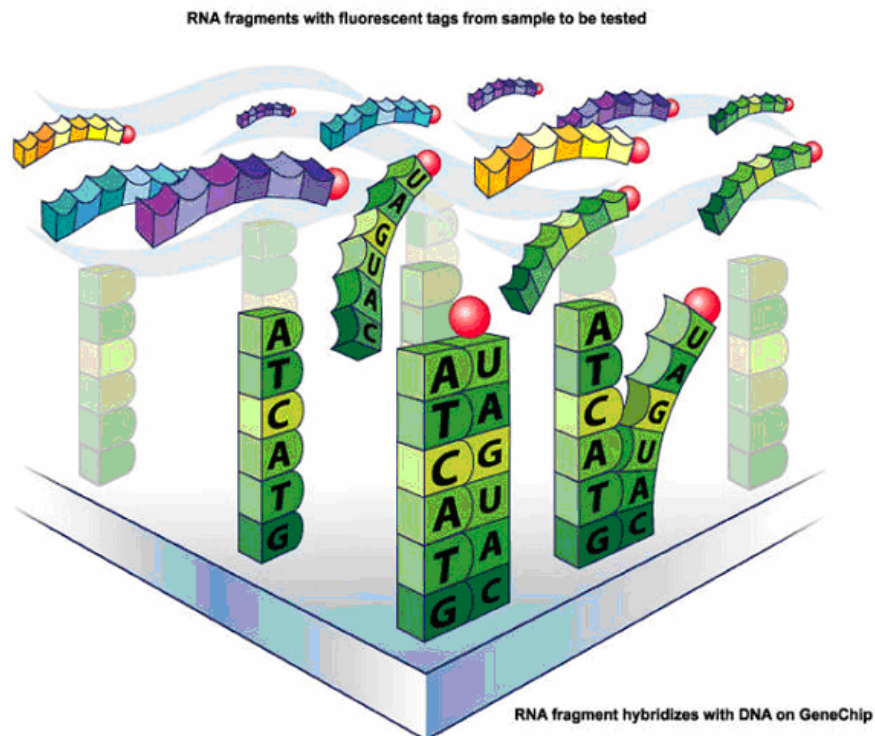


Microarray Technology Lab Manual



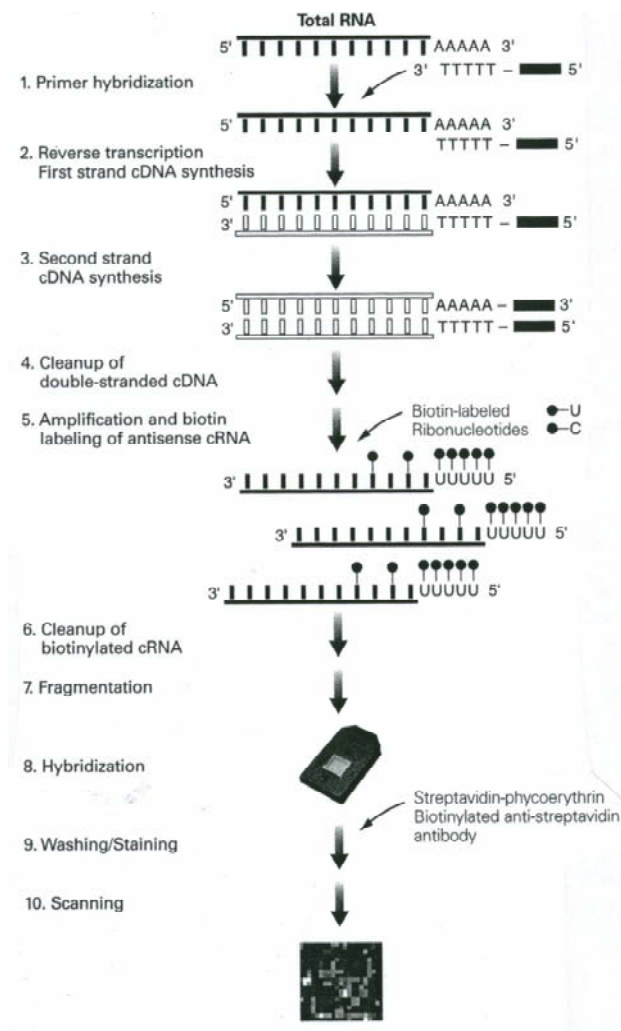
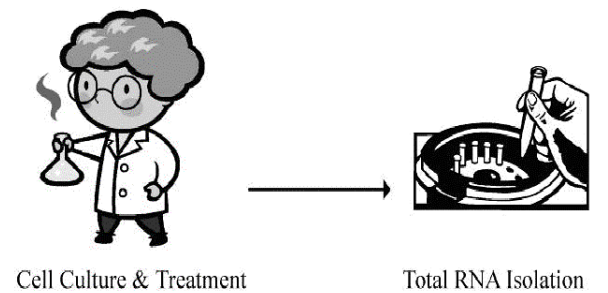
Instruction Team:

Ahmad Chaudhry, Scott Tighe, Tim Hunter, Pat Reed, and Anjie Watson

Lab Manual Ver.10-2005

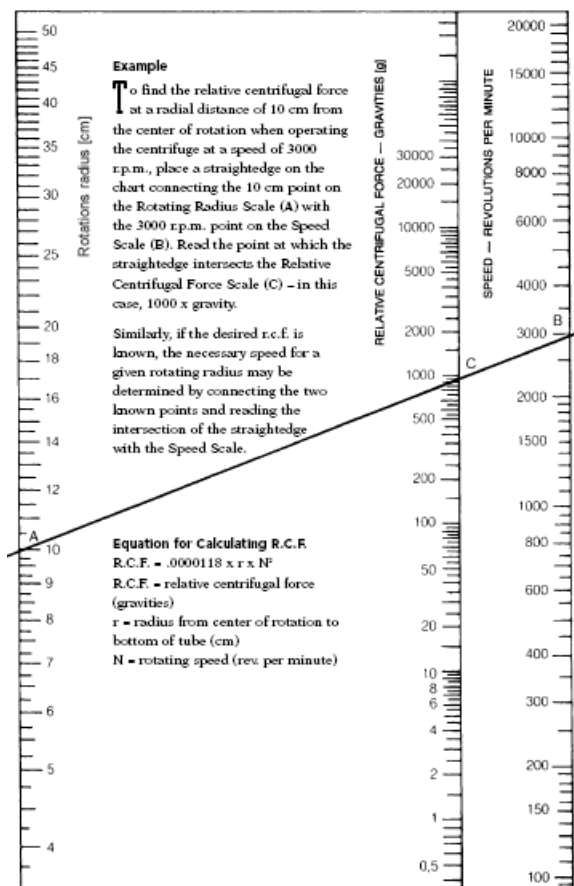
Overview

In this module, students will learn how gene expression in yeast (*Saccharomyces cerevisiae*) is changed after exposure of to dimethyl sulfoxide (DMSO), a common environmental contaminant and solvent. Control yeast are exposed to water and "treated" yeast are exposed to 10% DMSO for 2 hours. RNA is then extracted and prepared for use on Affymetrix® GeneChips. The final synthesis product is taken back to the UVM Microarray Core Facility and hybridized to the yeast GeneChips. The data is uploaded into special bioinformatic data analysis software where students are taken through complex data analysis procedures.



Special Notes- Important!

- 1] RNase-free water is DEPC water and the terms maybe used interchangeably throughout the protocol.
- 2] Record all data in notebooks.
- 3] Label the tops and sides of tubes with:
 Sample ID/Initials
 Date
 What is in the tube...ie cDNA, RNA, etc.
 Concentration
- 4] Check off boxes on the lab manual as you go.
- 5] Read Technical Discussion section before each day. This is fair game for quiz questions.
- 6] MSDS safety sheets are available for each chemical in the front of the room.
- 7] RPM on a centrifuge does NOT equal G-force. See the conversion chart below
- 8] Use RNase Zap anytime you want, better safe then sorry!



Microarray Data Sheet

Name: _____ Sample ID(used on tubes): _____ Date: _____

1) Total RNA data:

Spec conc: _____ 260/280: _____ Agilent Bioanalyzer Quality: _____

2) cDNA synthesis and clean-up:

Vol RNA used: _____ ug RNA used^[B] _____ Resuspension Volume _____ Visible Pellet _____

3) RNA InVitro transcription:

Volume of cDNA used in IVT reaction: _____

4) cRNA clean-up:

Final resus.vol^[C]: _____ Concentration: _____ Total amt RNA(ug): _____^[A]

Final Adj RNA concentration: _____ Formula= $A-B/C$

5) cRNA Fragmentation:

Amt Fragmented: _____ Conditions: _____

Attach Gel images and Spec data below

Set-up before day 1: Instructor

Note: The broth culture must be inoculated about 18-24 hours before the treatment procedure of day 1.

Note : The treatment procedure must occur 2 hours before class

Protocol for Preparing Yeast Cultures for Microarray Analysis **(24hr prior to day 1)**

Necessary Supplies

Autoclave (tape, tinfoil)

Inoculating loop (or sterile swabs) and Bunsen burner

Sterile flasks containing stir bars that are the same size: (1) 500 ml flask, (2) 125 ml flasks

(2) Stir plates

5 and 25 ml pipettes (sterile) Tape

and sharpies for labeling

Supplies provided by VGN

Yeast Stain *Saccharomyces cerevisiae* [NRRL Y-12632 or ATCC 18824]

YPD nutrient media (Difco powder form)

Dimethyl Sulfoxide (DMSO)

Sterile DI Water

Notes:

The broth culture must be inoculated 24 hours before the treatment procedure of day 1. Incubation should be conducted in a room that is 70-73°F.

Plan the treatment of the cultures so that the 2 hour treatment is complete 30 minutes AFTER the start of class on Day 1.

Please read through all instructions before proceeding.

A) Making YPD media and sterilizing [2] 125 ml flask with stir bars.

- ____ 1) Prepare 250 ml of half strength YPD broth in a 500ml flask (use 6.8gm/250ml). Place a stir bar in the flask, wrap tin foil loosely around the top, label the flask, and autoclave under standard conditions (15 psig at 121°C) for 15 minutes.

- ____ 2) Also autoclave (2) 125 ml flasks each containing the same size stir bar. Wrap top in foil.

It is important not to autoclave LONGER than 15 minutes as the sugars will caramelize and degrade with extended autoclaving.

B) Inoculating the parent culture

- ____ 1) Remember to use sterile technique throughout procedure. Bacterial contamination will be undetectable throughout the rest of the experiment and will adversely affect the final results.

- ____ 2) The parent culture should be inoculated 24 hrs before class (Day 1).

- ____ 3) Wearing gloves, touch the sterile end of the inoculating loop to a yeast colony on the plate. Your aim is to pick up a small amount. Alternatively, if a liquid culture is used, a 10 ul aliquot can be transferred to the new media using a P20 micropipet with a sterile aerosol resistant tip.

- ____ 4) Uncap and tilt the 500 ml flask at a 30 degree angle [or so] and inoculate the YPD broth with the loop (yeast).

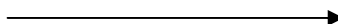
Remember that you want to minimize the time the flask is open AND you do not want your hands or sleeves over the flask opening. Broth is easily contaminated and it will not be possible to detect this until the very end of the experiment.

- ____5) Flame the mouth of the flask and place the tinfoil cap back on the flask. Replace the tinfoil around the mouth of the flask, but not too tightly as it is necessary for oxygen to get in. It is important that the yeast grow aerobically as respiration is the metabolic process that builds cell mass.

- ____6) Place flask on stir plate and stir approximately 24 hours at a medium speed at room temp (22-25°C). Do not have the speed so high that there is excessive splattering; some foaming is OK, but keep it minimized.

C) Treatment of yeast cultures (must be started 1.5 hr before class on day 1)

- ____1) Aseptically transfer 22.5 ml of the yeast broth culture into two (2) sterile 125 ml flasks each containing a stir bar using a sterile 25 ml pipette.



- ____2) To each flask, aseptically add 2.5 ml of sterile DI H₂O for the Control flask and 2.5ml Dimethyl sulfoxide (DMSO) for treated flask.

- ____3) Place each flask on separate stir plates and stir at room temperature for 2 hours at [as close as] the same speed as possible. Cultures should be ready for harvest 30 min after the start of the first class period. Therefore, DMSO is added 1.5 hours before the start of the class.

DAY 1

Isolating Total RNA From The Yeast, *Saccharomyces cerevisiae*, Using Enzymatic Lysis Protocol.

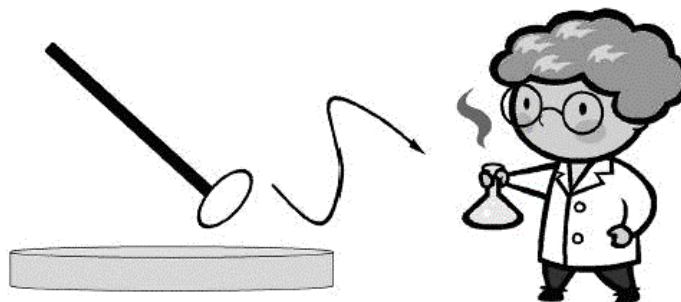
Technical Overview:

Today we will be treating the yeast *Saccharomyces cerevisiae* with dimethylsulfoxide [DMSO], an important by-product in several industrial processes including paper manufacturing. It will therefore be interesting to see what affect DMSO has on eukaryotic cells. To evaluate these effects, we have chosen yeast as the eukaryotic system. The DMSO treated yeast, and an untreated control will be lysed using a combined procedure employing a lyticase enzyme and a buffer containing guanidinium isothiocyanate after the 2-hour treatment procedure. The first step uses lyticase which degrades the poly- β -1,3 glucan bonds in the cell wall of fungi, resulting in the formation of spheroplasts (a fungal cell without a cell wall). The second step uses RLT buffer (guanidinium isothiocyanate) that ruptures the cell membrane thereby allowing recovery of total RNA on a common silica gel spin column manufactured by Qiagen.

Please read through all laboratory procedures prior to lab.

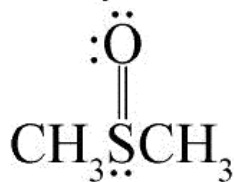
Day 1: DMSO Treatment & RNA Isolation

Last
Night

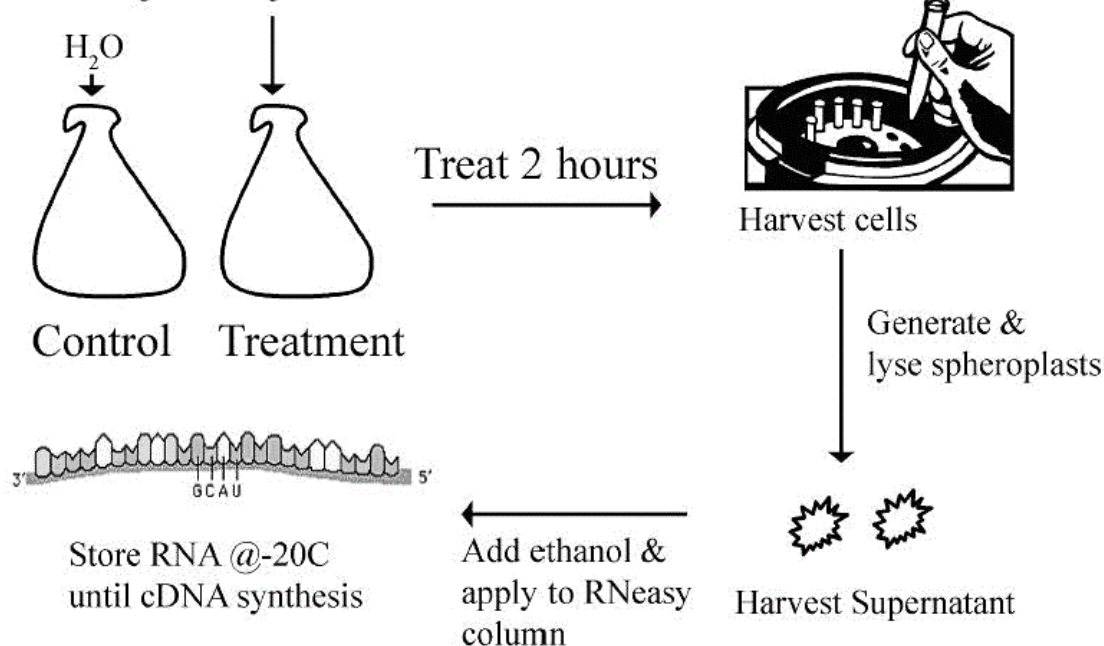


The yeast was aseptically inoculated to yeast extract peptone dextrose broth

Dimethyl Sulfoxide



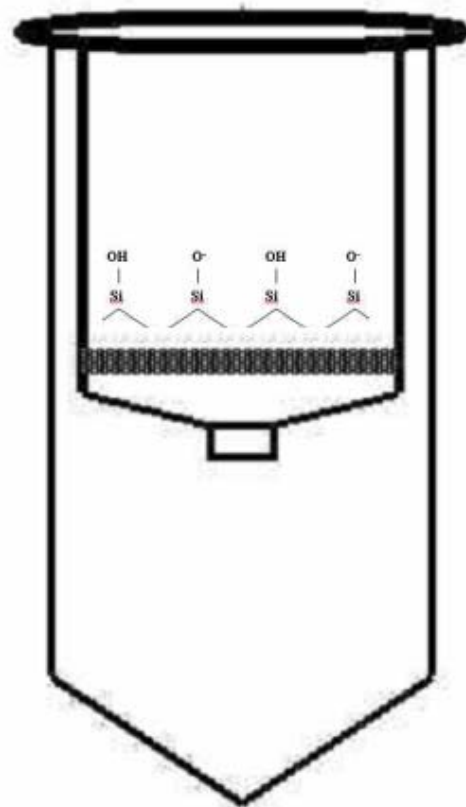
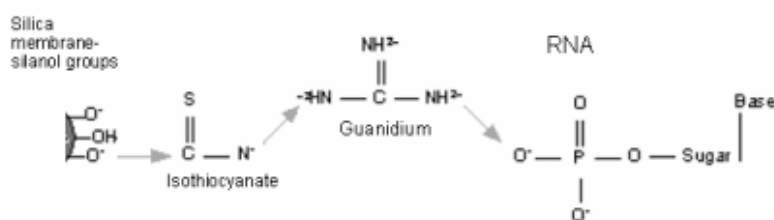
A highly polar organic liquid that is used widely as a chemical solvent. Because of its ability to penetrate biological membranes it is used as a vehicle for topical medications.



The Silica Column Chemistry

Guanidium isothiocyanate (GITC) is a chaotropic salt at about 5 M in the RLT buffer. This solution at a reduced pH (6.4) and used in conjunction with ethanol and water disrupts the molecular characteristics of water and forces the RNA to be less soluble. This promotes nucleic acids to electrostatically bind to the silica. A stoichiometric balance of GITC, ethanol, pH, and water controls the binding of either RNA or DNA to the silica. Therefore, changes in this stoichiometry will select a majority of either one or the other. This not a 100% selective procedure and DNase treatment is required. The use of ethanol in rinse RPE buffer maintains the bond between the silica and RNA. The recovery of RNA off the membrane is accomplished with water because RNA is very soluble in water and can no longer maintain an electrostatic bond with the silica once the water structure has been re-established.

The RNA is electrostatically bound to the silanol groups of the silica membrane using an isothiocyanate-guanidium salt bridge to the negatively charged phosphate backbone of the RNA.



Isolating total RNA from *Saccharomyces cerevisiae* using Enzymatic Lysis

Materials:

Axygen MCT150C 1.7ml tubes

P1000, P200, and P20 pipets

Yeast Lytic Enzyme-VWR # IC15352710-MP Biomedical 153527-10,000 units)

100 % Ethanol

DEPC water (aka RNase-free water)

Qiagen Mini RNeasy kit

Qiagen DNase Kit

Vortex

Microcentrifuge

Spectrophotometer

Eppendorf UVettes

2 ml microcentrifuge tubes

- ____ 1. Prepare an RNase-free work zone. This will be demonstrated by the instructor.

- ____ 2a. Prepare fresh lytic enzyme at 10 Units/ul by adding 1 ml of DEPC water directly to the lyticase vial. Vortex well and invert several times to insure complete mixing. This solution is stable for 12 hours.

10,000 U Lyticase
1 ml DEPC Water

Instructor will perform this task.

- ____ 2b. Prepare fresh DNase I solution using the Qiagen DNase kit and store on ice.

10 ul DNase I
70 ul Buffer RDD
80 ul per reaction

Instructor will perform this task.

- ____ 3. Spin down 1.5 ml of yeast culture at 4000x g [6800rpm] for 4 minutes using an Axygen 1.7 ml tube in a microcentrifuge at room temperature.

- ____ 4. Carefully remove supernatant (without disturbing pellet) using a P1000 micropipet, discard supernatant. **** **Repeat step 3. if indicated by instructor.******
- ____ 5. Add 1 ml of sterile DEPC water, vortex, and spin again at 4000 x g for 4 minutes, and then remove and discard supernatant. This time be sure to remove as much of the liquid as possible from the yeast pellet. You may need to use a smaller pipet to get any remaining liquid.
- ____ 6. Add the following to the yeast pellet and gently vortex:
- | | |
|----------------------------|--------|
| SG buffer | 100 ul |
| Lyticase solution (10U/ul) | 10 ul |
- ____ 7. Incubate for 30 min at room temp (22-25°C).
- ____ 8. Gently swirl to tube every 10 minutes to generate spheroplasts. Spheroplasts must be handled gently.
- ____ 9. Add 350 µl b-RLT buffer to lyse spheroplasts and vortex **vigorously** for 1 minute. Ensure your tube is tightly capped by holding it closed while vortexing.
- ____ 10. Add 250 µl of 100% ethanol to the tube and briefly vortex. **Do not centrifuge.** A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.
- ____ 11. Apply the entire sample to an RNeasy mini column. Close the tube gently and centrifuge for 15 sec at full speed.
- ____ 12. Discard the flow through tube and place the RNeasy column into a new 2 ml capture tube.

- ____13. Add 350 μ l RW1 buffer to the RNeasy column to wash the column. Close the tube gently and centrifuge for 15 sec at full speed.
- ____14. Apply 80 μ l of DNase solution to the middle of the Qiagen column membrane. Incubate at room temp for 15 min.
- ____15. Transfer RNeasy column to a new 2 ml capture tube.
- ____16. After the 15 minute incubation, add 350 μ l RW1 buffer to the RNeasy column and spin at full speed for 15 seconds.
- ____17. Discard the flow through tube and place the RNeasy column into a new 2 ml tube.
- ____18. Pipet 500 μ l RPE buffer onto the RNeasy column to wash the column. Close the tube gently and centrifuge for 15 seconds at full speed.
- ____19. Discard the flow through tube and place the RNeasy column into a new 2 ml tube.
- ____20. Add another 500 μ l RPE buffer to the RNeasy column. Close the tube gently and centrifuge for 15 sec at full speed.

- ____ 21. Place the RNeasy column in a new 2 ml collection tube and centrifuge in a microcentrifuge at full speed for 1 minute to “dry” the silica membrane.
- _____
- ____ 22. To recover the RNA, transfer the RNeasy column to a new 1.7 ml microcentrifuge tube. Pipet 30 ul of DEPC water **directly onto the very center** of the RNeasy silica-gel membrane **DO NOT TOUCH THE SILICA GEL MEMBRANE**. Look closely as you perform this step. Use both hands when pipeting, one on top and one down by the tip to guide the pipet. Make sure the water was evenly distributed on the membrane.
- _____
- ____ 23. Centrifuge in a microcentrifuge at full speed for 1 min.
- _____
- ____ 24. Carefully remove the 30 ul which is recovered in the 1.7 ml tube and pipet it back onto the center of the silica membrane of the same column. Place column back in to the same 1.7 ml tube and spin again for 1 minute at full speed. This double elution ensures that the entire membrane was extracted.
- _____
- ____ 25. Transfer the recovered RNA to a new 1.7 ml tube and label it with date and sample name. **Write clearly!!**
- _____
- ____ 26. Transfer 4 ul of RNA to a new tube for transport back to UVM for Nanodrop quantification and RNA assessment. Label this tube carefully with date, name, and RNA.
- _____

- ____27. Keep the samples on ice and quantify samples using the spectrophotometer at a 1 to 50 dilution (1ul sample + 49ul H₂O). This will be demonstrated by the instructor.

[Sample concentration:_____] [Write this on your tube]

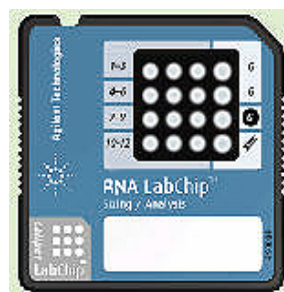
- ____28. Evaluate the RNA by agarose gel electrophoresis. This will be done as a class. Pre-run the gel for 2 minutes and turn off. Add 14 ul of loading dye to each well on the E-gel. Add 1ul of each sample to each well and pipet up and down to mix. Run the gel for 20 minutes.

- ____29. Samples should be labeled properly for return to UVM and analyzed using the Agilent Bioanalyzer 2100. This instrument is used to determine the condition of the RNA. Only fully intact RNA is eligible for microarray analysis. Why?

Store at -20C

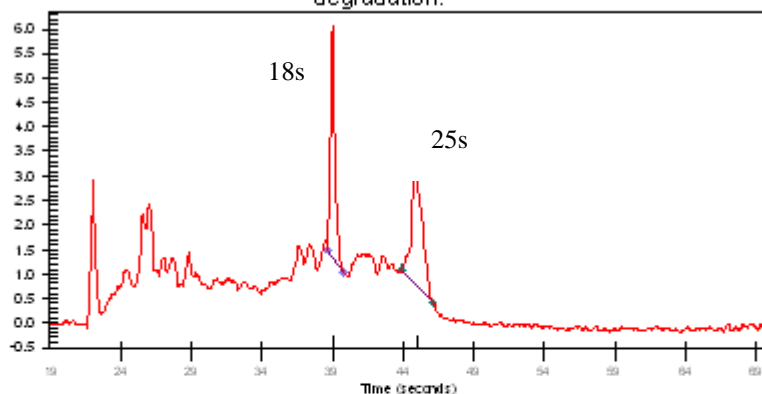
RNA Analysis using the Agilent 2100 Bioanalyzer

The Agilent bioanalyzer 2100 is an automated instrument that employs the use of Lab-on-a-Chip technology for analyzing RNA. This provides highly resolved data regarding the integrity of the RNA. The system is similar to an agarose gel where RNA is added to a well containing a gel and high-resolution electrophoresis is conducted through microfluidic channels. The use of an intercalating dye allows the RNA to be detected with an Nd-YAG diode-pumped laser.



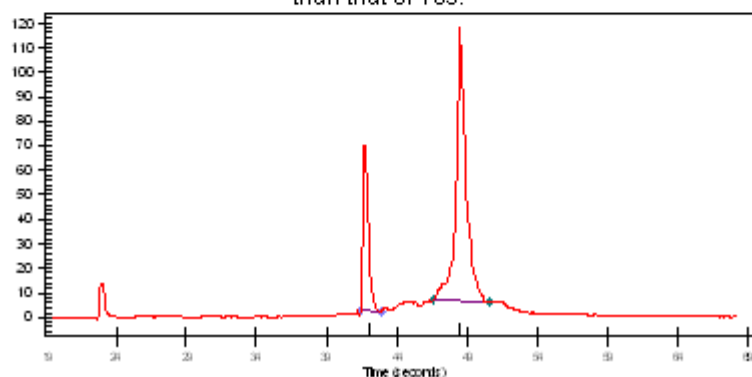
Moderately Degraded RNA

Elevated and "rough" baseline [dirty]. Significantly reduced 25s ribosomal peak indicating degradation.



Fully Intact RNA

Clean flat baseline and tall sharp peaks for 18s and 25s ribosomal RNA. 25s equal to or higher than that of 18s.



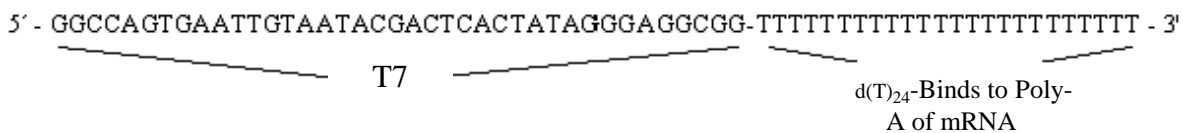
DAY 2

Microarray Target Preparation- cDNA synthesis

Technical Overview:

Today you will be starting to prepare the target for the genechip analysis. This is a lengthy procedure and will be broken down over several lab periods. In short, you will be starting with mRNA, which comprises 0.5-2% of the total RNA in typical eukaryotic cells. First, you will generate the first complementary DNA (cDNA) strand from the mRNA. This is called the first strand cDNA synthesis. This step utilizes a primer with a sequence of 24 T's in a row and is called oligo d(T), which binds to the poly A tail of eukaryotic mRNA. The primer also contains the consensus-binding site for T7 RNA polymerase (T7 promoter sequence). Once the primers have been hybridized to the poly-A mRNA, reverse transcriptase is added and the first strand of cDNA is synthesized. It is important to note that the 5s, 5.8s, 18s, and 25s rRNA do not have a poly-A tail and will not be primed or synthesized in this procedure.

The sequence of T7 Oligo d(T)₂₄ is:



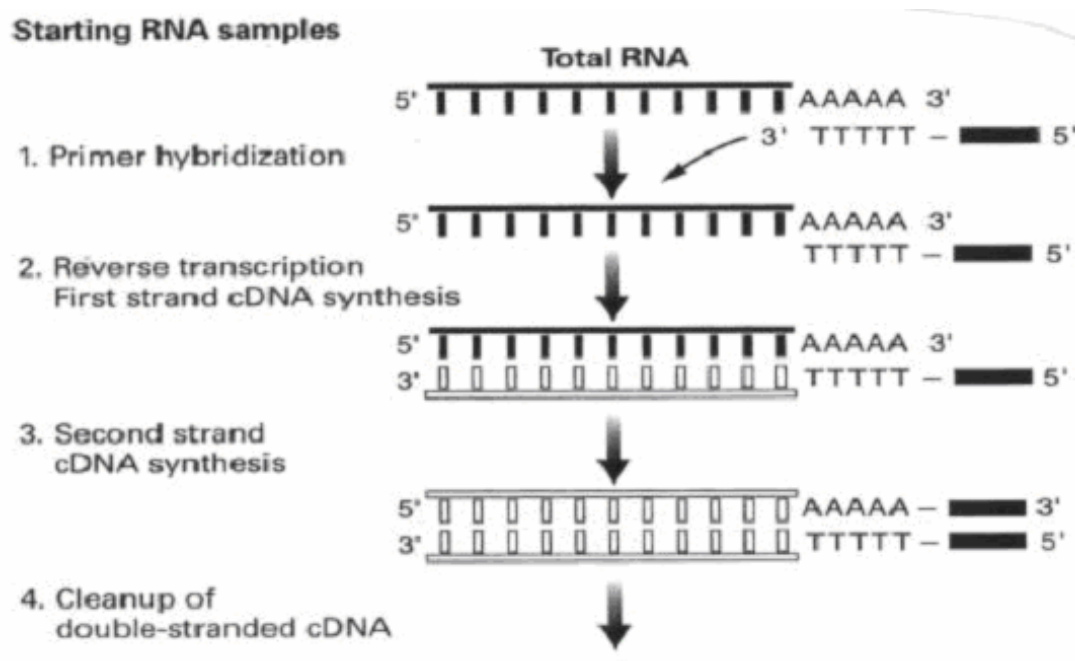
The next step in the cDNA reaction is the synthesis of the second cDNA strand from the first cDNA strand. This is done by adding RNase H, DNA polymerase I, and DNA ligase. The RNase H causes many strand nicks in the phosphodiester bonds of the bound mRNA strand creating the availability of 3'OH groups. DNA polymerase uses these 3' OH groups as a priming site; thus synthesizing another cDNA strand until it reaches

another nicked site. The DNA polymerase can only displace RNA, not DNA. After cDNA has been synthesized from all the nicks, the areas between the nicks need to be filled in or ligated. This is accomplished using DNA ligase. After the two hour synthesis process, all the RNA has been replaced with cDNA and ligated to form one large cDNA strand. However, because the DNA polymerase does not synthesize completely to the end of the strand, a T4 DNA polymerase is added. This ensures the synthesis of the T7 promoter site from the original T7 oligo d(T) primer used in the first synthesis reaction.

Synthesis of cDNA:

The starting material for your target preparation will be high quality, fully intact, total RNA from yeast. The minimum concentration to start with is 100-270 ng/ul. After isolation and purification of the yeast RNA, the concentration is determined by absorbance at 260 nm on a spectrophotometer (1 O.D.= 40 µg/mL RNA). The A260/A280 ratio should be approximately 1.8-2.1. We are checking the quality of the RNA by running an aliquot on the Agilent Bioanalyzer 2100 (performed prior to class at UVM core) and running on an agarose gel (EGEL) prior to starting the assay. More appropriately, a denaturing formaldehyde gel would be used, but we will not have time to perform this.

Overview of cDNA Synthesis Reactions



DAY 2

Microarray Target Preparation- cDNA synthesis

First Strand cDNA Synthesis

Note: Spin all reagent tubes before starting as some have only 1 ul in them!!!!

- ____ 1. Adjust 1-3 ug (determined by instructor) of RNA to 11ul with DEPC water in a RNase-free 0.5ml tube. Keep the tube on ice.

- ____ 2. **Primer Hybridization:** Combine the following reagents, vortex, and spin in microcentrifuge [full speed] for 5 second and put in a thermocycler at 70°C for 10 minutes.

T7 oligo d(T)24	1ul	
RNA(1-3 ug)	11ul	→Incubate 70°C 10 min. then
		place on ice.

- ____ 3. Add the following reagents **IN ORDER** to the above tube, vortex, and spin in microcentrifuge [full speed] for 5 second and put in a thermocycler at 50°C for 60 minutes.

First Strand Buffer 5X	4 ul	
0.1M DTT	2 ul	
10mM dNTP	1 ul	
Superscript III	1ul	→ Incubate 50°C for 60 minutes

- ____ 4. After the 60 minute incubation, place on ice.

Second Strand cDNA Synthesis

- ____ 1. Make the following master mix in a separate tube. Keep it on ice.

DEPC Water	91 ul
5x Second Strand Buffer	30 ul
dNTP(10mM)	3 ul
<i>E.coli</i> DNA Ligase (10U/ul)	1 ul
<i>E.coli</i> DNA Polymerase I (10U/ul)	4 ul
<u><i>E.coli</i> RNase H (2U/ul)</u>	<u>1 ul</u>
Total volume added to First Strand tube	130 ul

Need a P2 micropipet for this step
--

- ____ 2. Vortex master mix and perform a 5 second quick spin in the microcentrifuge [full speed].

- ____ 3. Add this 130 ul second strand master mix to first strand tube. Vortex and perform a 5 second quick spin. Incubate for 2 hours at 16°C in thermal cycler.

The next two steps may be performed by the instructor

- ____ 4. At the end of 2 hours and while the sample is still at 16°C, add 2ul of T4 DNA polymerase and incubate for exactly 5 minutes. Mix well. Do not incubate longer then 5 minutes or the quality of the cDNA may decrease due to the 3' to 5' exonuclease activity of the polymerase.

- ____ 5. At the end of the 5 minute incubation, add 10 ul of 0.5 M EDTA to stop the reaction. Store sample at -20°C.

Day 3

Cleaning the cDNA and setting up the IVT

Technical Overview:

Cleaning the cDNA:

After synthesis of the cDNA, it will need to be purified before proceeding to the next step. This will be accomplished using a solution of phenol-chloroform-isoamyl alcohol (PCI) at a specific pH. The underlying principle is that both phenol and chloroform cause proteins/enzymes to become denatured and subsequently soluble in the organic phase, while the cDNA will remain in the aqueous phase. This is performed by mixing the cDNA sample with PCI and transferring the full volume to a phase lock gel tube. The phase lock gel tube is centrifuged allowing separation of the aqueous and organic phases with a gel barrier. This allows for easy recovery of the cDNA in the aqueous phase. It is important to note that the pH of the PCI is important because this will determine which layer the cDNA will be retained in (the aqueous or organic phase). To ensure the cDNA is in the aqueous phase, the pH of the PCI needs to be basic, which is accomplished through a layer of TRIS pH 8.3 over the PCI solution.

The aqueous phase is further purified through a precipitation step using ethanol [an alcohol] and ammonium acetate [a salt]. Because DNA is negatively charged, salt is used to mask the charge, causing it to precipitate. The ethanol is used in conjunction with the salt because it is non-polar and DNA will not readily dissolve in it. This reaction is sometimes done at -20°C to encourage maximum DNA precipitation. After the precipitation, the cDNA is centrifuged to a pellet with a visualization agent called Pellet Paint, an additive that contains glycogen (a co-precipitate) and a dye. This helps form a high quality visible pellet on the bottom of the tube. The pellet is then washed several times with 80% ethanol, dried, and resuspended in DEPC water.

The In vitro Transcription (IVT):

The IVT step uses the T7 promoter site that is attached to the T7 Oligo d(T)₂₄ primer used in the first strand cDNA synthesis. A T7 RNA polymerase binds to this site and copies the opposing cDNA strand, synthesizing an antisense cRNA using the two standard nucleotides, A and G and two modified nucleotides; biotinylated uracil and cytosine. This generates a complementary cRNA [antisense] strand to the original mRNA with biotinylated nucleotides. These biotinylated nucleotides will be needed in the next step for staining of the RNA using streptavidin phycoerytherin.

Safety Considerations for Phenol Chloroform Isoamyl:

Note: Phenol Chloroform Isoamyl Alcohol is at a ratio of 25:24:1 and saturated with 10 mM Tris-HCl pH 8.3. Phenol is an organic acid and care must be used when handling. Phenol can burn skin and be absorbed into the circulatory system and cause health problems. Chloroform is an organic solvent and should be used in a hood.

■ **Phenol**

- Corrosive
- Local anesthetic
- Can severely damage eyes and skin
- Can cause severe burns of the skin and eye
- Poisonous if ingested-can be fatal

■ **Chloroform**

- Can cause irreversible corneal injury
- Fatal if swallowed
- Flammable

■ **Isoamyl Alcohol**

- Flammable
- Poisonous

Day 3

Precipitating the cDNA

- ____ 1. Centrifuge a Phase Lock Gel tube at full speed for one minute. **DO NOT VORTEX.**

- ____ 2. Add 162 ul of the **bottom layer** from the Phenol Chloroform Isoamyl Alcohol (PCI) to the cDNA synthesis reaction and vortex for 2 seconds. Hold cap tightly as leaking can occur during the vortex.

Note: PCI tends to leak out of the pipet so work quickly.

- ____ 3. Transfer the cDNA-PCI mixture to the phase lock gel tube. **DO NOT VORTEX** the phase lock gel tube.

- ____ 4. Centrifuge at full speed for 2 minutes.

- ____ 5. Transfer the top layer to an 1.7 ml microcentrifuge tube.

- ____ 6. Add the following to the 1.7 ml microcentrifuge tube and vortex.

Ethanol (100%)	405 ul
NH ₄ OAc (7.5M)	80 ul
Pellet Paint	1 ul

____7. Centrifuge at 12000 x g [full speed] for 20 minutes at room temp.

____8. GENTLY remove the tube from the centrifuge being careful not to disturb the cDNA pellet. The pellet should be pink and approximately the size of a grain of salt. Put on ice and immediately proceed to next step. Keep your tube on ice.

Is there a pellet? _____

****At this point a tiny pink visible pellet should be present on the bottom of the tube. If not, see the instructor.****



Cleaning the cDNA Pellet:

- ____ 1. Using a micropipette, carefully remove the liquid from the tube being very careful not to disturb the pellet. Tip the tube to enable removal of as much liquid as possible. Remember that the pellet is your sample!

- ____ 2. Add 500ul of ice cold [-20C] 80% ethanol to the tube with the pellet. Gently cap tube and invert slowly several times. Watch your pellet very closely. Place tube back in rack and let the pellet settle to the bottom of the tube for a minute. Alternately, you may centrifuge the tube at full speed for 15 seconds to get the pellet back down to the bottom of the tube. [See video on website for this technique]

- ____ 3. Using a 1 ml micropipette, carefully remove the ethanol being very careful not to disturb the pellet. Tip the tube to enable removal of as much liquid as possible.

- ____ 4. Repeat steps 2 and 3 with a new aliquot of 80% ethanol.

- ____ 5. Finally, remove all of the ethanol. Centrifuge the tube again [full speed] for about 5 seconds and using a smaller pipet such as a P20 or P200, remove the last few microliters. The goal is to remove as much ethanol as possible without disturbing you pelleted cDNA.

- ____ 6. Place the tube with the pellet in a drying box for 10 minutes to evaporate all of the ethanol. The dried pellet is easily lost once it is dry. Be **very** careful to handle the tube gently. Close the cap gently. Visualize the dried pellet to confirm it is present in the tube.

- ____ 7. Resuspend the pellet in 22 ul of DEPC water and put on ice.

In Vitro Transcription (IVT): Synthesis of biotin labeled cRNA from cDNA using the Enzo Kit

- ____ 1. Using the ENZO bioarray kit, a master mix for the entire class will be prepared as follows. The instructor will prepare this mix or designate someone from the class. This must be done in an RNase-free area free from air currents.

	<u>Amt/sample</u>	<u>#Samples</u>	<u>Total</u>
Reagent 1 [10x Reaction buffer]	4 ul		
Reagent 2 [10x Biotin nucleotides]	4 ul		
Reagent 3 [10x DTT]	4 ul		
Reagent 4 [10x RNase Inhibitor]	4 ul		
Reagent 5 [20x T7 RNA polymerase]	2 ul	_____	_____
Total Volume	18 ul		

Instructor will perform this task.

NOTE: Be sure to make extra for this step. Add enough for 1 more reaction

- ____ 2. Combine the following in a 0.5 ml microcentrifuge tube and pipet up and down several times to mix. Spin in centrifuge full speed for 5 seconds.

Clean cDNA	22 ul
<u>Enzo master mix [from above]</u>	<u>18 ul</u>
Total Volume	40 ul

- ____ 3. Incubate the above mixture at 37°C for 16 hours in the thermocycler. Store the sample at -20 C after the 16 hour incubation is complete (to be done by instructor).
- _____
- _____
- _____

Day 4

Cleaning and fragmenting the biotinylated cRNA

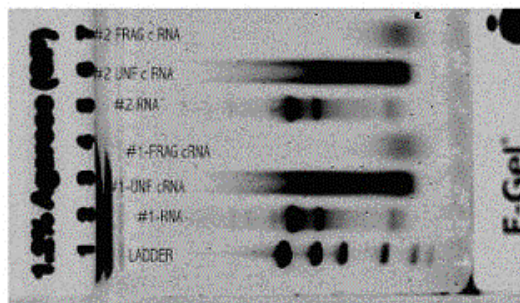
Using the Qiagen RNeasy system, the biotinylated cRNA must be purified. The cRNA needs to be free of enzymes, dNTP's, reducing agents such as DTT [dithiothreitol] and resuspended in DEPC water. The clean cRNA can then only be accurately quantified. Remember that enzymes and dNTP's also have an absorbance at 260 and/or 280nm that will affect the quantitation of the molecule of interest. That is why [for instance] you cannot quantify a raw PCR reaction before and after thermocycling, because all components are still there, just assembled differently.

The cleaning is performed by adding beta-mercaptoethanol, ethanol, and a buffer to the cRNA mix to reduce the proteins. These reagents aid in the solubilization of proteins and prepares the cRNA for binding to a silica-based spin column (pink basket of the RNeasy kit). The cRNA is washed and eluted using DEPC water. The cRNA is then quantified using a spectrophotometer at 260nm for nucleic acids and 280nm for proteins. A 260/280nm ratio of 1.8-2.1 indicates a clean cRNA sample that can be used for subsequent steps. 260/280 ratios of less than 1.8 indicate that the cRNA is contaminated with too much protein and should be purified again.

cRNA Fragmentation:

10ug adj cRNA + fragmentation buffer $\xrightarrow[30']{94C}$ fragmented cRNA

Run fragmented cRNA on precast E-Gel to assess fragmentation procedure and cRNA quality. The quality of cRNA is essential to obtaining valid results.



Day 4

Cleaning the biotinylated cRNA using the Qiagen RNeasy Kit

- ____ 1. Transfer the cRNA sample to a 1.7 ml microcentrifuge tube and add 60 μ l of DEPC water and 350 μ l BME-RLT and vortex.
- ____ 2. Add 250 μ l ethanol (96–100%) and lightly vortex again.
- ____ 3. Apply the sample (700 μ l) to an RNeasy mini column placed in a 2 ml collection tube (supplied). Close the tube gently and centrifuge for 15 s at full speed.
- ____ 4. Remove the pass-through liquid from the tube and reapply it to the same RNeasy mini column again. Close the tube gently and centrifuge for 15 s full speed. Transfer the RNeasy column into a new 2 ml collection tube and discard the flow-through and collection tube.
- ____ 5. Pipet 500 μ l of RPE buffer onto the RNeasy column. Close the tube gently, and centrifuge for 15 s at full speed to wash the column. Transfer the RNeasy column into a new 2 ml collection tube. Discard the flow-through and collection tube.
- ____ 6. Add another 500 μ l RPE buffer to the RNeasy column. Close the tube gently and centrifuge for 15 s at full speed to dry the RNeasy silica-gel membrane.
- ____ 7. Transfer the RNeasy column into a new 2 ml collection tube and centrifuge in a microcentrifuge at full speed for 1 min.

- ____ 8. Transfer the RNeasy column into a new RNase-free 1.7 ml microcentrifuge tube.
- _____
- _____
- _____
- ____ 9. To recover the RNA from the membrane, pipet 30 μ l of DEPC water onto the RNeasy silica-gel membrane and wait 1 minute. Make sure the DEPC water contacts the entire membrane. Do not touch the membrane. Close the tube and centrifuge for 1 min at full speed to elute.
- _____
- _____
- _____
- ____ 10. Remove the eluted RNA and apply it to the membrane again. Close the tube gently and centrifuge for 1 min at full speed to elute. This double elution results in a slightly higher cRNA yield.
- _____
- _____
- _____
- ____ 11. Transfer the cRNA to a new microcentrifuge tube. This is because the lid of the tube that was used is now contaminated. Why?
- _____
- _____
- _____
- ____ 12. Quantify samples using the spectrophotometer. Add 49 μ l of water to a Uvette and add exactly 1 μ l of sample. (1 μ l sample + 49 μ l H₂O). This will be demonstrated by the instructor.

[Sample concentration:_____]

Calculating the Adjusted cRNA Concentration

Calculation of the adjusted cRNA concentration must be done in order to subtract out the original untranscribed RNA [i.e. tRNA, 5s, 18s, and 25s rRNA]. The cRNA is fragmented using a metal-induced hydrolysis reaction to create cRNA fragments of 35-250 bp to be used in hybridization to the yeast GeneChip. Both the unfragmented and fragmented cRNA are visualized on an agarose gel. This is important because we need to ensure that the unfragmented cRNA is not degraded from contamination during the handling steps leading up to this point.

____13. Calculate the Adjusted cRNA concentration using the following formula:

$$ADJ\ cRNA = \{Amount\ of\ clean\ cRNA\} - \{Total\ amt\ RNA\ used\ at\ start\}$$

Example: 52.9ug – 2ug = 50.9ug or 50,900ng

A) What is the concentration of your cRNA sample per ul: _____ ng/ul

B) How many uL do you have: _____ uL

C) How many ug of total RNA did you recover on day 1: _____ ug

D) How many ng did you recover on day 1 after converting ug to ng _____ ng

$$\frac{[A \times B] - D}{B} = \text{adjusted cRNA concentration}$$

Fragmenting the cRNA for Target Preparation

Affymetrix recommends that the cRNA used in the fragmentation procedure be sufficiently concentrated to maintain a small volume during the procedure. This will minimize the amount of magnesium in the final hybridization cocktail. The cRNA must be at a minimum concentration of 600 ng/μL. The fragmentation buffer contains a basic solution of TRIS acetate, magnesium acetate, and potassium acetate which causes a metal induced RNA hydrolysis at high temperature.

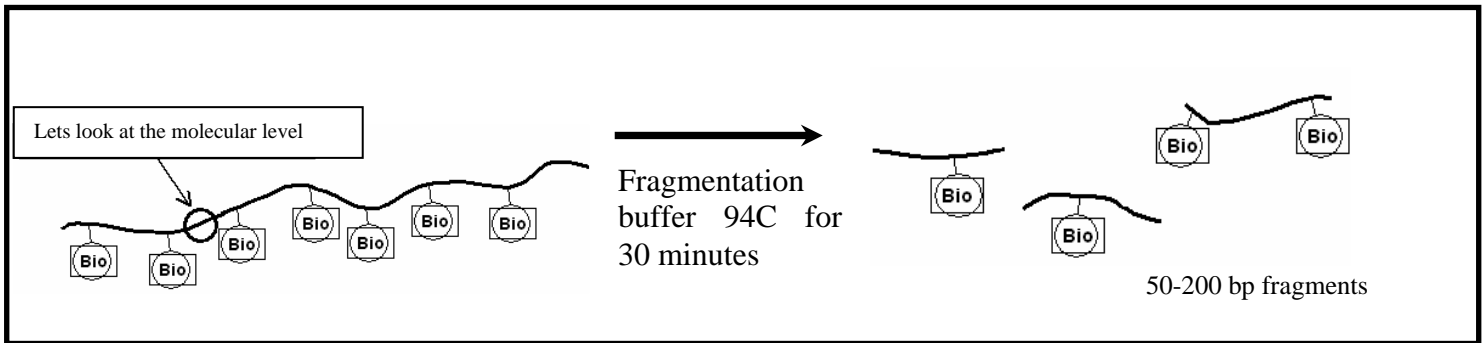
____ 1. Setup the following fragmentation reaction in a 0.5 ml centrifuge tube.

Adj cRNA concentration: _____ **ng/ul** **10ug=**_____ **ul**

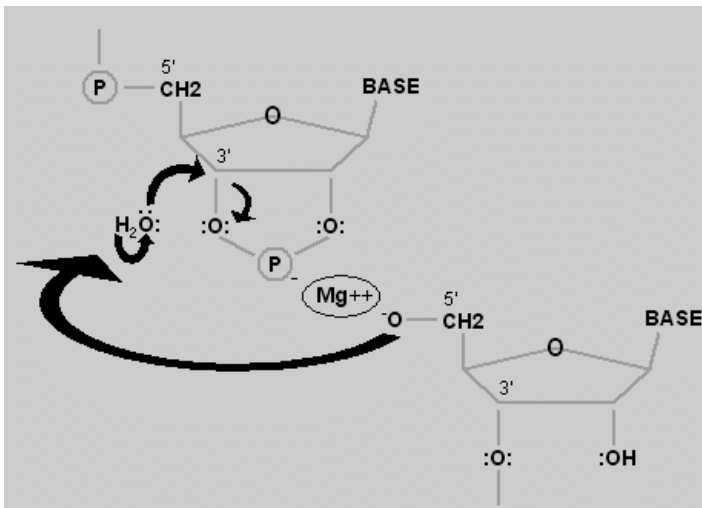
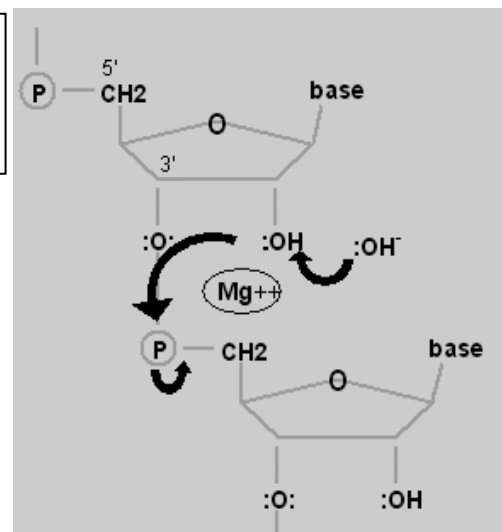
	<u>Volume</u>	<u>Volume (ul)</u>
10 ug of adj cRNA	1-16 ul	
5x fragmentation buffer	4 ul	
DEPC water	(as needed to make the final volume 20 ul)	
Total reaction volume	20 ul	20 ul

____ 2. Vortex and centrifuge briefly. Incubate at 94°C for 30 minutes in a thermocycler. Put on ice following the incubation.

Fragmentation: Hammerhead Cleavage

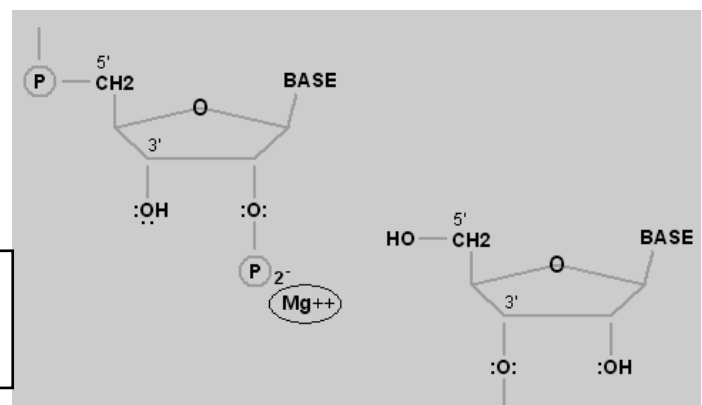


This is a heat-catalyzed hydrolysis reaction, mediated by magnesium ion



The intermediate product is a 2', 3' cyclic nucleotide. The next step is hydrolysis at the 3' carbon.

The cyclic nucleotide is now a 2'-nucleotide. Hydrolysis at the 2' carbon produces the 3'-nucleotide.

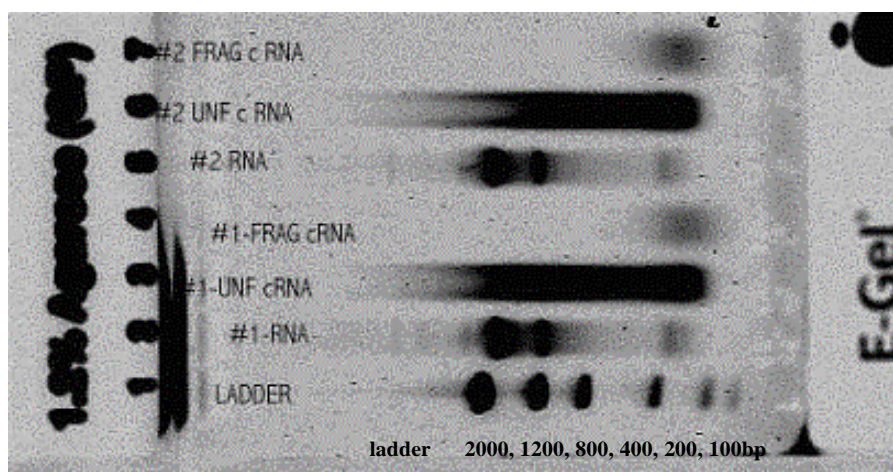


Evaluating The Samples on a Standard Agarose Gel

This procedure will evaluate your unfragmented and fragmented cRNA. Samples will be loaded on a precast E-GEL and allowed to run for 20 minutes.

- ____ 1. Remove a 2% double comb E-Gel from pouch and place on E-Gel base. Pre-run the gel for 2 minutes by pressing the button continuously for 10 seconds. You will see the light start to blink indicating it is in a pre-run mode. It will beep and stop after 2 minutes.
- ____ 2. Add 14 ul of loading dye to each well on the E-gel.
- ____ 3. Add 1ul of each sample to each well and pipet up and down to mix well. Run both the fragmented and unfragmented cRNA.
- ____ 4. Load 2 ul of Ladder
- ____ 5. Run the gel for 20 minutes.
- ____ 6. Visualize the E-gel on a transilluminator. Take pictures.

Note: The fragmented sample should be a smeared band at the bottom of the gel indicating fragments of RNA between 35 and 250bp. The unfragmented sample should be a smear from the top to the bottom of the gel. The total RNA [if you run them] should be two bands at the middle of the gel indicating the 25s and 18s ribosomal RNA units. See picture below.



Notes:

To Be Performed at the University

These steps will be performed at the University of Vermont Microarray Core Lab and will be show as a powerpoint presentation.

Preparing the Hybridization Mix

The hybridization mixture contains several internal controls that provide information about the success of the hybridization procedure. These controls are the B2 oligo for which there are several hundred probes along the outer edge of all expression arrays and a checkerboard pattern in each corner. These predefined patterns provide signals for the Affymetrix® Microarray Suite software to perform automatic grid alignment during image analysis. They can also be used to align the grid manually. The fluorescence intensities for control oligo B2 are not used for analyzing data. The bioB, bioC, and bioD are biotinylated gene fragments much like your sample which represent the biotin synthesis pathway from the bacteria *E. coli*. The cre control is the recombinase gene from P1 bacteriophage. These biological controls are prepared at standardized concentrations and signal intensities obtained on these genes provide information on how well the hybridization, washing, and staining procedures have performed.

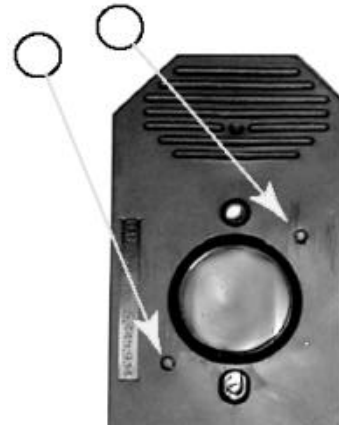
____1. Prepare the following:

Fragmented cRNA	[10ug]	?
Control B2 Oligo		3.3 ul
20x Eukaryotic Control mix [bio B, bio C, bio D, Cre]		10 ul
Herring Sperm DNA [10mg/ml]		2 ul
Acetylated BSA [50mg/ml]		2 ul
2x Hybridization Buffer		100 ul
Water	[QS to 200ul]	____

____2. After preparing the hybridization mixture, store the mixture at -20°C.

Hybridization to the Yeast 2.0 GeneChip

After the hybridization mixture has been made, it is denatured at 94C for 5min and injected in to the genechip through the injection ports on the back of the chip [see diagram below]. The genechip is then placed in a hybridization oven and the biotinylated target that you prepared is allowed to bind [hybridize] to the sequence specific probes on the array.

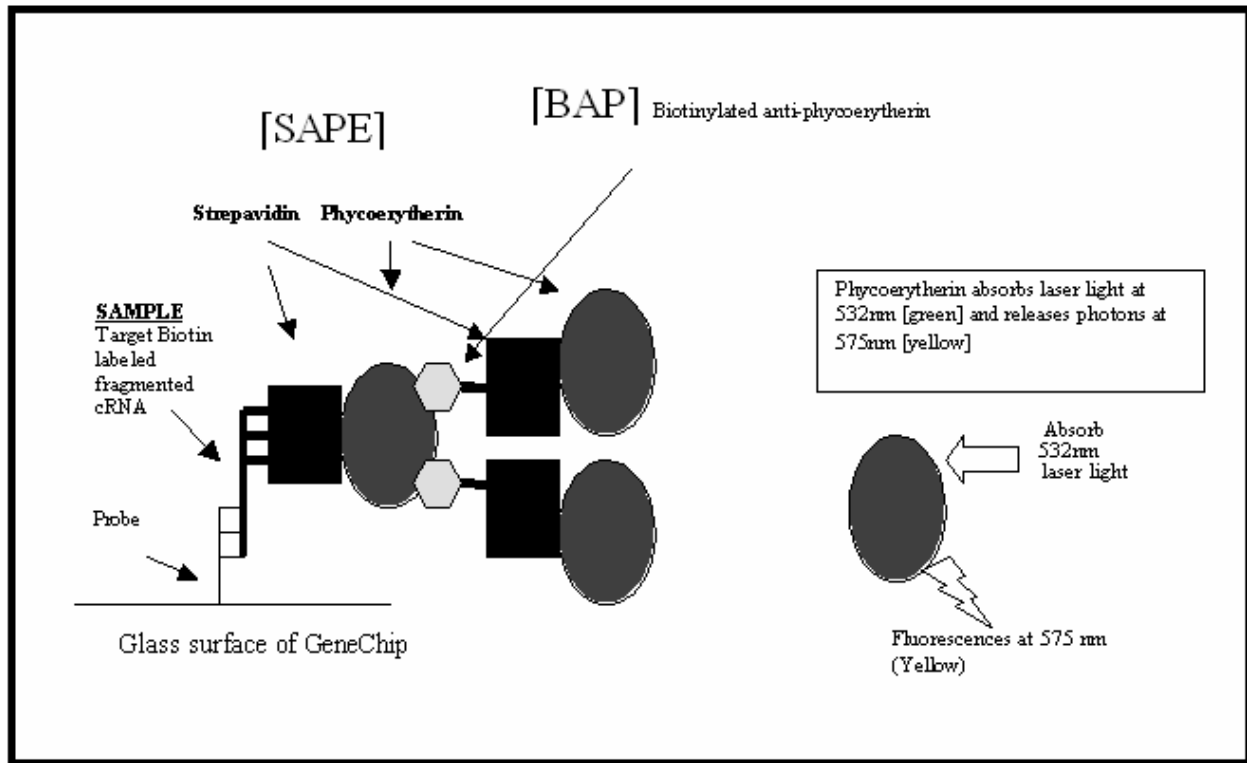


Staining the Yeast 2.0 Genechip

The staining procedure involves using a fluidic station designed to accommodate GeneChips. Two solutions are prepared; a strepavidin- phycoerytherin dye (SAPE) and a biotinylated anti-phycoerytherin antibody (BAP). Before placing the GeneChip into the fluidic station, the fragmented cRNA hybridization mixture is manually removed and replaced with buffer.

The chip is placed in the fluidics station and an automatic process of washing and staining occurs for about 2 hours. The procedure starts by first staining with SAPE. The SAPE will bind the biotinylated cRNA that is hybridized to the probes on the chip. It is washed and then stained again with BAP. BAP will bind to the SAPE. The chip is washed again and another SAPE staining is carried out to bind to the BAP. The double staining procedure is employed to amplify the signal intensities. This allows for the detection of low transcript mRNAs that might otherwise not be detected. After staining, the sample is washed and ready for scanning.

A schematic of the staining chemistry



Scanning the Yeast 2.0 Yeast GeneChip

The GeneChip is scanned after the wash and staining protocols are complete. The chip is placed in the high resolution GS3000 scanner which is equipped with a diode-pumped, frequency-doubled Nd-YAG laser (532 nm) and the 3.5 μ m beam is scanned back and forth across the glass surface at a pixel resolution of approximately 2.5 μ m. Fluorescence is detected using a high sensitivity photomultiplier tube [PMT]. This enables high resolution detection of fluorescence from the probe set feature [pixel] which is only 11 μ m square. There are approximately 1 million probe areas on each chip!



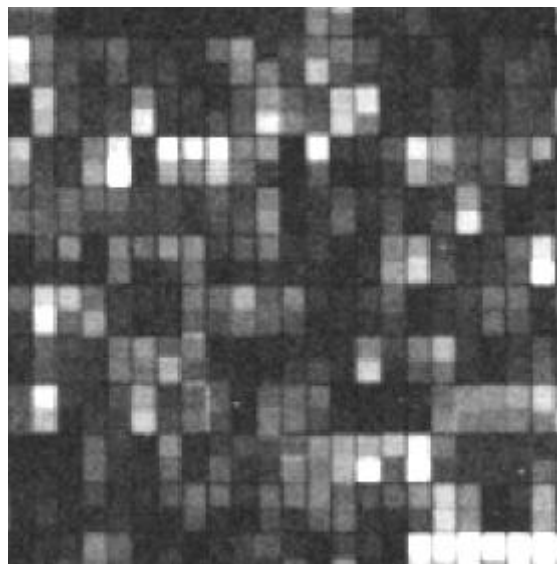
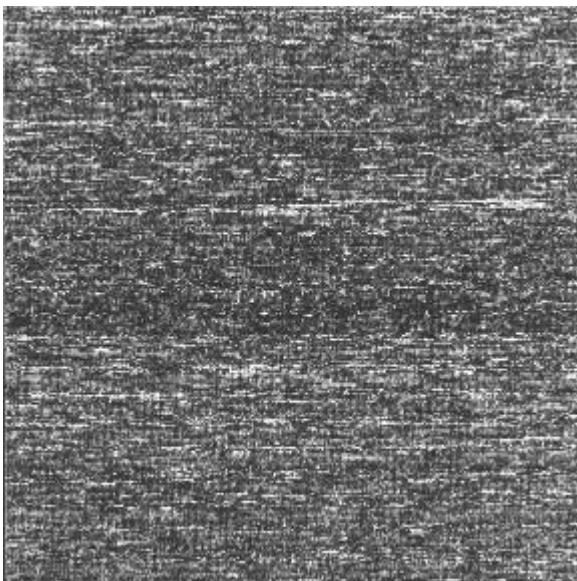
GeneChip



The Affymetrix GeneChip System

Images from the GeneChip scanner

The image on the left is all 1,000,000 probes. The image to the right is zoomed up 20,000 times to show approximately 500 probes. White indicates strong signal [high level of a mRNA in original sample], while dim or no signal indicate little to no mRNA in original sample.



Bioinformatics

After collecting and analyzing the image data, the results are archived and burned on to a CD. Because the data files are so large, it is necessary to analyze it using special microarray analysis software. This software can handle tens of thousands of data points at one time and data analysis can be time consuming. Therefore, it is critical that the data meet specific quality control specifications before performing this extensive analysis.

Notes:

Example Expression Report From MAS 5.0

Date: 04:46PM 01/16/2004
 Filename: UVM -test-Yeast.CHP
 Probe Array Type: Yeast S98
 Algorithm: Statistical
 Probe Pair Thr: 8
 Controls: Antisense

Alpha1: 0.04
 Alpha2: 0.06
 Tau: 0.015
 Noise (RawQ): 2.970
 Scale Factor (SF): 1.367
 TGT Value: 150
 Norm Factor (NF): 1.000

Background:	Avg: 84.82	Std: 2.37	Min: 77.40	Max: 90.40
Noise:	Avg: 3.16	Std: 0.15	Min: 2.70	Max: 3.80
Corner+	Avg: 163	Count: 32		
Corner-	Avg: 11225	Count: 32		
Central-	Avg: 6612	Count: 9		

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets:	14010	
Number Present:	5227	37.3%
Number Absent:	8406	60.0%
Number Marginal:	377	2.7%
Average Signal (P):	648.4	
Average Signal (A):	32.6	
Average Signal (M):	112.0	
Average Signal (All):	264.5	

Housekeeping Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
18S_RRNA	18.3	A	67.8	P	82.9	P	56.33	4.54
ACTIN	2983.0	P	7075.1	P	3976.6	P	4678	1.33
GAPDH	436.2	P	1217.7	P	1692.1	P	1115	3.88
DROS-EIF-4A	4443.5	P	10464.4	P	7337.3	P	7415	1.65

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')
BIOC	331.0	P	155.8	P	243.38	P
BIODN	244.2	P	1490.2	P	867.20	P
CREX	2554.0	P	5413.1	P	3983.52	P

