.50	1 – 30
0.75	0.8 – 12
1.00	0.5 – 10
1.25	0.4 – 7
1.50	0.2 – 3
2 - 5	0.01 – 0.5

1. For a 0.7% (w/v) gel, dissolve approximately 0.175 grams of agarose powder in 25 mL of 1X TAE in an Erlenmeyer flask or other microwaveable vessel. Each lab member is responsible for maintaining his/her own supply of 1X TAE. 1X TAE can be prepared from the 50X TAE concentrate (a common lab resource).

2. Add the 25 mL of 1X TAE into the flask containing the 0.175g of agarose powder. Gently swirl the mixture a few times to disperse the agarose powder throughout the TAE. The agarose should not dissolve in the TAE at this point. Loosely plug the mouth of the flask with a couple of Kimwipes to prevent excessive water loss during microwaving.

3. Microwave the flask in the lab microwave for 70-80 seconds. Remove the flask with a hot glove. If you still see undissolved agarose in the flask, microwave it for an additional 20 seconds. The final heated solution should be clear and colorless (no agarose powder should be visible). Let the flask cool briefly on the benchtop.

4. While your molten agarose is cooling, set up an electrophoresis tray and place a well comb in it. The well comb creates the holes in the gel where you will add your DNA samples. If you are planning on running samples larger than 15 µL on the gel (about what one large-toothed comb well will hold), you can carefully tape together multiple teeth on the well comb together to create a larger well. Alternatively, you can run a larger gel with wider teeth. For smaller volumes of liquid, the smaller-toothed comb wells can hold about 7 µL.

5. When the agarose cools such that you can hold the flask easily in the palm of your hand, pour the solution into an electrophoresis tray. Be careful not to generate any bubbles in the molten agarose. Bubbles can be removed by sucking them up using a pipet.

6. Immediately rinse out the flask that contained the hot agarose with water. Residual agarose will solidify quickly in the flask.

7. Let the solution cool and solidify into a gel. This takes roughly 20 minutes. Once the gel has solidified, gently remove the well comb from the gel.

8. Transfer the gel tray into an electrophoresis chamber. The side of the gel with the wells should be facing towards the black side (anode) of the chamber. Fill the chamber with 1X TAE until TAE just covers the gel.

9. Prepare each of your DNA samples in the following way:

   x µL DNA Sample
   10 – x µL 1X TAE
   2 µL 6X Loading Dye (found in the 4°C refrigerator)
   12 µL Total

   For larger samples (x > 10 µL), simply scale up the recipe to accommodate all of your sample. If you plan on loading your samples into wells generated by the small-toothed comb, scale down this recipe by half (to make a total of 6 µL of sample).

10. Prepare a 12 µL (or 6 µL if using the smaller wells) DNA ladder standard at a concentration of 0.25 µg/lane. For the NEB 1kb ladder, this comes out to 0.5 µL ladder, 9.5 µL 1X TAE, and 2 µL of 6X loading dye.

11. Carefully pipet your prepared DNA samples and the DNA ladder standard into the wells on your gel.

12. Gently seal off the electrophoresis chamber with the chamber cap and plug the cables extending from the chamber cap into a VWR AccuPower power supply.

13. Turn on the power supply and set it to deliver 90 volts for 60 minutes. Leave the current setting on the power unit alone. Hit the Run button to start running the gel.

    Alternative: For more throughput or shorter run times, 2 half gels (2 sets of combs per gel) can be run at a setting of 110V, 30 min. Note, the gels run only half as far so there is a hit in resolution. However, with the optics on the imager, the effects of this are negligible. As a general rule of thumb, the time affects the migration distance – shorter times equal shorter migration distances and lower resolutions. Conversely, the voltage controls the migration speed. Higher voltage increases the migration speed and increases the resolution. However, higher voltage also heats the system potentially leading to smearing of bands (increased diffusion) or DNA damage (if it's to be extracted later) so play with these settings carefully.

14. While the gel is running, check the log sheet beside the sink closest to the chalkboard to determine if you need to prepare a fresh ethidium bromide (EtBr) solution. EtBr is light sensitive and generally only works well as a DNA stain for 24 hours. EtBr solutions older than 24 hours should be poured into the EtBr waste jug and a new EtBr solution should be made. Prepare fresh solution in the EtBR chamber by combining 100 mL of deionized water with 5 µL of EtBr concentrate. Gently swirl the fresh EtBr solution until you no longer see any redness from the EtBr. EtBr is a DNA stain and also is a known

mutagen. Take care not to get any EtBr on your skin or clothes, and change gloves after working with EtBr.

15. Once the gel has finished running, remove the gel tray and gently slide the gel off of the tray and into the EtBr chamber. Gently swirl the chamber to completely submerge the gel in the EtBr solution. Let the gel sit in the EtBr chamber for 15 minutes. It is ok to let your gel sit in the EtBr solution for several hours if needed.

16. Visualize your DNA on the Alpha Innotech imager.

Gel Extraction

Gel extraction is used to isolate and concentrate pieces of DNA from an agarose gel.

1. Follow the directions in the Qiagen or Zymoclean gel extraction kit for isolating your DNA from an agarose gel.

2. Store your extracted DNA in the -20^{o}C freezer for up to a year.

Running a Polymerase Chain Reaction (PCR)

PCR amplifies a piece of DNA flanked by two primers. PCR can be used to amplify a specific piece of DNA up to a concentration where you can manipulate or otherwise work with it. The piece of DNA to be amplified may be a whole plasmid, a DNA fragment, or a gene from an entire genome.

1. Order two DNA primers from Sigma's Custom Products on ECat.

2. Dilute your primers to a concentration of 100 μM using deionized water (10x the number of nmols in μL, e.g. 52 nmol = 520 μL). The number of moles of primer DNA in your vial should be printed on the side of the primer vials you received from Sigma-Genosys.

3. Check the appropriate manual of the polymerase you are using to determine the optimal PCR mix and conditions for your PCR reaction. The PCR kit manuals are located in the drawer below the PCR machine. In general, your PCR reaction will consist of the following repeated 30-35 times:

   Denaturing: $\geq$ 95^{o}C for 5 - 30 s

   Annealing: 45 - 60^{o}C for 5 - 30 s

   Extension: Usually 72 ^{o}C for ~15 - 30 s per 500 base pairs in DNA sequence to be amplified. The extension temperature should be higher than the melting temperature of both of your primers.

Note, these times and temperatures are extremely polymerase dependent so be sure to consult the manual before beginning.

4. Start the PCR reaction and wait for it to complete. Standard PCR reactions generally take about 2-3 hours.

5. Following completion of the thermal cycler run, run a gel with 1-5 µl of your PCR reaction in a well. This is to confirm that you have the desired band amplified and to determine if there are other bands that may result from non-specific annealing and amplification.

6. After verification of your PCR reaction, you may need to do one (or more) of the following post-processing steps:
    - pGEM-T Easy vector ligation. This will put an A-tailed amplified fragment into a vector from which it can be easily subcloned later. For ***Taq*** amplified DNA you can use the fragment immediately. For fragments amplified from hi-fidelity polymerases (e.g. *Pfu*) , the fragment needs to be first A-tailed using dATPs and Taq at 70°C (consult the pGEM manual for protocol)
    - Gel extraction. If the PCR reaction contains more than one band or if the amplification was from a plasmid DNA template, you will need to cut your band out of a gel to separate it from contaminating DNA before using it further (for example, in a pGEM-T Easy ligation).
    - Qiagen PCR clean-up. Follow the Qiagen PCR cleanup protocol to isolate your amplified DNA away from your primers and the other components from the PCR reaction.

7. If you plan to digest your fragment for use directly in a ligation reaction, it is important that it be properly cleaned to increase your chances for success. This is because *Taq* polymerase has been found to bind tightly to the ends of amplified DNA, interfering with subsequent digestion. It is also not efficiently removed with the silica-based columns that we use. Please see the following references for a detailed discussion of the steps necessary to maximize your ligation efficiency:
    - Wybranietz and Lauer. (1998) *BioTechniques*. **24(4)**: 578-580. (online)
    - Sambrook et al. *Molecular Cloning Manual*, Chapter 8, p. 8.25 (in our lab)

    In general, you will need to perform the following steps:
    a. Proteinase K digestion (To remove *Taq* polymerase)
    10 mM Tris-HCl (pH 8.0)
    EDTA 5mM
    SDS 0.5%
    Proteinase K 50 ug/ml
    60 min at 37°C

    b. Heat inactivation (to inactivate Proteinase K)
    68°C for 15min

c. Purification of DNA fragments
   Extract once w/ phenol/chloroform
   Extract once w/ chloroform
   EtOH precipitation
   Wash w/ 70% EtOH
   Dissolve in deionized water

d. Use QIAquick PCR purification kit (to remove primers)

e. Restriction enzyme digestion

f. QIAquick Nucleotide Removal kit or gel extraction (to purify restriction digest)

8. Store your amplified DNA (purified or not) in the -20^{o}C freezer for up to a year.

Digestion of DNA using Restriction Enzymes

Restriction enzymes cut DNA at specific sequences. Each restriction enzyme (EcoRI, HindIII, etc.) cuts DNA at a different specific nucleotide sequence. You can digest your DNA sample with one or two restriction enzymes. When planning a digestion reaction, you should consult a sequence map to help select which enzyme(s) to use to achieve your desired experimental goal.

1. A 10-µL single enzyme digestion should contain:

   x µL DNA sample
   8 – x µL Deionized water
   1 µL 10x Restriction enzyme buffer
   1 µL Restriction enzyme
   10 µL Total

   where x is the µL of DNA sample you wish to digest (0 µL < x ≤ 8 µL). Restriction enzymes are found in the cryoboxes in the -20^{o}C freezer, while restriction enzyme buffers are found on the general shelf in the 4^{o}C refrigerator. The digestion mixture should be prepared in a 0.6-mL tube. The restriction enzyme should be added last. Never add the restriction enzyme to a solution without its appropriate buffer (even momentarily), as doing so may dramatically decrease the enzyme's activity. The restriction enzyme should be kept at sub-zero temperatures until added into the digestion mix. Do not remove the enzyme from the storage box in the freezer to take it to your bench. Instead, take the whole cold box with you, and remove the enzyme for the only the time needed to extract your aliquot(s).

   For a 50-µL single enzyme digest:

x μL DNA sample
40 – x μL Deionized water
5 μL 10x Restriction enzyme buffer
5 μL Restriction enzyme
50 μL Total

The restriction enzyme buffer used in the digestion depends on the restriction enzyme. A chart of restriction enzyme activities in each of the four New England Biolabs restriction enzyme buffers is found on the -20°C freezer. Note that this chart also lists the temperatures at which the enzyme works best (usually 37°C). This information can also be found on the New England Biolabs website (http://www.neb.com/nebecomm/EnzymeFinder.asp)

For a double digest, the following recipe (per 10 μL) applies:

x μL DNA sample
8 – x μL Deionized water
1 μL 10x Restriction enzyme buffer
0.5 μL Restriction enzyme #1
0.5 μL Restriction enzyme #2
10 μL Total

To determine the best buffer for the double digest, use the chart at the bottom right corner of the poster on the -20°C freezer or the Double Digest Finder on the NEB website (http://www.neb.com/nebecomm/DoubleDigestCalculator.asp?). If the enzymes you'd like to use are not compatible, two sequential single-enzyme digests can be performed.

2. Incubate the digestion at the appropriate temperature (usually 37°C) for 30 minutes to 3 hours. It is usually ok to let your digestion go longer than three hours, but it should not go on longer than a whole day. Shorter incubation times may result in incomplete digestions.

3. Run the digestion on an agarose gel (see the protocol for DNA electrophoresis above). Include a sample of undigested (uncut) DNA on the gel. Running your digested DNA on a gel separates it from the salts and enzymes used in your digestion. For digestions of 15 μL or less, use a single gel well. For larger digestions, you will need larger well holes. Larger holes in the gel can be made by carefully taping together multiple teeth on the well comb (so that liquid can no longer fill the gap between the teeth) before placing the comb in molten agarose. In this manner, you can create double, triple, etc. sized wells. A double well can hold about 35 μL. A triple well can hold about 60 μL.

4. Once your gel has run, stain it with EtBr for 15 minutes and visualize the gel on the Alpha Innotech imager. Check to make sure you see DNA fragment(s) of the correct size(s).

5. Follow the directions in the Qiagen or Zymoclean gel extraction kit if you need to isolate a DNA fragment from the agarose gel.

6. Store the digested DNA in the -20°C freezer for up to a year.

DNA Ligation Reaction

In a DNA ligation reaction, the T4 DNA ligase enzyme joins two pieces of DNA with compatible sticky or blunt ends.

1. Thaw a tube of 10X ligase buffer (found in the -20°C freezer) in the 4°C refrigerator. Do not thaw the buffer at room temperature. If you haven't done so already, make a small aliquot (~100 µL) to keep in your personal -20°C storage. It typically takes about 30 minutes to completely thaw the buffer at 4°C.

2. While the buffer is thawing, prepare the following 8 µL mixture of DNA in a 0.6 mL tube:

   x µL DNA Sample #1
   y µL DNA Sample #2
   8 – x – y µL Deionized Water
   8 µL Total

   where x and y are the amounts (in µL) of the two DNA fragments that you wish to ligate together. Generally if you are ligating two equally-sized pieces of DNA together, you should use roughly equal amounts of the two samples. If you are ligating an insert into a plasmid vector, and if the vector is at least 3-4 times larger (in base pairs) than the insert, a vector:insert ratio of 1:5 is generally ideal. If your DNA samples are dilute or blunt ended, you should devote the whole 8 µL of reaction volume available towards your DNA samples. For very concentrated, sticky ended samples, 1 µL will usually suffice.

   You can qualitatively estimate the concentration of the DNA fragments by running them on a gel. Absorbance at 260 nm can be used to quantify the fragment concentration more precisely.

3. Once the ligation buffer has thawed, vortex vigorously until any precipitate is completely dissolved and the buffer is well-mixed. The precipitate is likely ATP which is required by DNA ligase. After vortexing, add 1 µL of 10X ligase buffer to the 8 µL DNA sample mix.

4. Add 1 µL of T4 DNA ligase to the reaction mixture. The ligase is found in a cold box in the -20°C freezer. Never add DNA ligase into a solution not containing the ligase buffer, as this may dramatically lower the ligase's activity.

5. Cap the tube and let the ligation reaction proceed at room temperature for at least 20 minutes. It is ok for a ligation reaction to go overnight at room temperature. If the ligation fails under these conditions, you may want to consider running the reaction at 16°C overnight.

Electrocompetent Transformations (Electroporation)

Transformations are used to get DNA, such as a plasmid or the products of a ligation reaction, inside cells. Electoporation uses electricity to facilitate the transformation and is more efficient than chemical ($CaCl_2$) transformation, but is also more expensive. Electroporation is generally used when trying to transform the products of a ligation reaction into cells.

1. Obtain a tube of electrocompetent *E. coli* cells (20 µL) from the -80°C freezer and place it on ice. If there are none left, refer to the "Guide to the Prather Lab for New Members" document for instructions on maintaining the lab stock of electrocompetant DH10B cells.

2. Place a sterile electroporation cuvette and the tube containing the DNA you are transforming on ice as well. Make sure the electroporation chamber has a 1mm pathlength.

3. In the biosafety hood, add your DNA sample to the 20 µL of cells. For transforming cells with a ligation reaction mixture, 1.5-2.0 µL of ligation reaction mix per 20 µL of cells is generally a good amount to add. For purified, whole plasmids, only 0.1-1.0 µL is needed. If you are transforming two whole plasmids at once use 0.1-1 µL of each plasmid. Use your pipette to **gently** mix the DNA and the cells – electrocompetent cells are very fragile.

4. In the biosafety hood, transfer the DNA/cell mixture into the chilled electroporation cuvette. Be careful not to pipet bubbles or air into the chamber. Put the cuvette back on ice.

5. In the biosafety hood, pipet 1 mL of SOC medium into a sterile culture tube. Do not put this tube on ice.

6. The following steps will be performed using the BioRad Micropulser electroporation unit. Set the unit to the "Bacteria EC1" setting for 1 mm pathlength cuvettes (use the "Bacteria EC2" setting when using a 2 mm pathlength chamber).

7. Slide the electroporation cuvette into the electroporation unit until the metal parts of the unit contact the metal parts of the cuvette. Press and hold down the "Pulse" button on the unit until you hear a tone.

8. **Quickly** remove the electroporation cuvette from the unit and resuspend the cells in the 1 mL of SOC from the culture tube and pipet the cells back into the culture tube .

9. Check and record the voltage and time constant for the transformation. If the time constant is really low (<2 ms) and/or you heard a loud pop when you pressed the pulse button the transformation likely arced. Arcing can occur if the salt content of the transformation mixture is too high or if bubbles are present in the cuvette. Cells won't survive an arc, so discard the transformation if you think an arc occurred. If you are having a problem with arcing, add less DNA sample to your cells in Step 3 and try the transformation again.

10. Incubate the culture tube in a shaker for an hour at 37^{o}C (or the appropriate temperature for your strain).

11. While the culture tube is incubating, take the agar plates you plan to spread the transformed cells on out of the refrigerator. If you transformed your cells with a plasmid that has blue/white selection you can add IPTG and X-gal to the plates at this time (if desired).

12. Plate the transformed cells on appropriate solid medium. Remember that an agar plate can only absorb about 100 µL of liquid. If you want to plate more cells than this concentrate the culture by gently pelleting the cells, decanting the extra SOC, and then resuspending the cells in the remaining liquid.

    When transforming cells with whole plasmids, plating 100 µL of the 1 mL culture will generally yield a lawn of cells. If you wish to obtain single colonies from whole-plasmid transformations you can either use a sterile loop to streak a small amount of the culture across a plate or prepare a 10^{-5} to 10^{-6} dilution of the culture in LB and plate 100 µL of this diluted mixture.

13. Incubate your plates at 37^{o}C (or the appropriate temperature) overnight.

Making Frozen Stocks of Cells for Long-Term Storage

1. Grow up a 2-4 mL culture overnight (to stationary phase) of your bacterial strain in appropriate media.

2. The next morning, add 30 µL of the overnight, stationary-phase culture to a fresh 3 mL culture. Incubate the culture to an OD_{600} of ~0.5-1.0 (i.e. to mid-exponential phase, typically this takes 3-4 hours in rich media).

3. Obtain three 2-mL cryogenic vials. Label each vial with your initials, the strain name, and the date stored. Label each vial with the strain storage number on the side and top of the vial. Sign out a strain storage number from the list on top of the office filing cabinet.

4. In the biosafety hood, fill the vial with a 1:1 mixture of cell culture and cold, sterile 30% (v/v) glycerol. Typically, this is accomplished by adding 900 µL glycerol to 900 µL of cell culture.

5. Cap the three vials, invert them a few times to mix, and place the vials in the -80^{o}C freezer. One vial should be stored in the "Main Storage" box in the -80^{o}C freezer. The other two vials are stored in the "Backup Storage" box, also in the -80^{o}C freezer.

6. Register your strains in the Strain Database by filling out the "strain entry" form in the "Lab Members Only" section of the group website.

Plasmid Miniprepping:

Miniprepping concentrates and purifies plasmid DNA from whole bacterial cells. The resulting purified plasmid DNA can then be used for transformation, digestions, and ligations. The purified plasmid DNA can also be stored for up to a year in the -20^{o}C freezer. Miniprepping refers to the preparation of small quantities of plasmid DNA, while megaprepping refers to larger-scale preparation of plasmid DNA.

To miniprep DNA, follow the instructions in the manual of either the Qiaprep Spin Miniprep Kit (Qiagen) or the Zyppy Plasmid Miniprep kit (Zymo). There is also usually a maxiprep kit in the lab that can be used to prepare larger quantities of DNA. Refer to the manual for instructions.