Assignment Nine

Molecular Techniques: Nucleic Acid Preparation, Gel Electrophoresis, Enzymatic Reactions (PCR)

Functional Genomics Research Stream • Freshman Research Initiative • by Dr. Patrick J. Killion

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General Introduction

IMPORTANT NOTE: Background material and explanation is purposefully omitted from this assignment text. For more background on the subject matter covered in this assignment be sure to see the lecture notes and the online course notebook, each found on the course website (course materials):  http://fg.cns.utexas.edu/

Time Considerations

Please be aware of the time constraints of the various sections of this assignment and plan your attack accordingly.

Section A - Safety Check Point

Please note the following MSDS information for Phenol and Chloroform. This is a caustic chemical that will be used during the nucleic acid preparatory steps of this assignment. **Extreme Caution Is Required**

General Information

Skin Contact

Harmful if absorbed through the skin. Direct skin contact may result in white, wrinkled discoloration, followed by severe burns. Phenol solutions may be absorbed through the skin rapidly to cause systemic poisoning and possible death.

Skin Absorption Harmful if absorbed through the skin. Direct skin contact may result in white, wrinkled discoloration, followed by severe burns. Phenol solutions may be absorbed through the skin rapidly to cause systemic poisoning and possible death.

Eye Contact

Causes eye irritation and possible burns. May cause chemical conjunctivitis and corneal damage.

Inhalation

May be fatal if exposed to high concentrations. May cause severe respiratory tract irritation and possible burns. Aspiration may lead to pulmonary edema. May also cause pallor, loss of appetite, nausea, vomiting, diarrhea, weakness, darkened urine, headache, sweating, convulsions, cyanosis, unconsciousness, fatigue, pulmonary edema and coma. May cause systemic effects. Inhalation at high concentrations may cause CNS depression and asphyxiation. Acts as a relatively potent anesthetic. Irritates respiratory tract and causes central nervous system effects, including headache, drowsiness, and dizziness. Exposure to higher concentrations may result in unconsciousness and even death. May cause liver injury and blood disorders. Prolonged exposure may lead to death due to irregular heartbeat and kidney and liver disorders.

Ingestion

Harmful if swallowed. May cause central nervous system depression, characterized by excitement, followed by headache, dizziness, drowsiness, and nausea. Advanced stages may cause collapse, unconsciousness, coma and possible death due to respiratory failure. May cause systemic effects. Causes digestive tract irritation with possible burns. Causes severe burning in mouth and throat, pain in the chest and vomiting. Large quantities may cause symptoms similar to inhalation.

Chemical Specific Information

Phenol

Human poison by ingestion. An experimental poison by ingestion, subcutaneous, intravenous, parenteral, and intraperitoneal routes. Moderately toxic by skin contact. A severe eye and skin irritant. Questionable carcinogen with experimental carcinogenic and neoplastogenic data. Human mutation data reported. An experimental teratogen. Other experimental reproductive effects. Absorption of phenolic solutions through the skin may be very rapid, and can cause death within 30 minutes to several hours by exposure of as little as 64 square inches of skin. Lesser exposures can cause damage to the kidneys, liver, pancreas, and spleen, and edema of the lungs. Ingestion can cause corrosion of the lips, mouth, throat, esophagus, and stomach, and gangrene. Ingestion of 1.5 g has killed. Chronic Exposures can cause death from liver and kidney damage. Dermatitis resulting from contact with phenol or phenol-containing products is fairly common in industry. A common air contaminant. Combustible when exposed
to heat, flame, or oxidizers. Potentially explosive reaction with aluminum chloride + nitromethane (at 110ºC/100 bar), formaldehyde, peroxydisulfuric acid, peroxymonosulfuric acid, sodium nitrite + heat. Violent reaction with aluminum chloride + nitrobenzene (at 120ºC), sodium nitrate + trifluoroacetic acid, butadiene. Can react with oxidizing materials. To fight fire, use alcohol foam, CO₂, dry chemical. When heated to decomposition it emits acrid smoke and irritating fumes.

**Chloroform**
Confirmed carcinogen with experimental carcinogenic, neoplastigenic, and tumorigenic data. A human poison by ingestion and inhalation. An experimental poison by ingestion and intravenous routes. Moderately toxic experimentally by intraperitoneal and subcutaneous routes. Human systemic effects by inhalation: hallucinations and distorted perceptions, nausea, vomiting, and other unspecified gastrointestinal effects. Human mutation data reported.

Experimental teratogenic and reproductive effects. Inhalation of the concentrated vapor causes dilation of the pupils with reduced reaction to light, as well as reduced intraocular pressure (experimental). In the initial stages there is a feeling of warmth of the face and body, then an irritation of the mucous membranes, conjunctiva, and skin; followed by excitation, loss of reflexes, sensation, and consciousness. Prolonged inhalation will bring on paralysis accompanied by cardiac-respiratory failure and finally death.

Chloroform has been widely used as an anesthetic. However, due to its toxic effects, this use is being abandoned. Concentrations of 68,000–82,000 ppm in air can kill most animals in a few minutes. 14,000 ppm may cause death after an exposure of from 30 to 60 minutes. 5000–6000 ppm can be tolerated by animals for 1 hour without serious disturbances. The maximum concentration tolerated for several hours or for prolonged exposure with slight symptoms is 2000–2500 ppm. Prolonged administration as an anesthetic may lead to such serious effects as profound toxemia and damage to the liver, heart, and kidneys. Experimental prolonged but light anesthesia in dogs produces a typical hepatitis. Explosive reaction with sodium + methanol or sodium methoxide + methanol. Mixtures with sodium or potassium are impact-sensitive explosives. Reacts violently with acetone + alkali (e.g., sodium hydroxide, potassium hydroxide, or calcium hydroxide), Al, disilane, Li, Mg, methanol + alkali, nitrogen tetroxide, perchloric acid + phosphorus pentoxide, potassium-tert-butoxide, sodium methylate, NaK. Incompatible with dinitrogen tetroxide, fluorine, metals, or triisopropylphosphine. Nonflammable. When heated to decomposition it emits toxic fumes of Cl⁻.

**Safety Sign Off**

Before you begin work on Section D of this assignment you must read the information above and consult with a research staff member on the proper location, transport and use of phenol, chloroform, or phenol:chloroform in the laboratory environment.

**Student Component**

*I have consulted with a research staff member and agree to abide by the rigorous safety standards of the lab with regards to the use of these reagents.*

Student Signature: ___________________________   Date: ___________________________

**Research Staff Component**

*I have consulted with the student and have instructed them on the location, appropriate waste disposal and the safe transport and use of this set of reagents.*

Expectations: Storage Location ___________   Waste Disposal ___________   Safety in Transport & Use ___________

Research Staff Comments: ________________________________________________________________
____________________________________________________________________________________
____________________________________________________________________________________

Research Staff Signature: ___________________________   Date: ___________________________
Section B - Clone Selection

Introduction

Each of you will be assigned a specific deletion strain of *Saccharomyces cerevisiae*. Each deletion will be relevant to cell cycle regulation in some set of ways; these ways will be discussed in future lectures and researched by you for your final laboratory report.

Background Knowledge

**Question 1:** What is the name of the strain you were assigned: ____________________________________

**Question 2:** Where in the yeast genome is this gene located: _____________________________________

**Question 3:** What is known about this strain: ________________________________________________
                                                                                             __________________________________
                                                                                             __________________________________
                                                                                             __________________________________
                                                                                             __________________________________
                                                                                             __________________________________

**Question 4:** Do you expect this strain to grow normally in YPD (as compared to wild type) - why, why not:
                                                                                             __________________________________
                                                                                             __________________________________
                                                                                             __________________________________
                                                                                             __________________________________

Strain Selection & Storage

Each strain has a plate streaked and stored in the laboratory 4°C refrigerator. You should find the correct plate and then use aseptic technique to streak a new plate of that strain on a new YPD plate. You may use either a flame-sterilized inoculation loop or a sterile toothpick; your choice.

The generalized steps are detailed below. For more detail you should consult the online course notebook or previous assignments where you have already performed these activities.

Experimental Procedure

1. Label the plate fully on the agar side before streaking.
   *You do not need to use tape - write directly on the plate (agar side, not the lid) with a Sharpie.*
   *Lids can come off and get lost - this is why we always write on the agar side.*

2. Streak the plate by selecting a colony from the laboratory stocks.

3. Incubate the plate overnight at 30°C.
   *This is now your plate for your research strain.*

4. Present your successfully streaked and incubated plate to a research staff member (next day) for a signature.
   *It is important to note that at this point in the semester the research staff member will be both expecting a fully
labeled plate as well as a correctly streaked one. If it is not possible for you to pick one of many colonies from this new plate for an overnight culture you will be required to try again. This is science my friends.

5. You will need this plate for the next section (Strain Growth & Pelleting). After you have used the plate for this purpose wrap it in Parafilm and store it in the 4°C refrigerator. The plate can be stored for a number of days or even weeks.

**Important Note:** Please note that you will want to occasionally check on this plate from now until the end of the semester. This is the research strain you are working with. If you feel that your plate ever becomes contaminated or overgrown select a colony and re-streak a new plate (then discarding the old plate).

**Safe Stop Note:** This is a safe stop point; you can safely stop here and begin the next step when ready to do so.

**Timing Note:** At this point you might note that you are doing a few simple steps and then waiting a day (for both Section B and Section C). It might be a good idea to take a look at Section D and the solutions and buffers required therein. During some of your downtime preparation of these solutions would be very smart.

**Research Staff Evaluation**

Expectations: Fully Labeled _______ Correctly Streaked _______ Many Colonies to Choose _______

Research Staff Comments: ________________________________________________________________
____________________________________________________________________________________
____________________________________________________________________________________

Research Staff Signature: ___________________________ Date: ___________________________
Section C - Strain Growth & Pelleting

Introduction

In the previous section you plated a specific deletion strain of *Saccharomyces cerevisiae*. You now need to grow this strain up in order to be able to do basic molecular protocols.

You will do growth in three stages. First you will grow a small overnight culture. The next day you will grow a significantly larger culture by diluting the overnight culture growing the larger culture until it is nearing the end of exponential growth. Finally, you will snap-freeze the cell pellet for storage before processing.

Details of how to accomplish these steps are below. Reference detail in is the online course notebook (protocols).

Growth Procedure

Perform the following sub-procedures to grow an overnight culture, grow a larger experimental culture, and then finally produce a snap-frozen cell pellet.

Overnight Culture Growth

1. Get your plate from the 4°C refrigerator.
2. Select a colony following the (aseptic) procedures in the online course notebook and inoculate a 15 mL overnight culture.
3. Incubate the overnight culture at 30°C (shaking incubator).
   *The next morning your overnight culture will be fully grown. Note that an overnight culture must be used the next day for the larger culture growth. Thus, you will want to plan accordingly to start your overnight any time on the day before you intend to do the larger culture growth step.*
4. Apply fresh Parafilm to your plate and return it to the 4°C refrigerator for storage.

Research Staff Evaluation

Expectations: Fully Labeled ________ Correct Volume _________

Research Staff Signature: ___________________________ Date: ___________________________

Larger Culture Growth & Growth Curve

1. Dilute your overnight culture so that an accurate OD600 measurement can be taken of it.
   *A 1/4 or 1/5 dilution is likely appropriate. Your best bet for reliability of OD600 measurement is to take two dilutions - 1/2 and 1/4 perhaps - and compare OD600 results. If the OD600 measurements correlate after correcting for dilution factor then you likely have two good measurements.*
2. Take an OD600 measurement of your diluted overnight culture and do the mathematics needed to inoculate a 40 mL culture at an OD of 0.10. Use the Spectronic 20D+ for all culture measurements of OD600 throughout.
3. Perform the dilution of your overnight culture to produce the 40 mL culture at a theoretical OD600 of 0.02.
4. Use the Spectronic 20D+ to verify that your dilution is near OD600 ~ 0.20.
   *An OD600 value of anywhere between 0.05 and 0.15 would be considered an acceptable starting point.*
5. Record a growth curve by allowing the larger culture to grow until an approximate OD600 of ~ 0.80.
   You should record OD600 measurements at intervals of approximately every two hours (not every hour).
   Do not allow the culture to grow above an OD600 of 0.90. It might be helpful to look up the doubling time of
   yeast but keep in mind that your strain might grow at a rate different than wild type. If you are short on time an
   OD600 of 0.60 is acceptable while an OD600 of 0.80 is best for optimal cell count.

Research Staff Evaluation

Expectations: Fully Labeled ________    Correct Volume _________ Notebook Start & Finish OD600 ______

Research Staff Signature: ___________________________   Date: ___________________________

Cell Pellet Preparation

Introduction

Before you can prepare genomic DNA or total RNA you will need to first separate your cells from their growth
medium. This operation typically involves the use of a centrifuge to spin the cells down - form a tight pellet of cells at
the bottom of a tube. Once the tight pellet is formed the medium is removed (poured off or pipetted out). Immediately
following removal of the medium the cells are often snap-frozen using liquid nitrogen.

Why?

Remember, most biological experiments are targeting at measuring intracellular activity. Intracellular could change or
be affected by removing the medium and then pausing too long before lysing the cells (breaking them open). Thus, cell
pellet preparation typically involves a very fast spin down of cells, a quick removal of medium, and then an immediate
snap-freeze in liquid nitrogen. It is like having the power to freeze the cells in time at a particular moment and then
study that moment.

Preparation for Spin Down

For this section you are going to be preparing both genomic DNA and total RNA from this culture of cells. Take the
culture you have and equally divide its volume between two 50 mL conical tubes. For example, if you have 40 mL of
culture you would divide the volume such that you put 40 mL in one tube and 40 mL in another. Label one tube “for
 genomic DNA” and the other “for total RNA”.

You are separating the cells because you will later re-suspend them in different solutions for nucleic acid preparation.

Spin Down Procedure

You should have two tubes of cell culture ready for spin down.

1. Pellet cells by centrifugation at 4000 rpm for 5 minutes (centrifuge in PAI 2.14).
   This step of the procedure can be performed with any volume in a 15 mL or 50 mL conical tube. Be sure that
tubes are always counter-balanced inside any centrifuge with matching dummy tubes filled with water to the
equal volumes. Please ask for assistance the first time you use this centrifuge. Also consult the online course
notebook for more details on centrifuge safety and operation.

2. Pour off the growth medium - in technical terms this is often called decanting the supernatant.
3. Using a low-volume pipette (perhaps 100 µL), remove the rest of the medium from around the cell pellet.

4. Snap freeze the cell pellet by carefully dipping the pellet-end of the closed tube into a liquid nitrogen bath.  
   *You must be very careful with this step. Please ask for assistance the first time you perform it.*

5. The cell pellets can now be stored in this tube in the -20°C freezer until you are ready to perform either genomic DNA or total RNA preparation.

**Safe Stop Note:** This is a safe stop point; you can safely stop here and begin the next step when ready to do so.

**Timing Note:** At this point you might note that you are doing a few simple steps and then waiting a day (for both Section B and Section C). It might be a good idea to take a look at Section D and the solutions and buffers required therein. During some of your downtime preparation of these solutions would be very smart.

**Research Staff Evaluation**

Expectations: Fully Labeled ________ Correct Pellet Look & Size ________

Research Staff Signature: ___________________________ Date: ___________________________
Section D - Nucleic Acid Preparation

Introduction

You now have two 50 mL conical tubes - each with a frozen cell pellet stored in the laboratory -20°C freezer.

Why two tubes? Well, why not? Actually, as always, there is a method to the madness.

You have two 50 mL tubes because this is going to allow you:

‣ multiple attempts at a successful genomic DNA preparation
‣ multiple attempts at a successful total RNA preparation

Successful Preparations: Quantity & Quality

What do we mean by successful? Two metrics: quantity & quality.

Much like a Columbian drug lord, your goal in nucleic acid preparations is to purify as much mass of a specific nucleic acid (DNA, RNA) as possible. Typically you are after micrograms of the good stuff (µg). This is quantity.

A dump-truck full is not success in itself. You are also going after quality as well.

How do we define quality? Two ways: purity and integrity.

Purity refers to the homogeneity of the solution at the end of the procedure. If we wished to prepare total RNA did we end up with nothing but RNA (no DNA contamination)? Great! That would be high purity.

Integrity refers to how intact the nucleic acid is. We generally have no expectation for “intactness” when it comes to genomic DNA as we expect chromosomes to shear when when being prepared. RNA preparation is a complete and total different story, however. RNA is the proxy by which we measure gene expression. Most assays that analyze RNA work best when the RNA is fully intact; the original transcript is still in the form it was in when first transcribed from genomic DNA. This is a tough challenge as there are all sorts of things in the cell and in the laboratory that exist solely to degrade RNA.

Getting Ready to Attack

Here we go. It is highly recommended that when attempting either the DNA or RNA prep you use only one of the tubes at a time. If your first attempt at either protocol fails, worry not - you will have an opportunity to talk it over with the research staff and another attempt with another tube.

Clean your work area thoroughly. Use ethanol to fully wipe down all surfaces on which you will be working. There was once a saying among American fighter pilots during the Cold War; you’ll never see the MiG that gets you. The same applies to lurking nucleases that will destroy your genomic DNA or total RNA. You have no idea what the Cold War or a MiG is do you?

Question 5: What is a MiG? __________________________________________________________________

Question 6: What was the scariest movie ever about the Cold War and did you see it? __________________

Question 7: What is a nuclease? __________________________________________________________
**Experimental Tube Preparation**

You now have two 50 mL conical tubes - each with a frozen cell pellet stored in the laboratory -20°C freezer. Go get those tubes and bring them to your now very clean workspace.

1. Re-suspend the “for genomic DNA” cell pellet in 4 mL of autoclaved sterile H₂O.
2. Re-suspend the “for total RNA” cell pellet in 4 mL of AE buffer.  
   *Please see recipe below or in online course notebook.*
3. Aliquot the 4 mL of each solution into labeled microcentrifuge tubes - each tube receiving 500 µL solution.  
   *In the end you should have 4000 / 500 = 8 tubes for genomic DNA and 4000 / 500 = 8 tubes for total RNA.*

This separation into microcentrifuge tubes is going to allow us to process for each nucleic acid using the microcentrifuge models we have in the laboratory. It also gives you backup tubes in case your initial attempts fail or you feel that you need more practice at the procedure.

**Important Note:** For the rest of the assignment you will be preparing genomic DNA and total RNA. The reason you have eight tubes of each is because it is *expected* that you will have failures and we need to repeat these preps. You will be pulling out only one of these eight tubes (of each type) at a time to attempt the nucleic acid preparation. If you fail in your first attempts discard and try again with another one of your tubes.

**Genomic DNA Preparation**

**Reagent Preparation**

There are several reagents that you will need to prepare in order to proceed with this portion of the assignment. They are detailed in online course notebook. You need to take time and care in the preparation of these solutions as their accuracy will have a very direct impact on the efficacy of the protocol in which they are used.

**Note:** All of these solutions should be prepared with sterile autoclaved H₂O. I recommend preparing them directly in 15 mL conical tubes - prepare about 12 mL and use the graduation on the conical tubes for measurement.  
**Note:** Solution volumes are not provided. You should evaluate the protocol(s) below and make an appropriate volume. Typically some amount store in a fully labeled 15 mL conical tube is appropriate.

- 10M Ammonium Acetate
- TE (pH 8.0)  
  100 mM Tris-Cl (pH 8.0)  
  10 mM EDTA (pH 8.0)
- TE (pH 8.0) with RNase  
  *Contains 20 µg / mL RNase*  
  *Stock solution, on stock shelves (do not make this solution)*
- Phenol:Chloroform (25:24:1 Phenol:Chloroform mixed with Isoamyl Alcohol)  
  *Stock solution, in 4°C refrigerator*
- Triton / SDS Solution, sterilized by filter  
  2 % (v/v) Triton X-100, 1 % (w/v) SDS  
  100 mM NaCl, 10 mM Tris-Cl (pH 8.0)  
  1 mM EDTA (pH 8.0)  
  *Stock solution, on stock shelves (do not make this solution)*
Protocol Execution

Note: Verify this procedure (every detail) for any changes in the online course notebook before proceeding.

You should be using one of your eight microcentrifuge tubes dedicated to genomic DNA preparation. The tube should contain 500 µL of cells suspended in sterile autoclaved H₂O. The other tubes should be left in the -20°C freezer.

1. Pellet the cells by centrifugation at maximum speed for 1 minute at room temperature in a microcentrifuge.
2. Decant the supernatant (discard).
3. Add 200 µL of Triton / SDS Solution to the pelleted cells.
4. Re-suspend the cell pellet by gently tapping the side of the tube.
5. Add 200 µL of Phenol:Chloroform (25:24:1) solution and 0.3 g of glass beads to the cells and vortex the cell suspension for 2 minutes.
6. Add 200 µL of TE (pH 8.0) and mix the solution by vortexing briefly.
7. Separate the organic and aqueous phases by centrifugation (maximum speed) for 10 minutes.
8. Carefully pipette the aqueous upper layer to a sterile microcentrifuge tube.
   Avoid pipetting any of the material at the interphase. You will likely want to leave 20% of the aqueous layer behind in order to do so. Please ask for assistance the first time you perform this procedure.
9. Add 1 mL of 100% ethanol to the aqueous solution, close the microcentrifuge tube, and gently mix the contents by inverting a few times.
10. Spin down using the cold centrifuge located in the clear 4°C refrigerator for 10 minutes at full speed.
   At the end of this spin you should see a small DNA pellet at a specific spot on the bottom of the tube. Ask for assistance in visualizing the pellet - this is extremely important.
11. Remove the supernatant with a drawn out Pasteur pipette or careful micropipetting.
12. Centrifuge the tube briefly (2 seconds) and remove the last traces of ethanol from the bottom of the tube.
   Allow the pellet to dry completely before proceeding.
13. Re-suspend the pellet in 400 µL of TE (pH 8.0) with RNase and incubate the solution for 5 minutes at 37°C.
15. Mix by inverting the tube several times.
16. Separate the aqueous and organic phases by centrifugation at maximum speed for 5 minutes at room temperature in a microcentrifuge and transfer the aqueous layer to a new microcentrifuge tube.
17. Add 80 µL of 10M ammonium acetate and 1 mL of chilled 95% ethanol to the aqueous layer.
18. Mix the solution by gentle inversion and store the tube for 5 minutes at room temperature.
19. Pellet the precipitated DNA by centrifugation for 10 minutes in a microcentrifuge.
20. Decant the solution (discard).
21. Wash pellet with 500 µL 70% ethanol, spin down quickly in a microcentrifuge, carefully remove ethanol.
22. Repeat the wash procedure in the step above two more times.
23. Dry the pellet completely by removing all ethanol and allowing the pellet to air dry (10 - 15 minutes).
   Most people usually open the tube and leave it upside down on a sterile KimWipe.
24. When the pellet is fully dry you can re-suspend it in 20 µL autoclaved sterile H₂O.
   Show a research staff member the pellet before you re-suspend it in H₂O.
Total RNA Preparation

Reagent Preparation

There are several reagents that you will need to prepare in order to proceed with this portion of the assignment. They are detailed in online course notebook. You need to take time and care in the preparation of these solutions as their accuracy will have a very direct impact on the efficacy of the protocol in which they are used.

Note: All of these solutions should be prepared with sterile autoclaved H₂O. I recommend preparing them directly in 15 mL conical tubes - prepare about 12 mL and use the graduation on the conical tubes for measurement.

Note: Solution volumes are not provided. You should evaluate the protocol(s) below and make an appropriate volume. Typically some amount store in a fully labeled 15 mL conical tube is appropriate.

- AE buffer
  - 50 mM sodium acetate (pH 5.2)
  - 10 mM EDTA
- 3M sodium acetate (pH 5.2)
- 0.5 M EDTA (pH 8.0)
- 20% SDS
  - Aliquot from lab stock - do not make your own. Aliquot cleanly!
- Acid Phenol (pH 4.5-5.5)
  - Stock solution, in 4°C refrigerator
- Chloroform
  - Stock solution, in 4°C refrigerator
- 65°C water bath or heat blocks
- Phase Lock Gel Heavy (2 of them, 2 mL variety)
- Chilled 95% & 70% ethanol
  - Make your own and store in the -20°C freezer (in a rack, labeled completely).
- Sterile H₂O

Protocol Execution

Note: Keep the experimental solution and / or cells on ice throughout the protocol

Note: Verify this procedure (every detail) for any changes in the online course notebook before proceeding.
You should be beginning with one of your eight microcentrifuge tubes dedicated to total RNA preparation. The tube should contain 500 µL of cells freshly suspended in AE buffer. The other tubes should be left in the -20°C freezer.

1. Add 43 µl 20% SDS, 500 µL acid phenol (careful!).
2. Mix well by vortexing.
3. Incubate for 1 hour at 65°C, vortexing every 10 minutes.
   
   *You can use either the heat blocks or the water bath for this incubation - keep tube rims clean!*

4. Incubate for 10 minutes on ice.
5. While waiting spin down one 2 mL Phase Lock Gel tube (30 seconds at 12-16,000 g).
   
   *This is preparing the Phase Lock Gel tube for use in a future step.*
6. Separate the organic and aqueous phases by centrifugation (maximum speed) for 10 minutes.
7. Carefully pipette the aqueous upper layer to a sterile microcentrifuge tube.
   
   *Avoid pipetting any of the material at the interphase. You will likely want to leave 20% of the aqueous layer behind in order to do so. Please ask for assistance the first time you perform this procedure.*
8. Add 500 µL acid phenol and mix well.
9. Again, separate the organic and aqueous phases by centrifugation (maximum speed) for 10 minutes.
10. Carefully pipette the aqueous upper layer to the spun down Phase Lock Gel.
11. Add 625 µL of chloroform to this aqueous solution and mix well.
    
    *Do not vortex the Phase Lock Gel tubes. Do not stab the gel with your pipette tip. Close tube and rotate.*
12. Centrifugate the Phase Lock Gel tube for 5 minutes at 12-16,000 g.
13. Decant the supernatant to a fresh sterile microcentrifuge tube. Phase Lock Gels are great!
14. Add 1/10 the current volume 3M sodium acetate (pH 5.2) and 2.5 volumes of 95% chilled ethanol. Mix well.
15. Centrifugate using the cold centrifuge located in the clear 4°C refrigerator for 30 minutes at full speed.
    
    *At the end of this spin you should see a small RNA pellet at a specific spot on the bottom of the tube. Ask for assistance in visualizing the pellet - this is extremely important.*
16. Decant the solution (discard).
17. Wash pellet with 500 µL 70% ethanol, spin down quickly in a microcentrifuge, carefully remove ethanol.
18. Repeat the wash procedure in the step above two more times.
19. Dry the pellet completely by removing all ethanol and allowing the pellet to air dry (10 - 15 minutes).
    
    *Most people usually open the tube and leave it upside down on a sterile KimWipe.*
20. When the pellet is fully dry you can re-suspend it in 20 µL autoclaved sterile H2O.
    
    *Show a research staff member the pellet before you re-suspend it in H2O.*

**Research Staff Evaluation**

Expectations: Fully Labeled _______  Correct Volume ________

Research Staff Comments: ____________________________________________________________

Research Staff Signature: ___________________________  Date: ___________________________
Section E - Nucleic Acid Quantification & Quality Evaluation

Once you have purified nucleic acids you want to ask the questions we began with:

- Is there sufficient and/or expected quantity?
- Is the quality of the preparation to expectations?

Let us engage procedures that allow us to do that now.

**NanoDrop Evaluation**

The NanoDrop has the capacity to inspect both quantity and quality. The purpose of the NanoDrop spectrophotometer is to quantify the concentration of your nucleic acid solution while only using a very small amount of your sample. Using only 2 µL of your sample, you can determine the concentration of your sample in ng/µL using light absorbance.

The NanoDrop consists of two important parts: the pedestal and the sampling arm. During operation, your sample will be placed on the pedestal and the sampling arm will be lowered before you conduct your measurement. When the measurement is initialized, the sampling arm and the pedestal will stretch the sample drop and send light through the sample. The absorbance at 260 nm will be taken by the spectrophotometer and will be sent to the computer.

**Question 8:** What is the expected ratio of 260/280 for pure DNA? ________________________

**Question 9:** What is the expected ratio of 260/280 for pure RNA? ________________________

The following screen is the main *Nucleic Acid* screen within the NanoDrop program. In it you can determine both the quantity of the nucleic acid you prepared and the quality. Both DNA and RNA have a curve that is very similar to the graphic below when the quality of the nucleic acid is determined to be pure. Note that the graph is absorbance by wavelength. Nucleic acids have a signature pattern by which they absorb over the range of 220 to 350 nm. That signature pattern is below. The ng/µL informs you as to the quantity of nucleic acid that was purified. In the case below it can be seen that 3250.1 ng/µL was purified. This of course is 3.25 µg/µL - a relatively strong concentration.
1. Select the Nucleic Acid option from the NanoDrop main menu and the screen in the figure above will appear.
2. Raise the sampling arm and take a KimWipe that is lightly wet with diH$_2$O and clean both the pedestal and sampling arm to avoid any residue build-up on the apparatus.
3. Using a L20 micropipette, place a 2 µL drop of diH$_2$O on the pedestal and lower the sampling arm.  
   *This is done by lightly pressing the plunger and allowing a drop of the liquid to form at the end of the pipette.  
   *This drop is much easier to place on the pedestal.*
4. Lower the sampling arm gently and do not press the pedestal.
5. Click “OK”.
6. You will now need to blank the NanoDrop so that a baseline measurement of “0” will read before you begin.
7. Wipe the pedestal and sampling arm with a KimWipe.
8. From the new screen select the DNA or RNA option depending on the sample being measured.
9. Add a 2 µL drop of the liquid that the DNA or RNA was re-suspended in, typically diH$_2$O, Tris or TE.
10. Lower the sampling arm gently.
11. Click “Blank”.
12. Wipe the pedestal and sampling arm again with a KimWipe.
13. Add 2 µL of the target sample and click “Measure” which is located in the top left corner.
14. Take a reading from the bottom right corner in ng/µL.
15. Wipe the sampling arm and pedestal with a KimWipe after use and leave with sampling arm in the down position.  Clean the pedestal very thoroughly!
16. Click “Print” to produce a label that can permanently added to your laboratory notebook.
17. Exit the program or repeat steps 13 through 16 for additional samples.

**Required:** In the space below affix trimmed printouts for your **best** DNA preparation and your **best** RNA preparation. If your quantity or quality is questionable (see research staff) for either sample (genomic DNA, total RNA) you must repeat Section D until you have a good result for both nucleic acids.  It is expected you will need multiple attempts.

Research Staff Signature *: ____________________________________   Date: ___________________________

* research staff is expected to be critical here for both quantity and quality criteria.
Running a nucleic acid in an agarose gel is a method primarily used to evaluate quality. The figure below shows that there is a strong signature of good RNA when run on a gel. The following information is from Ambion.com.

Intact total RNA run on a denaturing gel will have sharp 28S and 18S rRNA bands (eukaryotic samples). The 28S rRNA band should be approximately twice as intense as the 18S rRNA band (Figure 1, lane 3). This 2:1 ratio (28S:18S) is a good indication that the RNA is intact. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will not exhibit a 2:1 ratio. Completely degraded RNA will appear as a very low molecular weight smear (Figure 1, lane 2). Inclusion of RNA size markers on the gel will allow the size of any bands or smears to be determined and will also serve as a good control to ensure the gel was run properly (Figure 1, lane 1). Note: Poly(A) selected samples will not contain strong rRNA bands and will appear as a smear from approximately 6 kb to 0.5 kb (resulting from the population of mRNAs, and depending on exposure times and conditions), with the area between 1.5 and 2 kb being the most intense (this smear is sometimes apparent in total RNA samples as well). Generally, 200 ng of RNA must be loaded onto a agarose gel in order to be visualized with ethidium bromide.

**Figure:** Intact vs. Degraded RNA. Two µg of degraded total RNA and intact total RNA were run beside Ambion's RNA Millennium Markers™ on a 1.5% denaturing agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample. The degraded RNA appears as a lower molecular weight smear.

**Required:** Affix images (through whatever means necessary) of your best RNA and DNA gel to the end of this assignment. Each lane needs to be clearly labeled and the gel must include a ladder. See Section F for procedure. You must repeat this section (and the total RNA portion of Section D) until you have a good gel image of intact total RNA.

**Research Staff Evaluation**

Expectations: 28S & 18S Bands Clear for Total RNA __ Genomic DNA Streak __ Stapled to Assignment __

Research Staff Comments: __________________________________________

__________________________________________________________

Research Staff Signature *: _____________________________ Date: __________________________

* research staff is expected to be critical here for quality criteria.
Section F - Gel Electrophoresis

Introduction

In this section you will be taking a small amount of your genomic DNA and total RNA and running them on an agarose gel to visualize the heterogenous species of nucleic acids therein separated by size. Note that you can easily run both genomic DNA and total RNA in the same gel (separate lanes, of course).

Consult the online course notebook and your course textbook for more information on the background and varied uses of gel electrophoresis. A generalized image of the process of making and running a gel is pictured below (textbook):

**Pouring the agarose gel:**  (A) Addition of agarose to $1 \times \text{TAE}$ running buffer. (B) After dissolving the agarose in a microwave, the gel solution is clear, with no transparent specks of agarose evident. (C) Once the gel solution has cooled to allow handling ($55^\circ$ to $60^\circ$ C), it can be poured. For the gel rig pictured, the gel tray is placed in the buffer chamber $90^\circ$ with respect to the usual running orientation, and the gel is poured. Rubber gaskets in the sides of the gel tray prevent leaking. Note the presence of the comb (arrow). This particular comb is double sided, with one set of teeth thicker than the other to allow a choice of narrow or thick wells.
Procedure

Gel Preparation

1. Measure out 30 mL of TAE using a graduated cylinder; pour into a 100 mL Erlenmeyer flask. Measure 0.30 g of agarose and add to flask to produce a ~ 1.0% agarose gel.
2. Heat the flask using a microwave to melt the agarose. Microwave for 15-30 sec and check to see if the agarose is completely dissolved. Swirl and mix the agarose. 
   *Note that we would need to weigh the mixture before microwaving, microwave, and then re-add evaporated water if we wished to ensure the gel was exactly 1.0%.*
3. Let cool for 3 minutes on the bench. Add 2.0 µL of Ethidium Bromide (EtBr) and swirl. Careful, carcinogen!
4. Pour onto assembled gel tray (with barriers in place to hold the contents of poured gel). Use a pipette tip to move any air bubbles to the side or the bottom of the tray. Insert the gel comb and let polymerize. Agarose will solidify after a few minutes and turn translucent, glossy. *Ask for assistance the first time.*

Sample Loading

1. Place tray with solidified gel into the gel box, and gently remove the comb from the gel. 
   *Tip: Run to red! Make sure that the wells of your gel are at the black Ru(negative) end of the gel box. DNA has a negative charge, and runs toward a positive charge (the red side).*
2. Pour TAE buffer into the gel box. Make sure that the entire gel is covered, and the wells are filled with buffer.
3. Pipet 1 µg of sample onto Parafilm (the side from which the paper was removed, aka the clean side).
4. Add autoclaved water to bring drop volume to ~ 10 µL.
5. Carefully add 1 µL of loading dye to each ~ 10 µL DNA sample.
6. Carefully pipet 7 µL of 1kb ladder into the first lane of the gel. 
   *Be careful not to pierce the bottom of the gel with the pipet.*
7. Load each of the samples into separate wells by pipetting, as described above. 
   *Tip: If your hand is shaking while loading the sample, stabilize the pipet with a finger from your other hand, or rest elbows on bench top and use both hands.*

Running the Gel

1. Place the lid of the gel box on properly (black with black, and red with red).
2. Run the gel at 100 Volts for about 45 minutes or until the blue dye has run about 1/2 to 2/3 across the gel.

Note that you will need two copies of each of your gels. One for your notebook and one stapled to the back of this assignment (see requirement in the next section).

Research Staff Evaluation

Expectations: Genomic DNA Gel ________ Total RNA Gel ________ In Notebook __________________

Research Staff Comments: ____________________________________________________________________
__________________________________________________________________________________________
__________________________________________________________________________________________

Research Staff Signature: ___________________________ Date: ___________________________
Section G - Proving the Deletion: PCR

At this point you have performed the following operations in this monster of an assignment:

- Plated a specific strain of *Saccharomyces cerevisiae* (a deletion strain).
- Grew an overnight culture.
- Grew a larger scale culture to a specific OD.
- Harvested genomic DNA and total RNA.
- Verified the quantity and quality of genomic DNA and RNA with the NanoDrop.
- Verified the quality of the genomic DNA and RNA with agarose gel electrophoresis.

We conclude this assignment with a final section dedicated to two final goals:

- Instruction in the principles and practicalities of enzymatic reaction-based experimentation.
- Proving the strain you are working with lacks the gene *putatively* deleted.

**Polymerase Chain Reaction**

PCR, also known as *polymerase chain reaction* is a functional way of amplifying a small amount of DNA into a quantifiable amount. Through the use of correct primers, the dNTP mix, and the DNA polymerase, PCR performs DNA replication in a short amount of time and very accurately. Conceptually, it is depicted in the figure below (text):
The polymerase chain reaction (PCR) was conceptualized by Kary Mullis in 1983 (Saiki et al., 1985; Mullis et al., 1986) and was rapidly put into practice. PCR has subsequently revolutionized molecular analysis in the life sciences to the point that Dr. Mullis received the 1993 Nobel Prize in Chemistry for his discovery. In fact, it has far exceeded the expectations of the inventor and the earliest developers. PCR has constantly evolved and amended itself to accommodate the specific needs of researchers. The simple elegance of PCR permitted the technique to be quite malleable and to be surrounded with supplemental techniques to enhance its capabilities.

The inherent power of PCR is its ability to exponentially amplify specific nucleic acid sequences in a short period of time. Deoxyribonucleic acid (DNA) amplification by PCR is achieved through multiple cycles of in vitro DNA replication. In its initial and simplest form, a DNA replication cycle for PCR proceeds as shown in Figure 10.2.1.

**Reaction mix composition.** The reaction solution within a tube contains the DNA template to be amplified (e.g., genomic DNA), two different species of short single-stranded oligonucleotides called primers, a DNA polymerase, the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), a salt with a divalent cation (i.e., Mg2+), and a buffer with a simple salt (i.e., KCl).

**Denaturation.** The solution is heated to a temperature for a period that promotes DNA strand dissociation from double-stranded to single-stranded form to prepare the DNA template for priming and subsequent DNA amplification (denaturation step).

**Annealing.** The temperature of the solution is brought down and held for a period sufficient to allow for hybridization of the primers to the single-stranded DNA template (annealing step).

**Extension.** The temperature is either kept constant (two-step PCR) or is raised for optimal DNA polymerase activity (three-step PCR) and is held for a period sufficient for all primed events to be fully extended, yielding the double-stranded products called amplicons (extension step).

**Repetitive cycling.** The denaturing, annealing, and extension steps are typically repeated 30 to 40 cycles.

In the original experiments to demonstrate proof-of-principle for PCR, the tube containing the reactants was manually transferred between water baths set and equilibrated to the desired temperatures. The added DNA polymerase was irreversibly inactivated during the denaturation step, making the process even more complicated; therefore, more enzyme had to be added manually just prior to each extension step. This was a very laborious and time-intensive methodology that was not amenable for routine use in the laboratory. Two key advances made PCR a technology that could be practiced in almost any laboratory. The first key improvement was the identification and implementation of thermostable DNA polymerases that eliminated the need for frequent manual addition of enzyme (Saiki et al., 1988). The second key advance was the development of an instrument called a thermal cycler that holds PCR reaction tubes and is programmed to adjust to specific temperatures for discrete periods of time.

**Proving the Deletion**

You will be using PCR with primers specific to your gene deletion to demonstrate that the putative gene deletion is actually true (the strain you are working with truly lacks the gene thought to be deleted). What if you were working with wild type *Saccharomyces cerevisiae*? Would you know if you did not prove it?
Experimental Procedure

Acquiring Primers

You will be provided primers for both your specific gene of interest as well as positive control primers. The positive control primers will of course be for a region of the yeast genome that is known to be present. Use of a positive control will allow you to differentiate between experimental failure and failure of your gene-specific PCR.

Check that you have the following primers before proceeding:

- Strain Specific Forward Primer: _____________ (check here)
- Strain Specific Reverse Primer: _____________ (check here)
- Positive Control Forward Primer: _____________ (check here)
- Positive Control Reverse Primer: _____________ (check here)

Question 10: What gene was used for the positive control? ___________________________________________

Question 11: Why is this gene a good positive control? ___________________________________________

Question 12: How does the combination of strain specific primers and PCR demonstrate the gene is deleted?
___________________________________________________________________________________________
___________________________________________________________________________________________
___________________________________________________________________________________________

Reaction Preparation

Note: The use of the lab thermocyclers may need to be scheduled to manage the availability of these resources. Please consult the online course notebook for instructions on how to reserve a thermocycler for a specific period of time.

Note that you will be running three parallel reactions:

1. one with your strain specific primer(s)
2. one with the positive control primer(s)
3. one with positive control primers but no template genomic DNA (negative control)

The execution of these three reactions in parallel will demonstrate the deletion of your gene of interest, the presence of a positive control gene, and the lack of contamination through no amplification in a negative control reaction. Consult the online course notebook for information regarding how your gene-specific primers work to demonstrate gene deletion - it is related to the process by which the actual gene deletion was first created.
Reaction Execution

The following instructions represent the process of running one PCR reaction. You will need to prepare three of these reactions (scale accordingly).

1. Mix the following solution in a 0.2 mL PCR tube making sure to mix the diH₂O first and the rest of the solution making sure to mix the Taq DNA polymerase last.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Reaction 1</th>
<th>Reaction 2</th>
<th>Reaction 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR Buffer</td>
<td>5 µL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µM forward primer</td>
<td>5 µL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µM reverse primer</td>
<td>5 µL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM dNTP (mix)</td>
<td>1 µL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 ng template DNA (genomic DNA)</td>
<td>x µL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taq DNA polymerase (1U/µL) (add last)</td>
<td>1.0 µL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diH₂O (use whatever volume y is correct to bring total reaction volume to 50 µL)</td>
<td>y µL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>50 µL</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Important Note: Keep Taq polymerase in freezer until needed and on ice at all times when out of freezer.

2. Lightly mix the solution and centrifuge down for 5 seconds.
3. Put the filled tubes in the thermocycler and turn the knob to the picture of a 0.2 mL tube to lock the lid.
4. Run the program “PCR” in the thermocycler by selecting the “PCR” program from the list of programs by going to “START” and pressing “SEL” and cycling to “PCR” and pressing “SEL”.

We could have asked you to program the thermocycler - it is actually very easy to do. Instead we simplified and homogenized matters by programming one PCR program that will be used by everyone.

The program consists of:

- Heat solution to 94°C for 2 minutes to denature DNA
- 35 cycles of:
  1. 94°C for 15 seconds: Denaturing Step
  2. 55°C for 30 seconds: Annealing Step
  3. 72°C for 60 seconds: Extension Step
- Incubate at 72°C for 5 minutes
- Store at 4°C until removed from thermocycler.
5. Stop the program by pressing the “START/STOP” button and remove the tube from the thermocycler and place it in the -20°C freezer.

**Expectations**

You will need to run your PCR-product on an agarose gel to interpret the result of the reaction.

An example from the course textbook is provided below.

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**Figure:** An example of gel electrophoresis of a PCR for a given gene using a 1.5% agarose gel (UNIT 7.2). Primers were designed to flank a region known to have insertion and deletion mutations. Lane M has a DNA ladder loaded (various sized DNA bands are pointed out in the figure). Lane 1 is an example the normal band (680 bp). Lane 2 shows both the normal 680 bp product and the presence of a smaller 517 bp deletion product. Lane 3 shows both the 680 bp amplicon and the presence of a larger 1033 bp insertion product. Lane 4 (labeled NTC) is the no-template control reaction run with all of the reagents used in the PCR reaction (the remaining portion of the master mix) except the template. The absence of any amplification in the NTC demonstrates that there was no contaminating template in the reagents master mix that would account for the amplification seen in lanes 1 to 3.

---

**Required:** Affix (below) a fully labeled picture of your best gel showing the results of your PCR reactions. It is expected that your gel will have one lane demonstrating deletion of the gene of interest, one lane demonstrating the positive control, and one lane demonstrating the negative control. A ladder lane is also required (four lanes total).
Assignment Submission

Sign Off

When you believe you have the assignment completed ask a staff member (Dr. Killion, any Functional Genomics Research Stream Undergraduate Mentor) look over your work and sign below.

You should be able to present the staff member:

› Complete laboratory notebook with all tables called for in this assignment.

What to do with your materials:

› Discard your liquid cultures (correctly).
› Discard your gels (correctly).
› Place your plates in the 4°C refrigerator.
› Keep your buffers and reagents in appropriate racks.
› Store your DNA in the -20°C freezer (completely labeled).
› Store your RNA in the -80°C freezer in PAI 2.14 (functional genomics box).

Date: ______________________________________________

Time: ______________________________________________

Staff Name:  __________________________________

Staff Signature: __________________________________

Submission

This assignment is due on April 14, 2009 - 2:30 PM.

This printed document is the only component that is needed for final submission.

Please answer the following questions:

I completed this work as an individual:

Signature: _______________________________ Date: _______________________________