SUPPLEMENTAL MATERIALS 1

Below are student handouts for each of the individual protocols discussed during the workshop. Each experiment is written with a student introduction and background followed by a protocol. Note: some protocols are written generally so they may be adapted as necessary.

Plasmid DNA Isolation

Introduction

Plasmids are small, circular pieces of double stranded DNA that exist in the cytoplasm of cells independent of the host's genome where they can be transmitted faithfully to daughter cells at division. Plasmids signal for the cellular machinery to initiate and complete their replication either in synchrony with the replication of the rest of the genome, or randomly. Plasmids have been found and studied in bacteria (prokaryotes) and yeast (eukaryotes). These pieces of DNA are not required for an organism to survive under normal conditions but can provide genetic information to give the cell an advantage under certain circumstances. For example, antibiotic resistance can be passed to a cell via a plasmid.

Our knowledge of bacterial plasmids has come a long way since the 1960s when bacterial geneticists discovered these small, circular DNA units. Since then, many different kinds of plasmids have been found in both prokaryotic and eukaryotic cells (where they reside in the nucleus). While the plasmids used in molecular biology are derived from natural sources, or parts of several naturally occurring plasmids, they often contain segments of DNA from yeast or other eukaryotic organisms, viruses, or sequences synthesized *in vitro*. These sequences are spliced together to form a new, recombinant structure that has the capabilities of its many parts. Scientists are then able to employ the plasmid as a vehicle or vector that can accept and propagate one or more fragments of DNA.

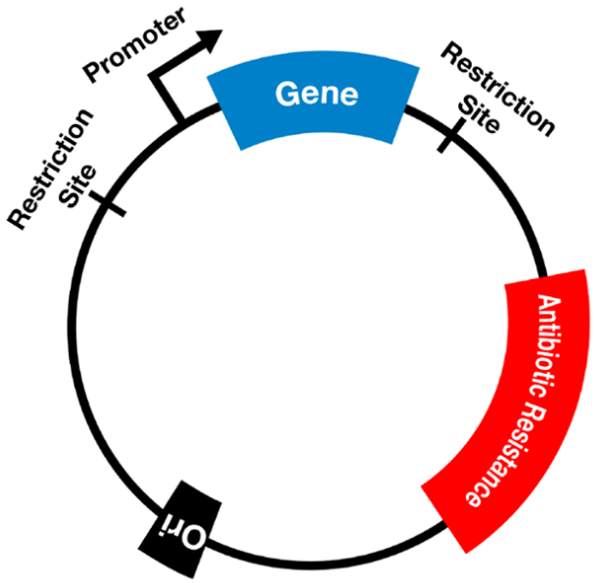

Figure 1 | A general schematic of a plasmid vector used in research. Note 3 key features of a plasmid: an Origin of Replication (ORI), Restriction Enzyme Sites, and a Selectable Marker.
https://blog.addgene.org/plasmids-simplified

There are several important features that must be present in the plasmid DNA in order for it to be maintained within the host cell (Figure 1). These include an origin of replication (ORI; where DNA replication begins) and a selectable marker (usually a gene encoding antibiotic resistance). To maintain a plasmid within the cell, it must contain an origin of replication, allowing the cell to replicate the plasmid and thus pass the plasmid on to daughter cells after cell division. Expression of the selectable marker allows for survival on selective media and elimination of non-transformed cells. The plasmids that you are isolating contain the ampicillin resistance gene (Amp^R). The ampicillin resistance gene codes for the enzyme ß-lactamase (penicillinase), which inactivates ampicillin and other types of penicillin by cleaving the B-lactam ring.

When using plasmid vectors for cloning, we need to cut open the vector to insert our gene of interest. To accomplish this, biologists make use of restriction enzyme sites located on the plasmid vector. Restriction enzymes are proteins that recognize and cut DNA at very specific sequences. Their "site" refers to the location in the DNA sequence where a given enzyme will recognize and cut.

Purifying Plasmids for Experiments

Whatever the purpose may be for which you purchased or even constructed a plasmid vector, it is likely to

require that you amplify the amount of DNA to some multiple of what you already have. It is for this purpose that the plasmid is held in bacterial cells – they are easy to grow in large numbers and, because their cellular organization is simple, the purification steps are quite straightforward. The plasmid DNA recovered from the cells must be pure: free of RNA, cellular proteins, and (most importantly) genomic DNA from the bacteria. When plasmid DNA has been recovered and purified, it can be stored for a long time at -20°C.

Several methods exist for the isolation and purification of the plasmid DNA. A maxi preparation requires many steps over several days, but it will produce a large amount of exclusively plasmid DNA that is essentially free of both RNA and genomic DNA. The mini method allows you to quickly recover a small amount of DNA. The purity will depend on your lab technique. We will be using a kit from the company Qiagen that allows you to obtain a fair amount of pure plasmid DNA in a short time. You will then assess the purity of your DNA using UV spectroscopy and agarose gel electrophoresis.

To understand how the plasmid isolation technique works, it is important to recognize the unique properties of plasmids that allow their separation from chromosomal DNA and other cellular components. The most obvious difference is size. The largest plasmids are only about 8% of the size of the bacterial chromosome (15 kb compared to 130 kb) and the majority of the other plasmids are much, much smaller. Second, plasmid DNA differs from the chromosome in its conformation during the isolation. Once a bacterial cell is broken open and subjected to the miniprep procedure, the normally circular and supercoiled chromosomal DNA becomes denatured into linear, relaxed single-stranded DNA pieces. In contrast to this, because plasmids are significantly smaller, they can be released as supercoiled, circular molecules. These differences in size and conformation upon the lysis of the cell are taken advantage of to allow the separation of plasmid and chromosomal DNA. The isolation protocol follows four fundamental steps:

1. Harvesting of bacterial cells.
2. Breaking open cells to release their contents.
3. Separation of plasmid DNA from chromosomal DNA and other cellular materials
4. Concentration of the purified plasmid DNA.

The main plasmids you are isolating for your project is pML104_NOP2 (Figure 2), or pML104_HAS1 (Figure 3). These plasmids were made by cloning sequence from our ribosome assembly factor (RAF) of interest into the pML104 vector between the SwaI and BclI restriction enzyme sites.

The manufacturer instructions for the QIAgen Miniprep kit are provided below (pg 5-9).

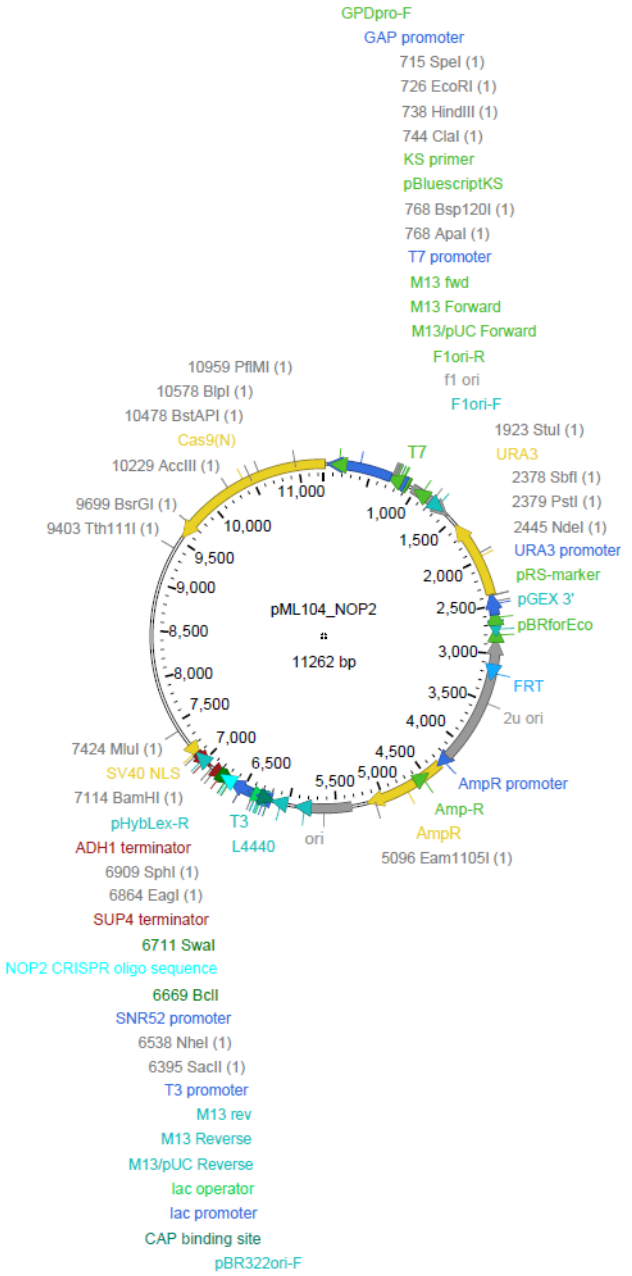

Figure 2 | Map of the plasmid pML104_NOP2. The name and size in base pairs of the plasmid are labeled in the middle of the map. Genes, promoters, terminators, and the origin of replication are represented by blocks and block arrows. Restriction enzyme sites are labeled in grey with their locations in base pairs. Map made using ApE by L. Doonan, 2022. Plasmid vector (pML104) designed by Laughery *et al* 2015.

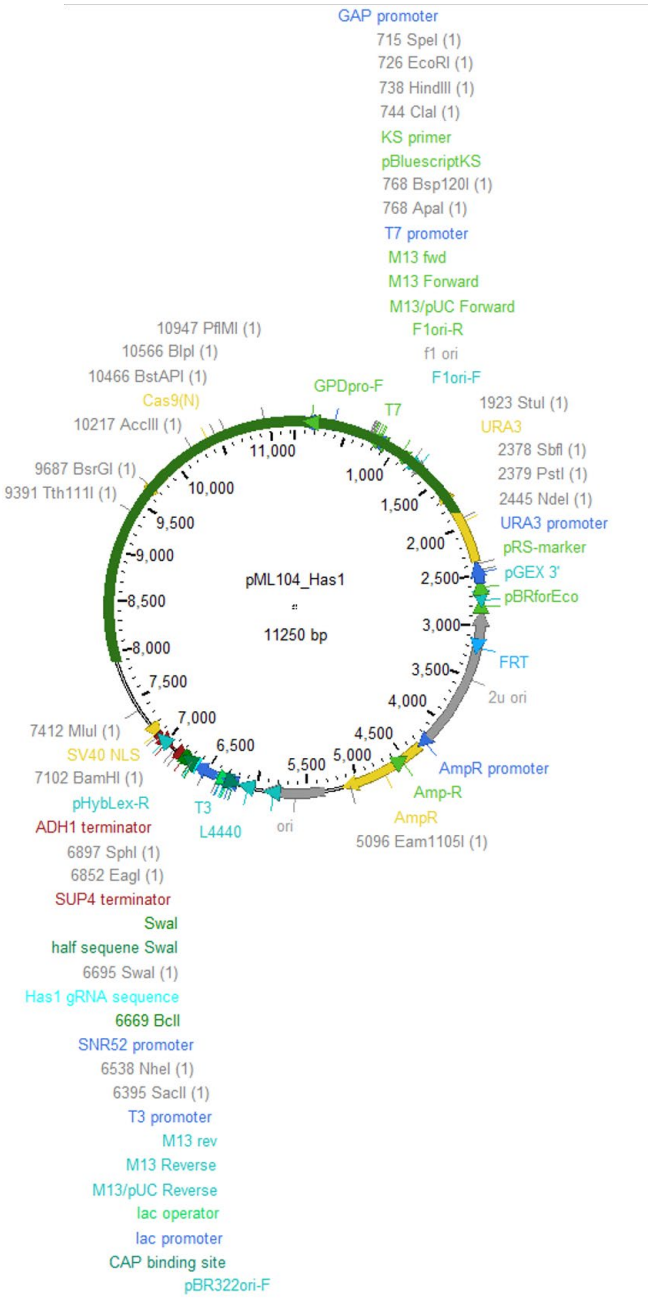

Figure 3 | Map of the plasmid pML104_HAS1. The name and size in base pairs of the plasmid are labeled in the middle of the map. Genes, promoters, terminators, and the origin of replication are represented by blocks and block arrows. Restriction enzyme sites are labeled in grey with their locations in base pairs. Map made using ApE by L. Doonan, 2022. Plasmid vector (pML104) designed by Laughery *et al* 2015.

LyseBlue reagent*

Use of LyseBlue is optional and is not required to successfully perform plasmid preparations. See "Using LyseBlue reagent" on page 11 for more information.

LyseBlue is a color indicator that provides visual identification of optimum buffer mixing. This prevents common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. This makes LyseBlue ideal for use by researchers who have not had much experience with plasmid preparations, as well as experienced scientists who want to be assured of maximum product yield.

DNA adsorption to the QIAprep membrane

QIAprep 2.0 columns, strips, and plates use a silica membrane for selective adsorption of plasmid DNA in high-salt buffer and elution in low-salt buffer. The optimized buffers in the lysis procedure, combined with the unique silica membrane, ensure that only DNA will be adsorbed, while RNA, cellular proteins, and metabolites are not retained on the membrane but are found in the flow-through.

Washing and elution of plasmid DNA

Endonucleases are efficiently removed by a brief wash step with Buffer PB. This step is essential when working with *endA*$^+$ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, to ensure that plasmid DNA is not degraded. The Buffer PB wash step is also necessary when purifying low-copy plasmids, where large culture volumes are used.

Salts are efficiently removed by a brief wash step with Buffer PE. High-quality plasmid DNA is then eluted from the QIAprep 2.0 column with 50–100 µl of Buffer EB or water. The purified DNA is ready for immediate use in a range of applications — no need to precipitate, concentrate, or desalt.

Note: Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water for elution, make sure that the pH value is within this range. Store DNA at –20°C when eluted with water since DNA may degrade in the absence of a buffering agent.

DNA yield

Plasmid yield with the QIAprep miniprep system varies depending on plasmid copy number per cell (see page 31), the individual insert in a plasmid, factors that affect growth of the bacterial culture (see page 31), the elution volume (Figure 1), and the elution incubation time (Figure 2). A 1.5 ml overnight culture can yield from 5 to

* LyseBlue reagent is only supplied with the QIAprep Spin Miniprep Kit since multiwell or automated formats do not allow visual control of individual samples.

15 µg of plasmid DNA (Table 1, page 11). To obtain the optimum combination of DNA quality, yield, and concentration, we recommend using Luria Bertani (LB) medium for growth of cultures (for composition see page 33), eluting plasmid DNA in a volume of 50 µl, and performing a short incubation after addition of the elution buffer.

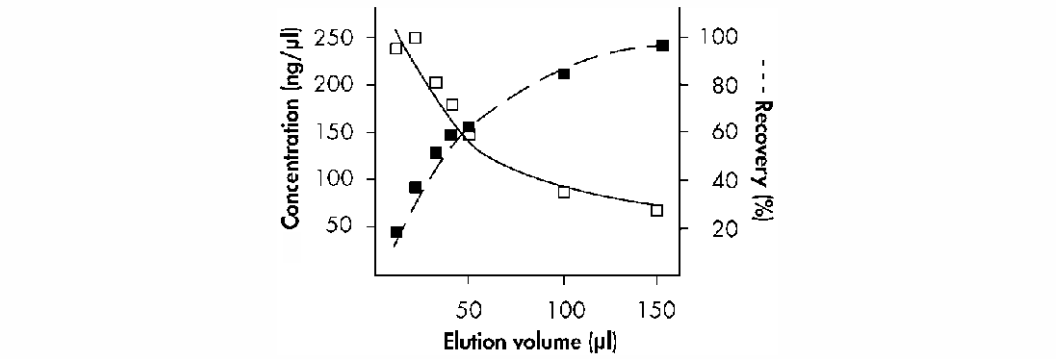

Figure 1. Elution volume versus DNA concentration and recovery. Using the QIAprep Spin protocol, 10 µg pUC18 DNA was purified and eluted with the indicated volumes of Buffer EB. The standard protocol uses 50 µl Buffer EB for elution, since this combines high yield with high concentration. However the yield can be increased by increasing the elution volume.

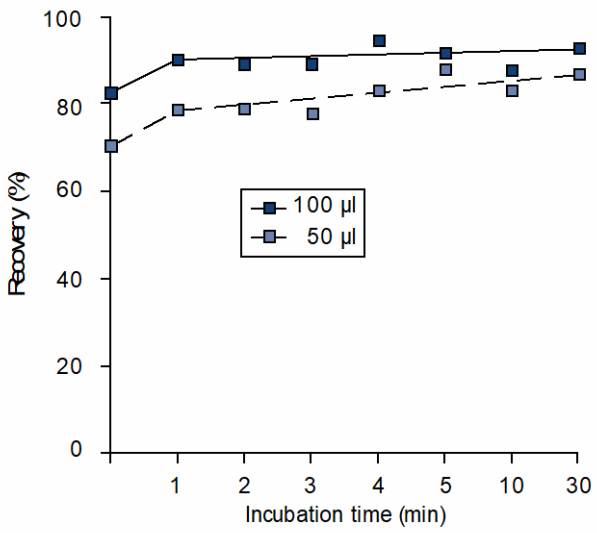

Figure 2. Incubation time versus DNA recovery. Using the QIAprep Spin Miniprep protocol, 10 µg pBluescript DNA was purified and eluted after the indicated incubation times with either 50 µl or 100 µl Buffer EB. The graph shows that an incubation time of 1 minute and doubling the elution buffer volume increases yield.

Table 1. Effect of different compositions of growth medium LB on DNA yield

Culture media	Yield
LB (containing 10 g/liter NaCl)	11.5 µg
LB (containing 5 g/liter NaCl)	9.5 µg

QIAprep Spin Miniprep Kit was used to purify DNA from 1.5 ml LB overnight cultures of XL1-Blue containing pBluescript®. Elution was performed according to the standard protocol (50 µl Buffer EB and 1 min incubation). Use of the recommended LB composition (with 10 g/liter NaCl, also see Appendix A, p. 43) provides optimal plasmid yield.

Using LyseBlue reagent

Using a simple visual identification system, LyseBlue reagent prevents common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, cell debris, and genomic DNA.

LyseBlue can be added to the resuspension buffer (Buffer P1) bottle before use. Alternatively, smaller amounts of LyseBlue can be added to aliquots of Buffer P1, enabling single plasmid preparations incorporating visual lysis control to be performed.

LyseBlue reagent should be added to Buffer P1 at a ratio of 1:1000 to achieve the required working concentration (e.g., 10 µl LyseBlue into 10 ml Buffer P1). Make sufficient LyseBlue/Buffer P1 working solution for the number of plasmid preps being performed.

LyseBlue precipitates after addition into Buffer P1. This precipitate will completely dissolve after addition of Buffer P2. Shake Buffer P1 before use to resuspend LyseBlue particles.

The plasmid preparation procedure is performed as usual. After addition of Buffer P2 to Buffer P1, the color of the suspension changes to blue. Mixing should result in a homogeneously colored suspension. If the suspension contains localized regions of colorless solution or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

Upon addition of neutralization buffer (Buffer N3), LyseBlue turns colorless. The presence of a homogeneous solution with no traces of blue indicates that SDS from the lysis buffer has been effectively precipitated.

Loading Dye

Loading Dye is provided for analysis of plasmid DNA samples using electrophoresis. It contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 to identify the dyes according to migration distance and agarose gel percentage and type. Loading Dye is supplied as a 5x concentrate; thus 1 volume of Loading Dye should be added to 5 volumes of purified DNA.

Protocol: Plasmid DNA Purification using the QIAprep Spin Miniprep Kit and a Microcentrifuge

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of *E. coli* in LB medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on pages 35–36.

Please read "Important Notes" on pages 13–18 before starting.

Note: All protocol steps should be carried out at room temperature (15–25°C).

Procedure

1. **Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.**

   Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. **Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.**

   Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

   If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

3. **Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.**

   To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥ 5 ml) may require inverting up to 10 times. The solution should become cloudy.

   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

4. **Centrifuge for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge.**

   A compact white pellet will form.

5. **Apply 800 µl of the supernatant from step 4 to the QIAprep 2.0 spin column by pipetting.**
6. **Centrifuge for 30–60 s. Discard the flow-through.**
7. **Recommended: Wash the QIAprep 2.0 spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.**

   This step is necessary to remove trace nuclease activity when using *endA*+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5®α do not require this additional wash step.
8. **Wash QIAprep 2.0 spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.**
9. **Discard the flow-through, and centrifuge at full speed for an additional 1 min to remove residual wash buffer.**

   Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.
10. **Place the QIAprep 2.0 column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep 2.0 spin column, let stand for 1 min, and centrifuge for 1 min.**

-- Notes --

Ultra Violet/Visible (UV/Vis) Spectroscopy

Any time you complete a plasmid prep, you should quantify the outcome – that is, determine the amount of DNA you isolated and its purity with respect to protein. One way that we measure these two characteristics is to complete a UV scan of your DNA sample. DNA and RNA maximally absorb at a wavelength of 260 nm. The ring structures of the aromatic amino acids, tryptophan, tyrosine, and phenylalanine, absorb at 280 nm. Finally, the peptide bonds of proteins absorb UV light maximally at 230 nm (Figure 1).

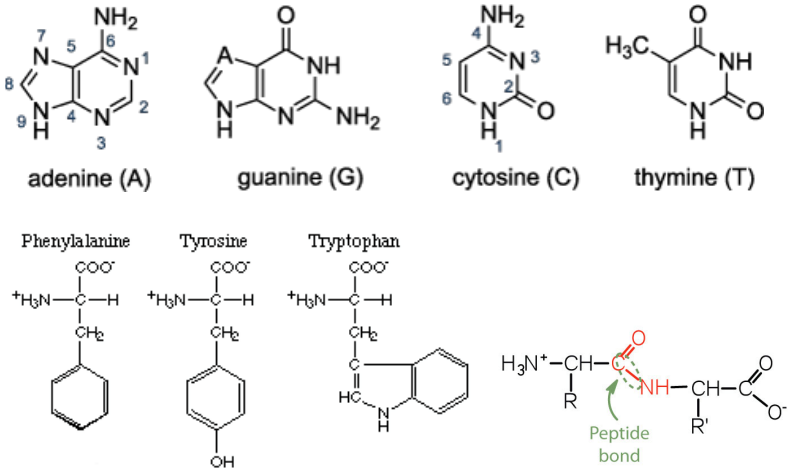

Figure 1 | The chemical structures of various ring compounds. Molecular structures of nucleic acids (top), aromatic rings of proteins (bottom left), and peptide bonds (bottom right).

Using this information, we can calculate the purity and concentration of our plasmid samples using the following equations.

$$Purity = \frac{Abs_{260}}{Abs_{280}} \qquad 1Abs_{260} = 50\ \frac{ng}{\mu L}\ DNA$$

The ratio of Abs_{260} to Abs_{280} gives the purity of the sample with respect to protein contaminants. For most molecular experiments and protocols, you will need a purity of 1.7 – 1.9. A purity <1.7 suggests protein contamination, which may interfere with future experiments. If your purity ratio is >1.9, this could suggest RNA contamination. While it is impossible to distinguish between DNA and RNA through spectroscopy, we do know that a molecule of RNA absorbs more light at 260 nm than a molecule of DNA. Because of this, RNA contamination can lead to high purity ratios. If your ratio is above 1.9, consult with your instructor about how to proceed.

Biochemists have worked out that double-stranded DNA at a concentration of 50 ng/µL has an absorbance of 1 at 260 nm. Furthermore, this relationship remains the same for any concentration of DNA. Thus, you can use the Abs_{260} to calculate the concentration of your sample.

When you scan your DNA sample to determine the purity and concentration you will want to record the absorbencies at 230, 260, and 280 nm. All 3 values can provide important information about the quality of your sample. In addition, you should print out the graph of the full scan of your sample for your notebook. The shape of the scan can also provide information about your sample. Figure 2 shows an example of a UV scan of very pure nucleic acids.

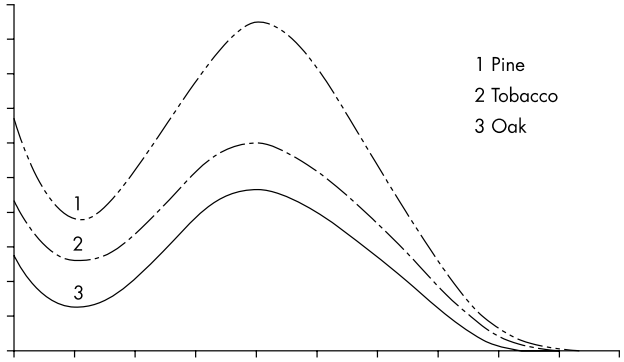

Figure 1 UV scan of DNeasy purified DNA diluted 1:5 in water.

Figure 2 | Example UV scan of pure DNA Notice the width of the peak at 260 nm. While nucleic acids maximally absorb at 260 nm, they have some absorbance from 230 – 300 nm. Qiagen DNeasy Plant Handbook, Pg. 48, www.quiagen.com, retrived 1/2014

Materials:

- 2 x Uvettes
- 10 µl Buffer EB (or whatever solution your sample is in)
- 500 µL sterile water

Protocol:

1. Blank the spectrophotometer with a 1:10 dilution of Buffer EB in water in an Uvette (unless already done by an instructor).

2. Directly in the Uvette, combine 10 µL of your plasmid sample with 90 µL of water (this is a 1:10 dilution).

3. Place the Uvette in the spec and scan the sample from 200-400 nm and print the resulting graph. Record the absorbencies at 230, 260, and 280 nm.

4. If you get a negative absorbance, talk to the instructor about what next steps you should take.

5. Calculate the concentration, yield, and purity (with respect to protein) of your DNA.

-- Notes --

Restriction Enzyme Digests

Introduction

Restriction enzymes or restriction endonucleases are proteins found in bacteria and archaea that cut foreign DNA at very specific sequences that are called recognition sequences. In this way, a cell can use a restriction enzyme as a defense against an invading virus. Restriction enzymes in bacteria are similar to innate immunity in humans. The enzymes are names for the species they were isolated from. For example, SwaI was purified from *Staphylococcus warneri*. The first restriction enzyme was discovered in the 1960s; since then thousands have been characterized in detail and approximately 300 are commercially available for use in research. These enzymes are powerful and vital tools for DNA modification in laboratories and molecular cloning.

Frequently, the recognition sequence of an enzyme is palindromic. The recognition sequences of BclI and SwaI are listed below. Note that the palindromic nature of the sequence only becomes apparent when you consider the complimentary strand:

BclI	SwaI
5'…T^GATCA…3'	5'…ATTT^AAAT…3'
3'…ACTAG^T…5'	3'…TAAA^TTTA…5'
5'…T GATCA…3'	5'…ATTT AAAT…3'
3'…ACTAG T…5'	3'…TAAA TTTA…5'

The carrot in each sequence indicates where the enzyme will cut, and the sequence below shows what the cut DNA would looks like. The type of cut resulting from BclI is called a "sticky end" because the 3' overhang regions are complimentary and can re-anneal and self-repair under the proper conditions. SwaI results in a "blunt end" because there is no overhang. Blunt ends are less likely to self-repair.

You may be asking yourself "If restriction enzymes cut DNA, how does a cell protect itself from having its own genome cleaved?" The answer is rather interesting. Bacteria use methylases to add a methyl group to nucleotides in their own genome. Methylated nucleotides are not recognized by restriction enzymes and thus prevent cutting. Not all restriction enzymes are sensitive to methylated bases, but some are. If this is the case, care needs to be taken to ensure that the recognition site for such an enzyme is demethylated when working with an enzyme experimentally.

Uses in Research

Restriction enzymes are used in molecular biology to cut DNA molecules in a precise and defined manner for the purposes of gene cloning and gene analysis. For example, human insulin can be produced in mass quantities because a plasmid was cut open with restriction enzymes and the insulin gene was inserted (in other words, the insulin gene was cloned into the plasmid). This plasmid can then be put back in bacteria (a process called transformation), which will now synthesize the insulin protein.

Analyzing the number and size of DNA fragments is extremely useful DNA analysis. If the sequence of your gene of interest or plasmid is known, restriction fragments can be predicted, and the resulting fragments can be analyzed to verify the identity of the cloned DNA. When the sequence is unknown, restriction digestion allows an initial characterization of the cloned DNA fragment and sub-cloning of the DNA for further analysis.

Using Enzymes in the Lab

Restriction enzymes need several things to function. All restriction enzymes (and many enzymes that interact with DNA) require the divalent magnesium cation (Mg^{2+}) in order to function. The Mg^{2+} sits in the active site of the enzyme to facilitate interaction between the enzyme and the DNA. If the Mg^{2+} is removed or unable to interact with the enzyme, the DNA will not be cut. Individual restriction enzymes have different requirements to function optimally. A buffer specific for each enzyme is used to adjust the pH and ionic strength to achieve maximum enzyme activity. Commercially available restriction enzymes are usually supplied with the necessary reaction buffer. In addition, different restriction enzymes have different optimal digest temperatures, meaning that they function optimally at specific temperatures. Many enzymes have an optimal temperature of 37°C, but some, like BsrI have an optimal temperature of 65°C. In addition, like many other types of enzymes, restriction enzymes are often sensitive to heat. They must remain on ice when in use and care should be taken to prevent denaturation.

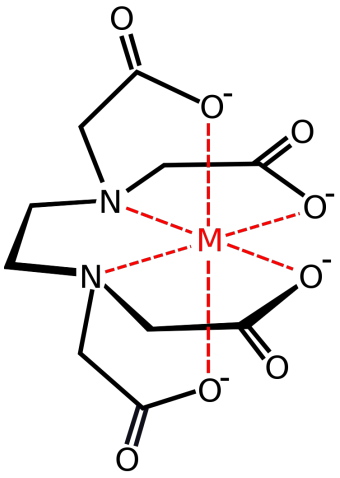

Figure 1 | EDTA chelates divalent cations. EDTA molecules work similarly to an arcade claw machine and "grab" Mg^{2+} ions in solution. The red dashed lines show the electrostatic interactions between nitrogen/oxygen atoms and the Mg^{2+}.
https://en.wikipedia.org/wiki/Ethylenediaminetetraacetic_acid#/media/File:Metal-EDTA.svg

Preventing Enzyme Activity

Most commonly used restriction enzymes are inhibited at cold temperatures. Keeping the enzymes cold when not actively in use prolongs their shelf life and prevents any unwanted activity. Because of this, enzymes are stored at -20°C and always kept on ice until ready to incubate with DNA. Restriction enzyme activity can also be prevented or stopped by adding ethylenediaminetetraacetic acid (EDTA). EDTA is a chelating molecule that sequesters a single Mg^{2+} cation in a solution (Figure 1). When the cation is bound by the EDTA, it cannot interact with a restriction enzyme, and the enzyme is therefore unable to cut DNA. EDTA chelates Mg^{2+} in a 1:1 molar ratio. Often, DNA is stored in a solution of buffer and EDTA to prevent its degradation.

Materials:

- Restriction enzyme(s)
- 1 mL ddH_2O
- 10X Restriction Enzyme Buffer (may need more than one depending on the digest)
- 10X DNA Loading Dye (Tris, EDTA, Bromophenol Blue, Glycerol)
- Heat block
- Ice

Protocol:

1. In a sterile microfuge tube, add the following components on ice (1 tube per reaction):

2 µL 10X Enzyme Buffer

1 µL Restriction Enzyme

200 ng Plasmid DNA

Q.S. 20 µL in sterile H_2O

Q.S. is an abbreviation for *Quantum Satis*, a Latin phrase meaning "just far enough". It is frequently used in scientific protocols to indicate the what the volume should be brought up to. Here, it means that the final volume of the reaction should be 20 µL, so after you add the buffer, enzyme, and DNA, you bring the total volume up to 20 µL using sterile H_2O.

Keep your reaction on ice until you are ready to incubate at the appropriate temperature

2. Spin the tube in a centrifuge for 5 seconds to collect the droplets at the bottom of the tube.

3. Mix the reaction thoroughly (if your enzyme can't reach your DNA, the enzyme can't cut it!). Flick the bottom of the tube with your finger, then spin down the droplets for 5 seconds to collect them at the bottom of the tube. Repeat this process two more times (for a total of 3 mixes).

4. Place your tubes in a heat block set to the appropriate temperature and incubate for 60 min.

This is a good time to set up an agarose gel if you need to

5. Stop the reaction using a method conducive with your next experiment with the digested DNA. If you are going to run an agarose gel, add 2 µL of the 10X DNA Loading Dye to the contents of each tube. Mix thoroughly as in step 3. Keep digests on ice until you are ready to load the agarose gel.

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Agarose Gel Electrophoresis

Introduction

Agarose gel electrophoresis is a technique used to analyze the size of the DNA fragments that are produced from restriction enzyme digests or polymerase chain reactions (PCR). Agarose (a carbohydrate isolated from seaweed) melts at 100°C and solidifies at 42°C. We can melt the powdered agarose at the higher temperature in water and buffer, pour it into a form, and allow it to solidify by simply letting it cool below 42°C. The gel is formed and run as a horizontal slab since it does not have enough structural strength to be used vertically.

The phosphate backbone of DNA gives it a uniform negative charge. Because of this, DNA will be attracted to a positive charge. If DNA is placed in an agarose gel with a negative electrode placed at the top and a positive electrode placed at the bottom, the DNA will migrate through the pores of the gel towards the positive electrode.

The concentration of agarose in the gel determines the size of the pores, which in turn dictates the size of the DNA fragments that can be resolved. We typically make 0.8 – 1.2% (w/v) gels. This means that 0.8 – 1.2% of the volume of the gel is composed of an equivalent weight of agarose. For example, if you were to pour a 1% 100 mL gel, 1% of 100 is 1 mL. Since 1 mL of water weighs 1 g, we can exchange that much water for agarose in our gel. Thus, 1 g of agarose dissolved in 100 mL of buffer gives us a 1% gel. Standard agarose gels separate DNA fragments from 0.5 to 25 kilobasepairs (kbp) in length. Varying the concentration of agarose changes the pore size and the range of resolution separating the various sizes of DNA fragments. The higher the percentage of agarose, the smaller the pore size that is produced. A 1% agarose gel will separate DNA fragments from 0.5 to 10 kb long. To determine the sizes of the DNA fragments, you will run a DNA ladder on the gel. This is a solution of known sizes of DNA. By comparing the distance traveled of the fragments in the ladder with those in your sample, you can determine the size of your fragments. Smaller fragments are able to travel faster through the gel than larger fragments and will appear at the bottom of the gel compared to the larger fragments.

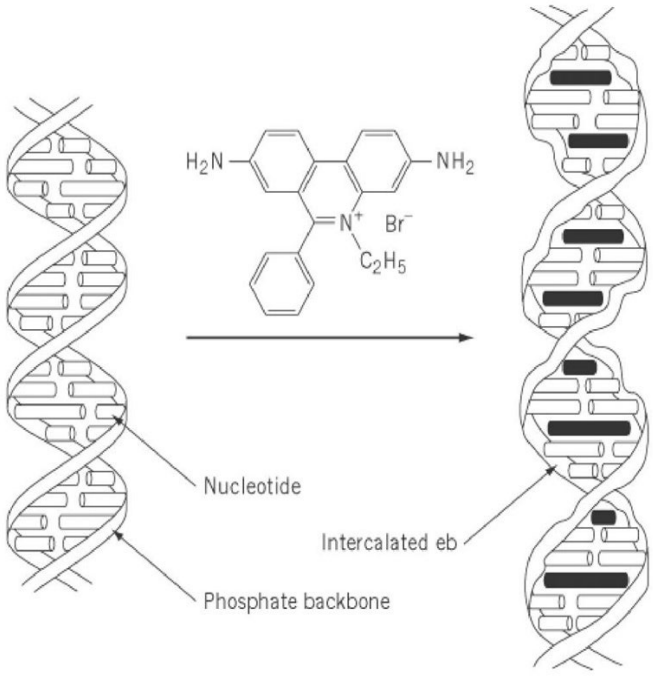


Figure 1 | Ethidium bromide is an intercalating dye. The 3 fused rings of EtBr share electrons across their double bonds. This gives the molecule a flat shape and allows it to slip between the base pairs of DNA. http://what-when-how.com/molecular-biology/ethidium-bromide-molecular-biology/

Before the gel material solidifies we must add ethidium bromide (EtBr) and a sample comb. The comb forms wells in the gel in which you can load your samples. EtBr (Figure 1) has a very high affinity for nucleic acids, and because of its flat shape, it readily slides in between the stacked base pairs. Since it is a fluorescent molecule, we can visualize nucleic acids to which it has bound. However, because of its interaction with DNA and RNA, EtBr is a powerful mutagen and therefore a carcinogen. Although the concentration that we use EtBr in the gel is very dilute, you must

wear goggles, a lab coat, and gloves while handling liquid agarose with EtBr. When finished with a gel it must be double bagged and disposed of in the chemical waste bin.

Using Gels for Analysis

In addition to analyzing the fragment size produced by the restriction enzyme digestion, gel electrophoresis will allow us to perform other quality checks. Recall that all nucleic acids, including RNA and genomic DNA, absorb at 260 nm. If RNA is present, it will appear as a large, intense band at low molecular weight. If genomic DNA is present, it will appear as a smear throughout the lane. We can also assess the integrity of the plasmid DNA. Nicked dsDNA will relax and consequently travel differently through the gel than pristine plasmid DNA.

The following steps describe how to pour and run 1% midi gel, consult the instructor for appropriate volumes if you will use a mini gel instead.

Materials:

- 1 g agarose
- 90 mL ddH_2O
- 10X TBE Buffer (0.89M Tris Base, 0.89M Boric Acid, 0.02M EDTA, pH 8.0)
- 1Kb DNA ladder/Mass Marker
- 10X DNA dye
- 0.5 mg/ml Ethidium Bromide (EtBr)
- Gel apparatus

Protocol:

1. At your bench top, assemble the casting tray and place it on a blue absorbent pad. Gels must be poured on an absorbent pad. Be sure you have a sample comb ready by the tray.

2. Using the electronic balance, weigh out 1 g of agarose and then place it in a 250 mL Erlenmeyer flask. At your bench, add 90 mL of ddH_2O to the agarose and microwave the flask until the sugar has completely melted.
3. Using hot-hands, remove the flask from the microwave and place in a 1L tripour beaker as secondary containment. Bring the flask back to your bench and add 10 ml of warm 10X TBE to the flask and gently swirl to mix.
4. With your flask in secondary containment and while wearing appropriate PPE, walk your flask to the instructor or TA so they can add EtBr. Return to your bench and swirl the flask gently to mix.

5. Carefully pour the agarose into the casting tray. Pop any bubbles that form with a sterile pipet tip. Insert the sample comb. The gel will take 20-30 min to solidify (it will become turbid).

6. While the gel is solidifying, prepare your samples to run on the gel.

a. Determine the appropriate volume of your sample to load on the gel (ex. 3-5 µL of uncut plasmid, the entirety of an enzyme digest, 5-10 µL of PCR product) and transfer to a fresh microfuge tube.

b. Add a volume of 10X DNA dye (glycerol, bromophenol blue, xylene cyanol FF, EDTA) to each tube such that the final concentration of dye is 1X (ex. 1 µL of 10X dye to 10 µL of sample)

c. Flick and spin three times to mix.

7. Once the gel is solid, place the casting tray in the electrophoresis apparatus and add 1X TBE buffer to the apparatus until the entire surface of the gel is covered with buffer. Carefully remove the comb.

8. Load your samples in an order that makes logical sense for your experiment.

a. It is common to load, from left to right, ladder → control → experimental samples.

b. If you plan on loading a mass marker, it is common to load one amount to the left of your samples and the other amount to the right of your samples.

9. Place the lid on your gel apparatus and plug the leads into the power source. Set the gel to run at an appropriate voltage for the gel. Consult your instructor for appropriate voltages.

-- Notes --

Polymerase Chain Reactions (PCR)

Introduction

PCR is short for polymerase chain reaction and was developed by Kary Mullis at Cetus Corp. in 1983. He received a Nobel Prize for this revolutionary work in 1993. PCR provides a way to amplify a segment DNA *in vitro* (a cell free system) and is essentially copying and amplifying a short piece of DNA from a small amount of template using DNA polymerase enzyme in a test tube. In fact, as little as a single DNA molecule can be enough to allow for the production of thousands to millions of copies of a DNA fragment. It is a simple and elegant technique that has revolutionized biological research. It can be used to test for the presence or absence of a DNA sequence or amplify a sequence for further testing. Applications of PCR include diagnostic testing, paternity testing, forensics, pathogen identification, and various other investigations in which larger amounts of DNA must be copied from small sized samples.

Components of a PCR Reaction

Template DNA is the DNA that will be copied and amplified during PCR. Template DNA can be double or single stranded; it can be a fragment, a plasmid, or genomic DNA. While this technique is sensitive enough to create a product from just a single copy of template, hundreds of copies are usually used in a given reaction. Using too much template can decrease the efficiency of your PCR reactions by increasing the probability that it re-anneals before primers can bind. Therefore, care and consideration should be given to the amount of DNA used as template.

Deoxynucleotide Trisphosphates or dNTPs are the building blocks of DNA and include adenine (A), thymine (T), guanine (G), and cytosine (C). A and T are complimentary bases, as are G and C. This means that they will anneal to each other across two strands of DNA. dNTPs are synthesized into a strand of DNA by DNA polymerase.

Taq DNA Polymerase The polymerase first used in PCR was isolated from the bacteria *Thermos aquaticus* and is often simply called Taq Polymerase. *T. aquaticus* is an extremophile that prefers to live at temperatures of 100°C. Its proteins are therefore very heat stable and will function optimally at near boiling. This characteristic will be quite valuable during the PCR cycling (described below).

Buffer *In vitro* replication requires an environment similar to that of a cell. Any commercially produced DNA polymerase comes with an appropriate buffer that contains salts, is at an optimal pH, and has a divalent cation as a cofactor. This means that the cation is required for proper function of the enzyme. Often, the divalent cation, Mg^{2+}, is the cofactor for polymerase. The function of Mg^{2+} with polymerase is very similar to its function with restriction enzymes.

Primers are short (15-30 nt) strands of DNA complimentary to the outer regions of the sequence you are trying to amplify. They are necessary to initiate DNA synthesis at the location of interest. In Figure 1a the region of interest to be amplified is highlighted in gray. In panel b, you can see where the purple forward primer (shown at the left) and the blue reverse primer (shown at the right) anneal to their complementary sequences on the bottom and top strands, respectively. DNA synthesis then occurs in the 5' → 3' direction (synthesized DNA in bold). For more information on how to design PCR primers, see Appendix D.

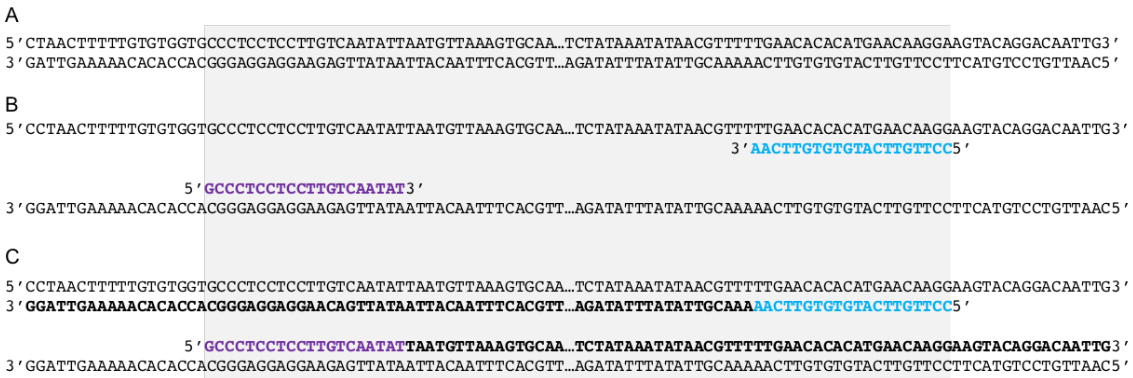


Figure 1 | Diagram of primer annealing. The grey box outlines the region to be amplified **(A)** Template dsDNA with the region to be amplified in italics. **(B)** The dsDNA is denatured and the forward (purple) and reverse (blue) primers anneal to their complementary regions. **(C)** DNA polymerase synthesizes a strand of DNA complementary to the template beginning at the primed regions (written in bold).

Reaction Cycles

The first step in DNA replication (Figure 2) is to generate ssDNA template from the original dsDNA sample. In cells this process is accomplished through the complex interactions of many different enzymes. In PCR we simply raise the temperature of the reaction mixture and denature the dsDNA. The initial denaturation is 5-10 min in length to ensure that all the template DNA has been completely melted into ssDNA. The next denaturation is the start of the repeated cycles and does not need to be as long (typically 30-60 s). Then, the temperature is lowered to 50-60°C for 30-60s in order to allow the primers to anneal to their complementary sequence on the template. The temperature is raised to 72°C to allow the DNA polymerase to bind the DNA and synthesize the new complementary sequence (extension). The latter 3 steps are repeated 25-30 times for an exponential increase in the desired product. Figure 3 illustrates this exponential synthesis and the resulting product size.

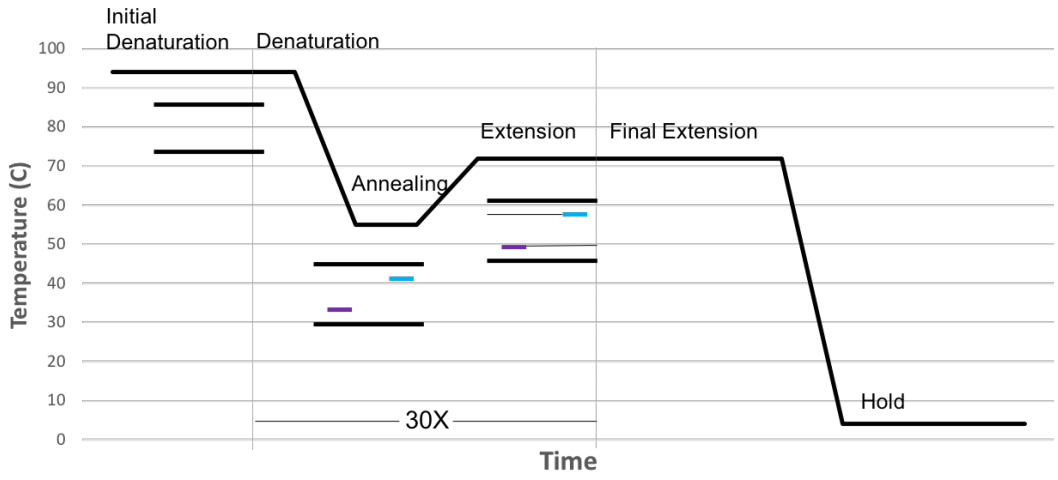


Figure 2 | Stages of a PCR Cycle. Note in the above diagram the initial denaturation and final extension steps. These are not repeated during the reaction. Only the middle steps, underscored by the “30X” are repeated during PCR. Also to note, the final step is a “hold” at 4°C. This means that the temperature will drop to 4°C and stay there indefinitely.

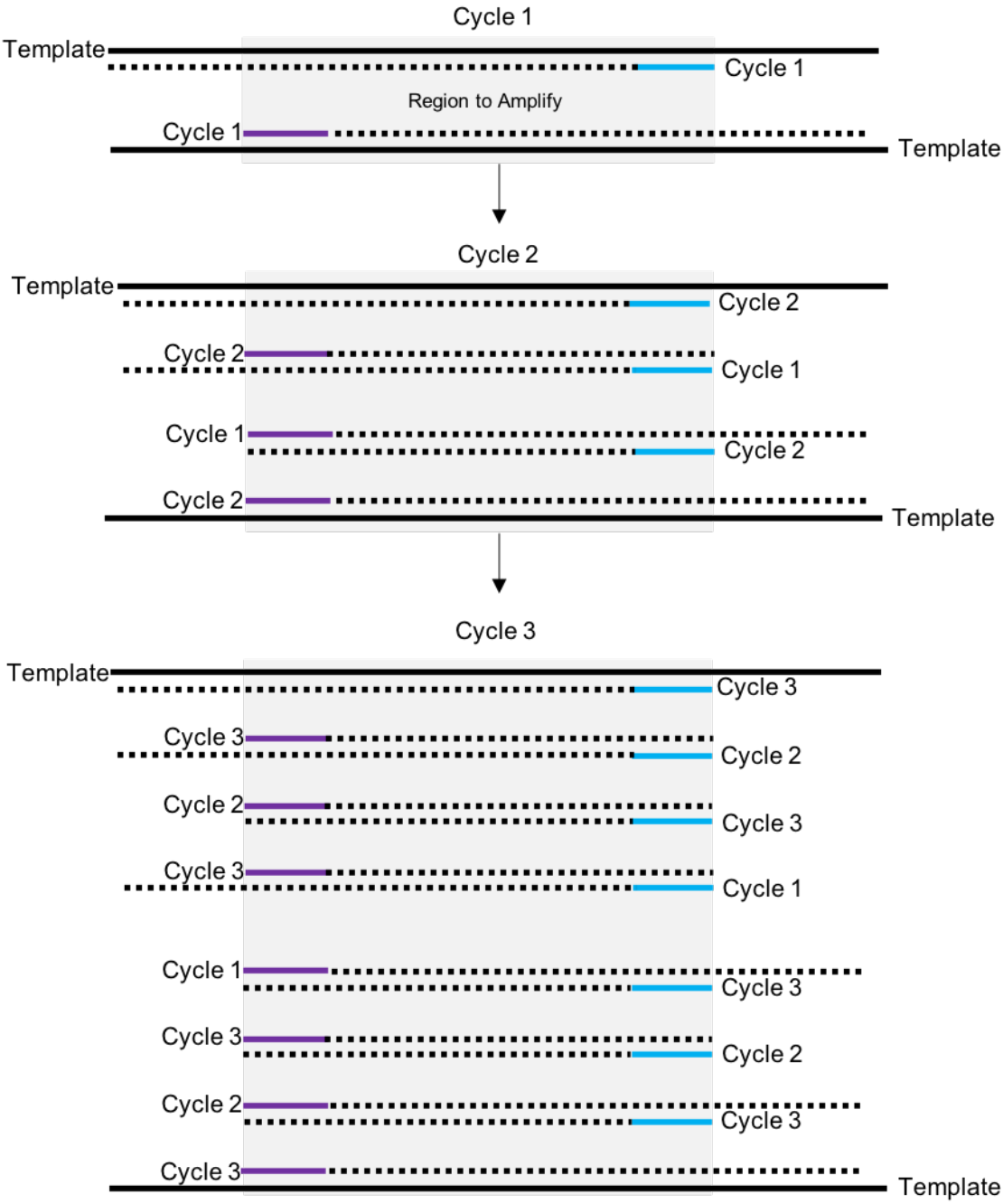


Figure 3 | Exponential amplification during PCR. The gray box highlights the region of DNA to be amplified by the PCR reaction. The bold black lines represent the original template DNA. The purple and blue lines indicate the forward and reverse primers. The dotted lines indicate the DNA synthesized during each cycle. The cycle during which each strand of DNA was synthesized is labeled. Notice how after just 2 cycles, the products are the size of the region to amplify.

Materials:

- 2X Master Mix (with 0.4 mM dNTPs)
- 1 mL ddH_2O
- Template DNA (10 ng)
- Taq Polymerase (2.5 U/µL)
- Forward Primer (10 µM)
- Reverse Primer (10 µM)
- Sterile 0.2 mL PCR tubes

Protocol:

1. Before you begin any PCR experiment, you will need to fully plan each reaction. The tables and charts below are designed to help you plan your experiment and should be recreated in your lab notebook each time you do PCR.

2. Primer and PCR Product Information

To determine the cycler conditions and know what you are looking for after the PCR reaction, you will need to calculate the melting temperature, annealing temperature, and predicted product size of your reaction. The primers to create the disruption-repair cassette are given in the Table 1. The portion in upper case is homologous to regions of the ribosome assembly factor (RAF) gene of interest in *S. cerevisiae.* The portion in upper case is therefore also complementary to regions of the RAF gene of interest within the tiling library plasmid template. The portion in lower case bold are either stop codons that will terminate the amplification of the RAF gene or start codons that will initiate amplification of the RAF gene.

a. To calculate melting temperature of the plasmids, you should only consider the regions that will anneal to the template (not lowercase bold start and stop codons). Use the equations listed below Table 1 to calculate the melting temperatures of each of your primers. Annealing temperatures are usually 2-5°C lower than the melting temperature of the primers. Once you calculate the melting temperature, calculate the annealing temperature. Use the 2-5°C range to find a single annealing temperature that will work for both your forward and reverse primers (since they will be used in the same reaction).

b. Finally, look to the sequence of your template to calculate the predicted product size. Remember, the polymerase will synthesize new sequence between the primers, but the final product will include the entirety of the primers used.

Table 1 | Primer Information *Note: the portion of the primer that is lowercase bold font are inserted stop codons (tta) or start codons (atg) to alter where translation begins and ends, uppercase underlined are complementary to untranslated regions to generate the deletions, and up percase are complementary to the RAF gene of interest.*

NOP2ΔNTD Forward	AT TCC CCT TGT GTT GGC TAA TAT TAG AAT TAC ATA TAC ATA TAA TAG GAA **atg** TCA AAA GCC AGG AAA TCT TTA TT			
NOP2ΔNTD Reverse	TTA TTT TTC GTT AGA AGG TCT TTT AGC			1667
HAS1ΔNTD Forward	AC TGT ATA CTA TAA TAA TTA GAT AAG CTG AGC AAT ATT AAC AGG AGA AGT **atg** TTC GAA GAA CTA AAG TTA TCA CAG C			
HAS1ΔNTD Reverse	TTA CTT ATG AGT TTT ACG TCT TTT GG			1442

If the primer length is ≤18nt: Tm = 2˚C(As+Ts) + 4˚C(Gs+Cs),

If the primer length is >18nt: Tm = 69.3˚C + 0.41(%GC) – (650.0/Length)

3. Reaction Composition

Before you begin pipetting anything, you should make sure that you have all necessary reagents at the appropriate concentrations. Table 2 is designed to help you keep track of the various components in the PCR reaction. Note that "2X" means that the solution is twice as concentrated as it should be for the final reaction. "1X" means that the solution is at working concentration. The 2X Master Mix provided already contains dNTPs (0.8 mM) and Mg^{2+} in addition to the appropriate pH and salt concentration for the Taq you will be using. You will need to calculate the volume of template DNA to add to the reaction for a total of 10 ng based on the concentration of your isolated template plasmid sample. Remember that "QS" is an abbreviation for a Latin term meaning "to bring to volume". The final volume of your PCR reaction should be 25 µL. You should use special, thin-walled 0.2 mL sterile PCR tubes to make up your reaction.

Reagent	Starting Concentration	Final Concentration	Volume to Add to Reaction
Master Mix (w/ dNTPs)	2X	1X	
Taq Polymerase	2.5 U/µl	2.5 U	
Template (10 ng)	_____ ng/µl	10 ng	
Forward Primer	10 µM	0.5 µM	
Reverse Primer	10 µM	0.5 µM	
ddH_2O	n/a	QS 25 µl	

Table 2 | Reagents needed for one 25 µL PCR reaction

Note: everything should be kept on ice/cold until ready to go on the thermocycler

4. Cycler Conditions

The exact thermocycler conditions can vary based on the size of your template, size of desired product, and properties of the primers. The times and temperatures for the initial denaturation, denaturation, and extension are provided. While these temperatures and times are good general guidelines, consideration should be given to the time of the initial denaturation depending on the type of template being used (DNA fragment, plasmid, genomic DNA, etc). Also, it is important to keep in mind that a Unit of polymerase is defined as the amount of enzyme needed to synthesize 1 kb of DNA per minute. The extension time should reflect the size of your desired product. Based on your calculations, fill in the appropriate annealing temperature and extension time.

Initial Denaturation:	94°C	5 min	
Denaturation:	94°C	30s	
Annealing:	___°C	30s	Repeat for 30 cycles
Extension:	72°C	___ s	
Final Extension:	72°C	10 min	
	4°C	Hold	

5. Setting up the Reaction

Now that you have determined the appropriate reaction composition, PCR cycler conditions, and know what your target PCR product size is, you may now begin setting up your reaction by pipetting each component into a thin-walled 0.2 mL sterile PCR tubes. Keep all reagents on ice. It may be helpful to check off each reagent as you add it to help keep track and prevent leaving out a critical component.

a. Add the appropriate reagent to the PCR tube in the following order: water, master mix, template, forward primer, reverse primer, polymerase.

b. Create a negative control reaction as well by leaving out a critical component such as template DNA or Taq.

c. Mix the reaction by flicking and spinning 3x to ensure homogeneity.

d. Place in the thermocycler with the appropriate conditions.

Questions to consider:

1. How would using 100 ng of template DNA instead of 10 ng affect you PCR reactions and product?

2. How might increasing the extension time to 10 minutes each cycle affect the PCR product?

3. What might happen if you skipped the initial denaturation step?

4. Would you have any product if your annealing temperature was the same as the melting temperature of the primers?

5. How could increasing the amount of Mg^{2+} in the reaction affect the product?

-- Notes --

Polymerase Chain Reaction Purification

Introduction

Following the amplification of product in a Polymerase Chain Reaction (PCR), there are multiple ways that the PCR product can be utilized. Usually, the first step is to conduct an agarose gel electrophoresis in order to verify the presence of PCR product. Following confirmation of the intended size of PCR product, the next step must be chosen carefully to complete the desired outcome. One possibility is to send the product for sequencing, to ensure that no mutations were incorporated during amplification. Another option is for cloning purposes. Perhaps the PCR product needs to be digested by restriction enzymes in order for it to be ligated (incorporated) properly into a vector to make a complete plasmid. A third option is to use the PCR product as a template for another round of PCR. Perhaps you did not get enough product in the first round of amplification. You could use the product as a template, since it is the intended product and will be recognized by your primers, to greatly increase the amount of PCR product generated.

Purifying PCR Product for Experiments

Whatever the purpose may be for your PCR product, it is likely to require that you purify the generated PCR product. While the PCR product may not be contaminated with extraneous proteins or cellular components, there is still a pool of reagents present from the PCR reaction. Excess polymerase, dNTPs, divalent cations, and even salts are often required to be separated from the PCR product before future experimentation, often in order to increase efficiency of the following experiments.

PCR purification can be conducted through various methods, depending on how the product is intended to be used. One of the most common methods, and the one we shall utilize, is the use of a PCR purification kit. The kit purifies PCR product in a very similar manner to a plasmid isolation kit, a method you have previous experience with. The PCR purification kit isolates DNA through the use of a column that selectively binds to DNA through its negatively charged backbone and washing away of unwanted components. One of the main differences, however, is that the PCR product is not contained inside a cell, such as *E. coli*. Therefore, we do not have to separate the DNA from its cellular host. We simply need to put it on a column, wash away unwanted contaminants, and elute it from the column. As with any DNA preparation, once the PCR product is isolated it should be kept on ice and stored at -20°C.

The manufacturer instructions for the GeneJET Purification kit are provided below (pg 2-3).

PURIFICATION PROTOCOLS

Note

- Read IMPORTANT NOTES on p. 3 before starting.
- All purification steps should be carried out at **room temperature**.
- All centrifugations should be carried out in a table-top microcentrifuge at **>12000 × g** (10 000-14 000 rpm, depending on the rotor type).

Protocol A. DNA purification using centrifuge

Step	Procedure
1	Add a **1:1 volume** of **Binding Buffer** to completed PCR mixture (e.g. for every 100 µL of reaction mixture, add 100 µL of Binding Buffer). Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
2 for DNA ≤500 bp	*Optional:* if the DNA fragment is ≤500 bp, add a 1:2 volume of 100% isopropanol (e.g., 100 µL of isopropanol should be added to 100 µL of PCR mixture combined with 100 µL of Binding Buffer). Mix thoroughly. **Note.** If PCR mixture contains primer-dimers, purification without isopropanol is recommended. However, the yield of the target DNA fragment will be lower.
3	Transfer up to 800 µL of the solution from step 1 (or optional step 2) to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through. **Notes.** If the total volume exceeds 800 µL, the solution can be added to the column in stages. After the addition of 800 µL of solution, centrifuge the column for 30-60 s and discard flow-through. Repeat until the entire solution has been added to the column membrane. **Close the bag with GeneJET Purification Columns tightly after each use!**
4	Add **700 µL** of **Wash Buffer** (diluted with the ethanol as described on p. 3) to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through and place the purification column back into the collection tube.
5	Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove any residual wash buffer. **Note.** This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.
6	Transfer the GeneJET purification column to a clean 1.5 mL microcentrifuge tube (not included). Add **50 µL** of **Elution Buffer** to the center of the GeneJET purification column membrane and centrifuge for 1 min. **Note** • For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended. • If DNA fragment is >10 kb, prewarm Elution Buffer to 65 °C before applying to column. • If the elution volume is 10 µL and DNA amount is ≥5 µg, incubate column for 1 min at room temperature before centrifugation.
7	Discard the GeneJET purification column and store the purified DNA at -20 °C.

Protocol B. DNA purification using vacuum manifolds

Step	Procedure
1	Perform DNA binding stage according to steps 1 - 2 in Protocol A on page 4.
2	Prepare the vacuum manifold according to the supplier's instructions. Place the GeneJET purification column(s) onto the manifold. **Close the bag with GeneJET Purification Columns tightly after each use!**
3	Transfer up to 800 µL of the solution (from step 1 or 2 as in protocol A) to the GeneJET purification column. **Note.** If the total volume exceeds 800 µL, the solution can be added to the column in stages. After each application, apply the vacuum and discard the flow-through. Repeat until the entire volume has been applied to the column membrane.
4	Apply the vacuum to draw the sample through the column. Switch off the vacuum after the entire sample has passed through the column.
5	Add **700 µL** of **Wash Buffer** (diluted with the ethanol as described on p. 3) to the GeneJET purification column.
6	Apply the vacuum to draw the solution through the column. Switch off the vacuum after the solution has passed through the column. Place the purification column back into the collection tube.
7	Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove any residual wash buffer. **Note.** This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.
8	Transfer the GeneJET purification column to a clean 1.5 mL microcentrifuge tube (not included). Add **50 µL** of **Elution Buffer** to the center of the GeneJET purification column membrane and centrifuge for 1 min. **Note.** • For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended. • If DNA fragment is >10 kb, prewarm Elution Buffer to 65 °C before applying to column. • If the elution volume is 10 µL and DNA amount is ≥5 µg, incubate column for 1 min at room temperature before centrifugation.
9	Discard the GeneJET purification column and store the purified DNA at -20 °C.

-- Notes --

Lithium Acetate Yeast Transformation

Adapted from O'Donnell Lab @ UPitt, Current Protocols in Molecular Biology, and Gietz & Schiestl 2007

Yeast cells, like many other eukaryotic and prokaryotic cells, can be persuaded to take up naked DNA from the surrounding medium. A transformation is the act of forcing a cell (of any type) to take up DNA or RNA. Our ability to introduce DNA directly into the cells offers experimenters an extra measure of control over the experimental organism. Since you do not need to "cross" cells to bring new genes in, you do not have to deal with unwanted new combinations of genes that would naturally result from normal genetic crosses.

The transformation process with yeast can be summarized in the following 4 steps:

1. Make the cells competent
2. DNA Uptake
3. Recovery
4. Plating on selection media

Make the cells competent

Yeast cells do not have a natural mechanism for taking up DNA. In fact, most species have evolved several mechanisms to prevent taking in foreign DNA as a means of combating infection. Treatments that make cells competent, or able to accept DNA, involve some biochemical shock to the cells. The method described here uses lithium acetate (LiAc) and polyethylene glycol (PEG) to make the yeast cells competent. The Li^+ cations from the LiAc help to neutralize the negative charges of the DNA and yeast cell wall and membrane so they do not repel each other. PEG works in a similar manner and in addition is thought to destabilize the yeast membrane due to its amphiphilic nature.

DNA Uptake

Once the cells are competent, they are ready to take up DNA. DNA can be forced into the cells through several different methods, but a commonly used technique is heat shock. This is exactly as it sounds, where the cells are briefly heated during which time their membranes become slightly more soluble and DNA is able to pass through. The heat shock also creates a temperature gradient from the inside to the outside of the cell, encouraging DNA to enter the cell. It is critical that the timing of the heat shock be tightly controlled to prevent damaging the yeast cells.

In addition to the DNA you are trying to transform, it is very common to include single-stranded (ss) carrier DNA. The ssDNA strands shield the DNA of interest while it enters the cell. Some amount of DNA will bind to the yeast cell wall and some amount of DNA will be digested by nucleases as it makes its way to the nucleus. ssDNA increases the odds that your DNA makes it into the cell and to the nucleus.

Recovery

After the heat shock, cells need a chance to recover under optimal growth conditions. The cell wall and membrane of the yeast cells have just been highly manipulated and are likely damaged. The cells' first priority will be to repair this damage. It is important to keep in mind that gene expression takes time. The cells not only need to express the genes for self-repair, but also express the genes that will allow them to grow on selection media. If you put the cells in a harsh environment immediately after heat shock, they will likely die.

Plating on selection media

Once the cells have fully recovered from the transformation process they can be plated on selection media. Often the selectable marker used for yeast transformations involves nutrient essential for growth. For example, *Saccharomyces cerevisiae* strain BY4741 cannot make uracil (genotype *ura*$^-$) and can only grow on media that contains uracil. If the plasmid that you transform into the cell contains a copy of the URA gene, then the cell will make uracil and will be able to survive on media that does not contain this nutrient (com-ura media). Selection medias allow you to grow only the cells that have taken up your DNA of interest.

Materials:

- YEPD Media
- Sterile ddH_2O
- 1X TE/LiAc buffer, pH 7.5 filter sterilized (0.1 M LiAc, 10 mM Tris, 1 mM EDTA)
- Carrier ssDNA (2 µg/µL)
- 50% (w/v) PEG 4000 or 3500, filter sterilized
- 100% ethanol
- Sterile 15 mL conical tubes
- Com-Ura plates

Protocol:

Grow and prepare yeast cells

1. Inoculate 10 mL YEPD media with a single yeast colony of the strain to be transformed. Grow overnight to saturation at 32°C.

2. The morning of the transformation dilute the culture to an OD_{600} of 0.25 in 10 mL. Incubate this in the air shaker at 200 rpm for 2-3 hours or until the OD_{600} reaches 0.5 to 0.6.

3. Harvest cells by centrifuging 3 min at 3000 rpm in a clinical centrifuge. Carefully remove the supernatant.

Wash cells with water

4. Wash the cells by resuspending in 10 mL of sterile ddH_2O.

5. Spin the cells as before and carefully remove the supernatant.

6. Resuspend the washed cells in 0.5 mL 1X TE/LiAc and transfer to a microfuge tube. Place on ice until ready to transform.

Transformation

7. Make up the transformation mixture in a microfuge tube by combining 100 µL of cells, 10 µL of ≤ 500 ng/µL plasmid DNA, 10 µL of carrier ssDNA, and 700 µL of 50% PEG solution.
    a. Note: these values are general guidelines for transformations. For your first transformation use the following: 100 µL of yeast cells, 3 µL of plasmid DNA, 12 µL of disruption repair cassette, 10 µL of carrier ssDNA, and 700 µL of 50% PEG solution.
    b. In future transformations, you will need to carefully consider the concentrations of plasmid and disruption repair cassette you have available and consult with your instructor.

8. Incubate the transformation for 30 min at 32°C with end-over-end rotation.

9. Heat shock the transformation at 42°C for 10 min and then quickly add 80 µL 100% ethanol. Continue to heat shock for an additional 5 min.

10. Pellet cells in centrifuge by spinning 5 min at 5000 rpm at room temperature and remove supernatant.

Recovery

11. Resuspend cells by adding 1 mL YEPD media directly into the microfuge tube.

12. Immediately transfer to a sterile tube containing 2 mL YEPD media.

13. Shake the culture tube in water bath for 1 hr to overnight at 32°C.

Plating

14. *OPTIONAL: Transfer cells to a 15 mL conical tube and pellet the cells by centrifuging 5 min at 5000 rpm at room temperature.*

15. *OPTIONAL: Remove supernatant and resuspend in 1 mL fresh YEPD by pipetting.*

16. Spread plate 20 µL and 200 µL onto the appropriate selection media.

17. Incubate plates at 32°C for 2-5 days.

Preparing carrier ssDNA

1. Dissolve 200 mg of salmon sperm DNA in 100 mL of sterile TE by stirring at 4°C for several hours
2. Dispense into 1 mL aliquots and store at -20°C.
3. For a transformation, thaw one aliquot and denature at 100°C for 5 min and immediately place in an ice/water slurry.
4. Denatured carrier DNA can be reheated at 100°C 3-4 times without significant loss of activity.

References

Gietz, R. D., & Schiestl, R. H. (2007). High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature protocols*, *2*(1), 31.

-- Notes --

Serial Dilutions and Spot Plating

Introduction

Simply generating mutants, bacterial or yeast colonies with possible genetic mutations, is not enough. Once the mutants are generated, they must be screened or selected for the mutation of interest. Screening allows all colonies to survive the mutagenesis process. One must then screen the surviving colonies to identify the ones with the desired phenotype. Selection is the process in which only the colonies with the desired genotype or phenotype survive the mutagenesis process. During mutagenesis, there is often a step that allows all survivors to grow whether they have the wanted phenotype or genotype or not. This is followed by a selective step that only allows the desired survivors to grow.

Growth Sensitivity

Characterization is the determination of how those mutations affect the organism of interest. There are many ways to characterize the mutations generated from a screen or selection. One way to characterize yeast mutations is to determine temperature sensitivity. Normally, yeast grows best between 30 - 32°C. However, yeast can also be incubated at other temperatures and will still grow; often growth is affected and not as fast as when it is grown at 32°C but the yeast are not dead. When yeast growth is affected by different temperatures, they are referred to as being temperature sensitive. Temperature sensitivity occurs when yeast are incubated at higher than normal temperatures and growth is significantly slowed or stopped; often this occurs at 37°C. Cold sensitivity occurs when yeast are grown at lower than normal temperatures and growth is significantly slowed or stopped; often this occurs at 16°C. Yeast can be grown at these various temperatures and monitored for growth; these sensitivities can indicate one characteristic of the mutation. However, not all mutations are temperature sensitive.

Microbial Growth Phases

In addition to growth on a solid agar media, bacteria and yeast can also be grown in liquid media that is agitated. In liquid media, bacteria and yeast will go through three growth phases: lag, log, and stationary. The lag phase (Figure 1) is the initial phase the cells will start in as soon as the culture is started. In lag phase, there is very little growth as the cells are adapting to their new environment and starting to produce proteins required for cell growth. The next phase is the log phase where the cells are actively growing and dividing. In log phase, the number of cells increases exponentially. Following log phase, the cells in the culture will enter the stationary phase where the amount of available nutrients and space no longer allow the total number of livings cells to increase. In stationary phase, cells are still growing and dividing, but the rate of growth and the rate of cell death is at an equilibrium. Bacterial and yeast cells can stay in stationary phase (if stored appropriately) for long periods of time.

A single colony of yeast or bacteria on an agar plate can have up to 10^8 cells. A liquid culture is measured

in numbers of colony forming units (CFU) per milliliter (mL) and can vary from only a few CFU/mL (lag phase) up to 10^{10} CFU/mL (late-log phase or stationary phase). There are several methods for assaying the density of bacterial liquid culture, including counting cells in a microscope using a hemocytometer and measuring optical density/turbidity in a Klett meter or spectrophotometer. One other commonly used technique for determining cell count is a viable count.

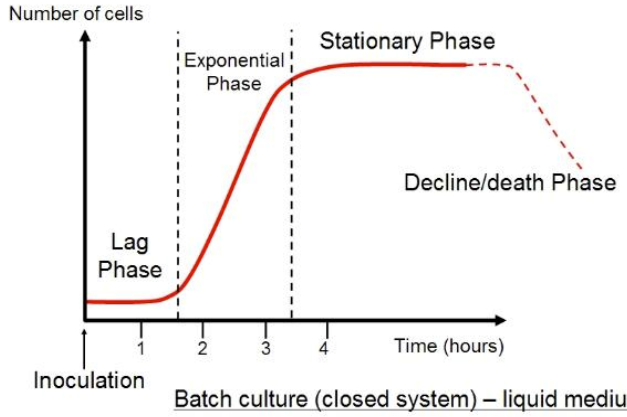


Figure 1 | Bacteria Growth Curve. Note the differences between lag, exponential (log), and stationary phase. cronodon.com/sitebuilder/images/Exponential_growth_curve-600x455.jpg

Serial Dilution

All of the methods of determining cell density require a countable number of colonies; often there are so many colonies present they cannot be differentiated from one another even by our equipment. Serial dilution is a technique to dilute cell density in order to get a countable number of colonies. Serial dilutions involve diluting cultures in succession until dilutions are reached which should produce a countable number of colonies. A countable number of colonies is 200 or less colonies. Serial dilutions generate several concentrations of cells/mL that will then be plated onto solid media. When a single cell is deposited onto the media, it will grow and divide to form a colony that can be detected by the naked eye. Each of the resulting colonies can be counted and then the concentration of the culture can be estimated by multiplying this number by the dilution factor. For example, plating 100 µL of cells from a culture at 10^8 cells/mL will result in 10^7 cells deposited and will produce a "lawn" of growth. However, diluting this culture to 10^3 cells/mL will allow 100 cells to be deposited onto a plate from a 100 µL aliquot and that will growth to produced 100, well defined colonies.

Materials:

- Sterile water
- Vortex
- Eppendorfs
- Yeast cultures
- Micropipettes

Protocol:

Growing cells to stationary phase

1. Inoculate 5 mL of appropriate media with a single yeast colony. Vortex to mix.

2. Let incubate overnight, shaking at 32°C.

Making Serial Dilutions

1. Obtain 5 Eppendorf tubes and label each with the appropriate strain name. Label the first as 10^{-1}; this will be your first dilution. Label the second tube as 10^{-2}; this will be your second dilution. Continue until you have reach 10^{-5}.
    a. Repeat this process as necessary until all samples that will be serially diluted and spot plated have been labeled.

2. Add 900 µL sterile water to each Eppendorf, sterilely.

3. Add 100 µL of appropriate yeast culture to the first tube; this is your 10^{-1} or 10-fold dilution.

4. Close the lid and vortex until well mixed.

5. Transfer 100 µL from the 10^{-1} Eppendorf to the 10^{-2} Eppendorf.

6. Close the lid and vortex until well mixed.

7. Repeat this process until the 10^{-5} dilution has been reach.

8. Repeat steps 3-7 for all yeast cultures.

Spot Plating

In order to characterize our yeast mutants, we will use a spot plating assay. Spot plating allows us to examine yeast growth at various temperatures for multiple colonies at the same time. Spot plating requires the use of serial dilutions; each serially diluted colony will be compared at the different temperatures to determine if there is a difference in growth. Mutants that are temperature or cold sensitive will show an obvious growth defect on the agar plates when compared to controls and other temperatures. Multiple dilutions will allow the identification of subtle differences, if any, when compared to controls and other temperatures.

The key to spot plating is the use of a plate grid. This grid allows the colonies to all be plated in the same way across each plate. Each column should have the same dilution of the various yeast while each row should contain the same colony with the serial dilutions. Simply place the agar plate, agar side down, on the template. You should be able to see the grid through the agar. You can then spot a small amount of serially diluted yeast onto the appropriate grid. Be sure to orient the plates the same for easy comparison; you may even want to indicate the top on each plate. Make copies as many of the plates as you have incubation temperatures; they will be incubated for the same amounts of time and monitored together over multiple days. This will allow for the identification of growth defects.

Materials:

- Serially diluted yeast cultures
- YEPD plates
- Spot plating grid
- Vortex
- Micropipettes

Protocol:

Spot Plating of Cells

1. Label a YEPD plate appropriately, including the temperature it will be incubated at.

2. Using the grid below, place the agar plate agar side down.
    a. Do not remove the lid until you are ready to spot yeast onto the plate. This will reduce the likelihood of contamination.

3. Vortex the 10^{-1} dilution of the yeast.

4. Beginning in the first row, micropipette 10 µL into the center of the first open grid square.
    a. Be careful not to spot too much yeast or allow the yeast to spill over into the next grid.
    b. Be sure to replace the lid after each spot to reduce contamination.

5. Vortex the 10^{-2} dilution of the same yeast.

6. Beginning in the first row, micropipette 10 µL into the second open grid.
    a. This should be in the same row as the first spot, directly next to it.

7. Repeat steps 5-6 for the remaining dilutions of the same yeast, vortexing well before spotting each dilution.

8. Repeat steps 3-6 in the second row for the next yeast strain.

9. Repeat step 8 for each yeast strain, until all dilutions are spotted on the same plate.
    a. Each row should contain the same yeast, with various dilutions.
    b. Each column should contain the same dilution from various yeast strains.

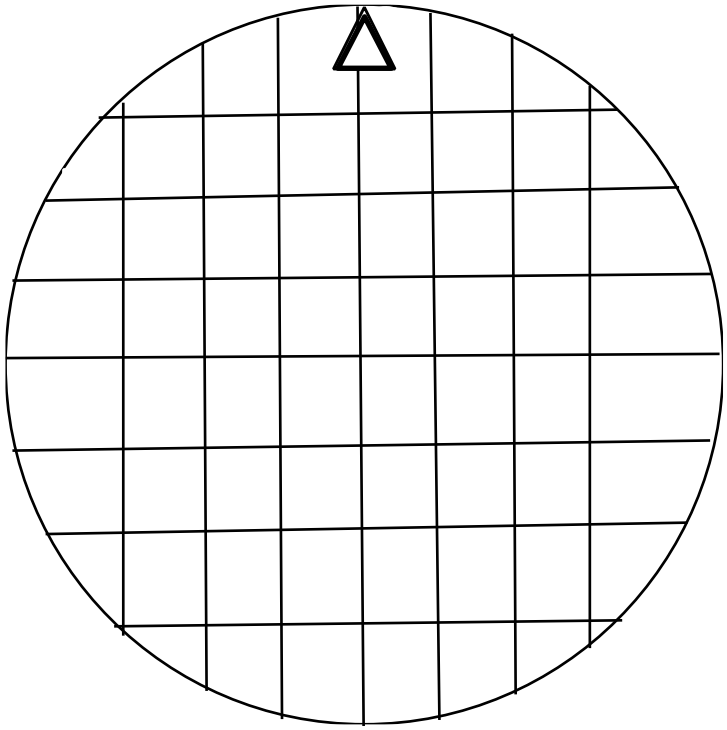
10. Repeat the entire process for each plate that will be incubated at each temperature.

11. Allow the plates to dry 5 minutes, agar side down with the lid on, to allow the yeast to adsorb to the surface of the plate.

12. Incubate each plate at the appropriate temperature.

13. Monitor the plates for signs of growth. Pictures may even help to identify growth differences.

Spot Plating Grid



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