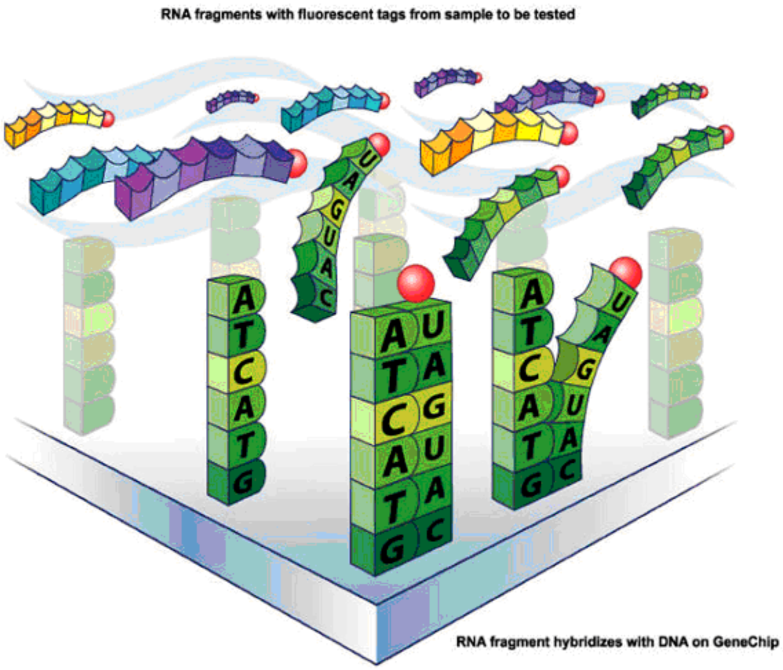
Microarray Technology
Lab Manual



Instruction Team:

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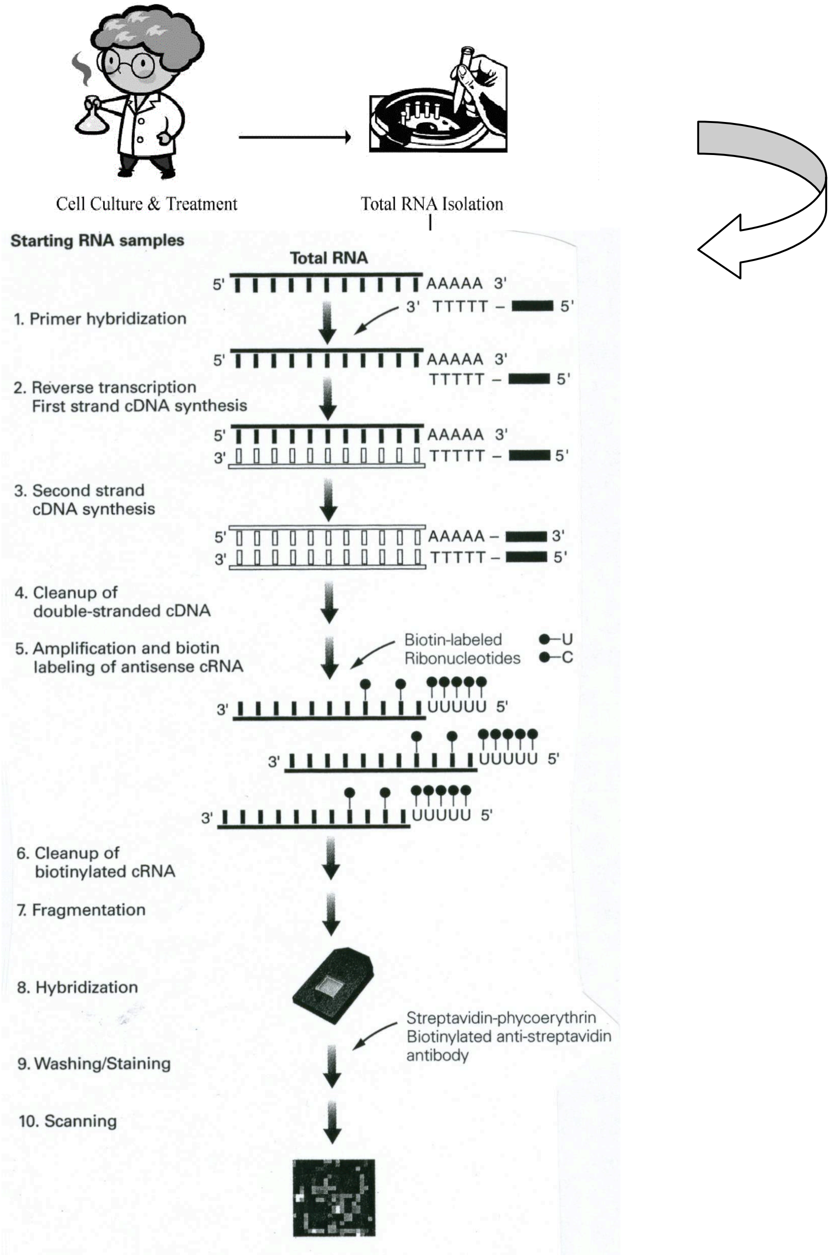
Manual: Arabidopsis 10/11

Overview

In this module, you will research the local adaptation and phenotypic plasticity of North American populations of *Arabidopsis lyrata* to warm and cold environmental conditions by examining changes in gene expression. Individuals from both NC (warm) and MA (cold) populations of *A. lyrata* will be grown under two different temperature regimes, 20°C (control) and 2°C (cold stress). Leaf material is collected from these plants to look at changes in gene expression associated with cold tolerance and adaptation. Total RNA is collected and is then converted to a labeled cRNA product through a multi-step process. The final synthesis product is taken back to the UVM Microarray Core Facility and hybridized to the Affymetrix Arabidopsis GeneChips. The resulting gene expression data are uploaded into bioinformatics data analysis software. You will select specific genes whose expressions have changed as a result of cold exposure and generate a report to the class**.**



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

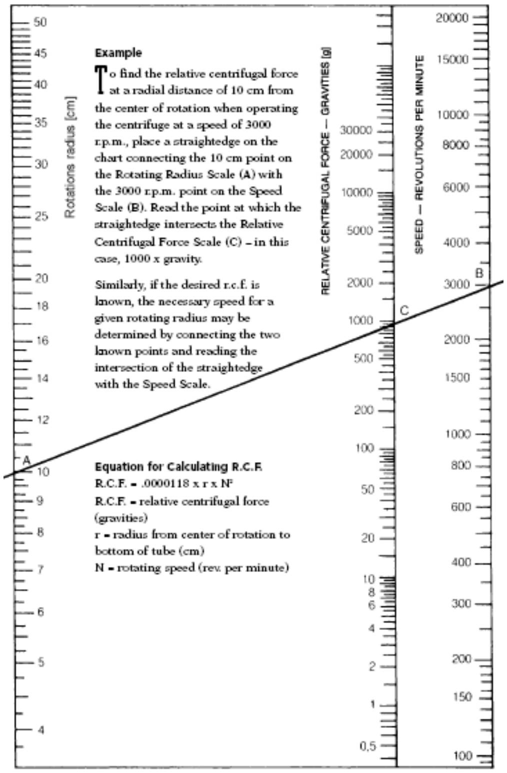
4] Check off each step in the lab manual as you complete it.

5] Read the 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!



Example

To find the relative centrifugal force at a radial distance of 10 cm from the center of rotation when operating the centrifuge at a speed of 3000 r.p.m., place a straightedge on the chart connecting the 10 cm point on the Rotating Radius Scale (A) with the 3000 r.p.m. point on the Speed Scale (B). Read the point at which the straightedge intersects the Relative Centrifugal Force Scale (C) – in this case, 1000 x gravity.

Similarly, if the desired r.c.f. is known, the necessary speed for a given rotating radius may be determined by connecting the two known points and reading the intersection of the straightedge with the Speed Scale.

Equation for Calculating R.C.F.

R.C.F. = $.0000118 \times r \times N^2$
R.C.F. = relative centrifugal force (gravities)
r = radius from center of rotation to bottom of tube (cm)
N = rotating speed (rev. per minute)

Microarray Data Sheet

Individual identification:

Name __________________________ (Group ID ______________________)

Experiment type____________________

1) Total RNA data from DAY 1

RNA conc:______________ug/ul: A_{260}/A_{280}:___________

2) cDNA synthesis and clean-up:

Volume RNA used: ________
µg RNA used[B] __________
Resuspension Volume________
Visible Pellet________

3) RNA InVitro transcription:

Volume of cDNA used in IVT reaction: ________

4) cRNA clean-up:

Final volume[C]: __________ ul
Concentration: ____________ug/ul
Total RNA yield ____________ug[A]

Final Adjusted RNA concentration: __________________ ug/ul

5) cRNA Fragmentation:

Amount Fragmented:___________

Attach Spectrophometer data

Attach gel images

Set-up before day 1: For course Instructor

Check off each step in the protocol after completing it.

Please read through all instructions before proceeding.

DAY 1

Isolating Total RNA from *Arabidopsis lyrata*

Technical Overview:

You will be researching differences in gene expression from two populations of *A. lyrata,* grown at 20°C (control) and 2°C (cold stress). Dr. Alongi has collected leaf tissue prior to lab during the dark cycle. During lab, total RNA is extracted from leaf tissue. The plant cells are lysed by grinding the tissue in a buffer containing the chaotropic agent, guanidinium isothiocyanate that dissolves the cell membrane. This sample is run through a QIAshredder to remove large cellular debris and homogenize the sample. All the RNA is collected using a silica gel spin column manufactured by Qiagen. (RNeasy system)

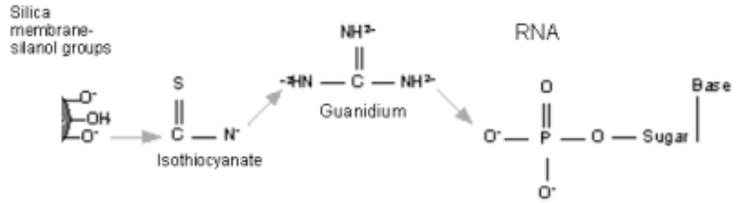
Please read through all laboratory procedures prior to lab.

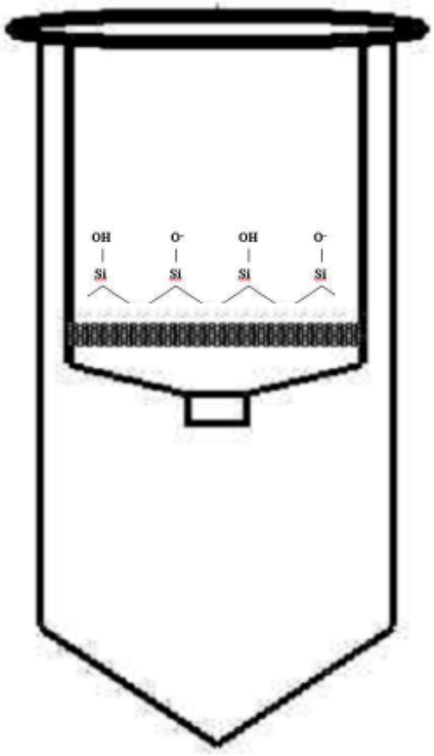
Check off each step in the protocol as you complete it.

The Silica Membrane Chemistry

Guanidium isothiocyanate (GITC) is a chaotropic salt present at a concentration of about 5 M in the RLT buffer. This solution (pH 6.4) used in conjunction with ethanol and water disrupts the molecular characteristics of water and creates conditions where RNA is less soluble. This promotes nucleic acids to electrostatically bind to the silica membrane of the spin column. 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. Since this not a 100% selective procedure a DNase treatment is used to destroy any DNA that is bound to the silica membrane. The use of ethanol in the rinse RPE buffer maintains the bond between the silica and RNA. RNA is eluted from the membrane with pure water, which removes the salts and breaks the electrostatic bond of RNA with the silica. RNA in an almost pure form is collected by centrifugation.

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 *Arabidopsis lyrata*

Materials:

Axygen MCT150C 1.7ml tubes
P1000, P200, and P20 pipets
100 % Ethanol
DEPC water (aka RNase-free water)
Qiagen Mini RNeasy kit
QIAshredder
Qiagen DNase Kit
Vortex
Microcentrifuge
Fast Prep or Bead Beater
2 ml microcentrifuge tubes (with ceramic ball and diamond dust)

____1. Prepare an RNase-free work zone using RNase ZAP. This will be demonstrated by the instructor.

Note: The Instructor will perform step 2 tasks.

______2. Prepare fresh DNase I solution using the Qiagen DNase kit and store on ice.

10 µl DNase I
<u>70 µl Buffer RDD</u>
80 µl per reaction

____3. Add 450 µl b-RLT buffer to the tube and cap tightly. Place the tube in the Fast Prep Tissue Homogenizer and **run for 20 seconds**. This procedure will grind and lyse the tissue.

3b. Label a new 1.7ml tube with your sample name.

____4. Pipet your sample into a QIAshredder spin column (lilac), contained in a 2ml collection tube. Centrifuge the column for 2 minutes at full speed. Transfer the supernatant from the collection tube into the new 1.7ml tube; do not transfer the pellet. This step will remove cellular debris and homogenize your sample.

____5. Add 0.5 volumes of 100% ethanol to the tube and briefly vortex (approximately 200 - 250 µl). Aprecipitate may form after the addition of ethanol, but this will not affect the collection of RNA.

____6. **Collecting the RNA:** Assemble an RNeasy spin column with a 2 ml collection tube. Label the spin column with your identification information. Carefully transfer all of the solution from step 10 to the spin column using a micropipette. Be careful not to touch the silica membrane with the pipette tip. Close the tube and centrifuge for 15 seconds at full speed. The RNA in the sample will adhere to the silica membrane of the spin column.

____7. Remove the spin column from the collection tube. Pipet the pass through liquid (the liquid from the collection tube) back onto the spin column and centrifuge again. Discard the collection tube (with the flow through fluid) and place the spin column into a new 2 ml collection tube.

____8. Add 350 µl RW1 buffer to the spin column. This solution is used to wash the RNA and remove salts and dissolved cellular debris. Close the tube and centrifuge for 15 seconds at full speed.

____9. **Degrading DNA:** Remove the spin column from the centrifuge, open the tube lid and add 80 µl of the DNase I solution to the middle of the spin column membrane. This step will degrade any DNA in the sample and dissociate it from the silica membrane. Close the column lid and incubate at room temperature for 15 minutes.

____10. Transfer the spin column to a new 2 ml collection tube.

____11. **Cleaning and washing the RNA**: Add 350 µl RW1 buffer to the spin column and centrifuge at full speed for 15 seconds.

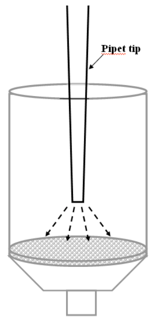
____12. Discard the collection tube and place the spin column into a new 2 ml collection tube.

____13. Add 500 µl RPE buffer to the spin column to wash the RNA on the silica membrane. Close the tube and centrifuge for 15 seconds at full speed.

____14. Discard the collection tube and place the spin column into a new 2 ml collection tube.

____15. Wash the silica membrane again by adding another 500 µl RPE buffer to the spin column. Close the tube and centrifuge for 15 seconds at full speed.

____16. Place the spin column in a new 2 ml collection tube and centrifuge in a microcentrifuge at full speed for 1 minute to ensure any residual liquid is removed from the silica membrane.



____17. **Recovering the RNA:** Label a 1.7 microcentrifuge tube with your identification information. Transfer the spin column to this tube. The lid of the microcentrifuge tube will be off to the side. Carefully pipet 30 µl DEPC water **directly onto the very center** of the silica membrane. **Do not touch the silica membrane with the pipet tip (see diagram)**. Look closely as you perform this step. Use both hands when pipetting, one on top and one down by the tip to guide the pipet. Make sure the water is evenly distributed on the membrane.

____18. Incubate the spin column at room temperature for 1 minute. Centrifuge at full speed for 30 seconds.

____19. Carefully transfer the 30 µl that is recovered in the 1.7 ml tube back onto the center of the silica membrane of the same spin column as in step 22 above. Place the column back in the same 1.7 ml collection tube and incubate for 1 minute at room temperature. Centrifuge for 1 minute at full speed. This double extraction ensures that the maximal amount of RNA is eluted from the membrane.

____20. Label two new 1.7 microcentrifuge tubes with your identification information. Transfer all of the recovered RNA sample to one of these tubes.

____21. Transfer 4 µl of this sample to the other labeled tube. This sample will used for quantification on the Nanodrop and the remainder will be transport back to UVM for RNA assessment.

____22. Place all samples on ice.

____23. Quantify the RNA:

Absorbance of sample:_________________ A_{260}/A_{280} ratio:__________________
Concentration of RNA in sample:________________ µg/µl

Record these data on the data sheet provided on page 5 of the manual.

____24. Evaluate the RNA quality using agarose gel electrophoresis. This will be done as a class.

____25. Set up the E-gel electrophoresis apparatus. 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.
Add 14 µl of water to each well on the E-gel. Add 1µl of each sample to each well and pipet up and down to mix well. Record which sample is in each lane. Run the gel for 20 minutes.

____ 26. Take a photo of the gel.

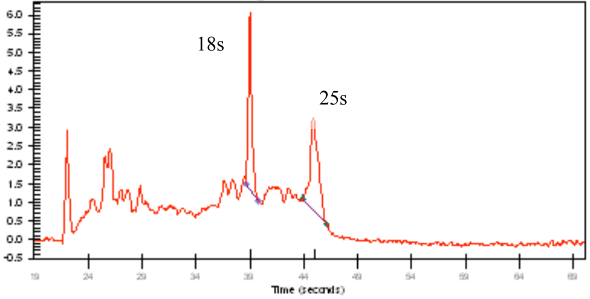
A sample of your RNA may be analyzed on the Agilent 2100 Bioanalyzer at UVM, which provides a move sensitive image of the RNA quality. (Described on the next page).

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.

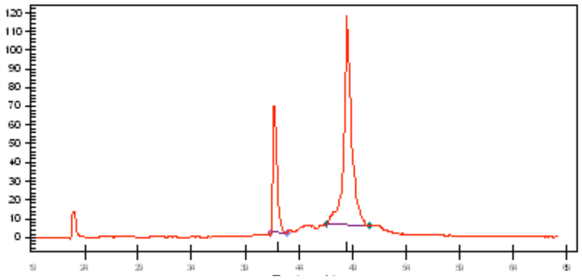
Moderately Degraded RNA

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



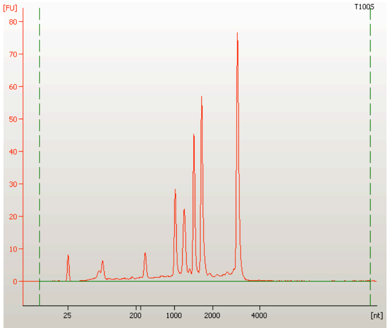
Fully Intact RNA

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



Fully Intact Plant RNA

Clean baseline with 18s and 28s rRNA,
additional peaks from chloroplast and mitochondrial rRNA are present.



Microarray Target Preparation- cDNA synthesis

Day 2 Technical Overview:

Today you will be starting to prepare the target cRNA for the Genechip analysis. This is a lengthy procedure and will be broken down over several lab periods. 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 strand (cDNA) from the mRNA. This is called the first strand cDNA synthesis. The mRNA molecule is oriented in a 5’ to 3’ direction. This step utilizes a primer with a sequence of 24 T's in a row and is called oligo d(T), This primer binds to the poly A tail of mRNA (the 3’ end). The primer also contains the consensus-binding site for T7 RNA polymerase (T7 promoter sequence) which is 36 bases long. The total length of the primer is 60 bases. Once the primer has hybridized to the poly-A tail of the mRNA molecule, reverse transcriptase, an enzyme that uses RNA as a template to synthesis DNA, is added and the first strand of cDNA is synthesized. The final product of this reaction is a hybrid molecule composed of one RNA strand (the original mRNA molecule) and one DNA strand. The first DNA strand is synthesized in a 3’ to 5’ orientation relative to the mRNA strand (see the diagram on page 20, step 2). It is important to note that rRNA and tRNA molecules in the sample do not have a poly-A tail and will not bind the oligo T primer and will not be used as a template for cDNA synthesis in this procedure.

The full sequence of T7 Oligo d(T)24 is:

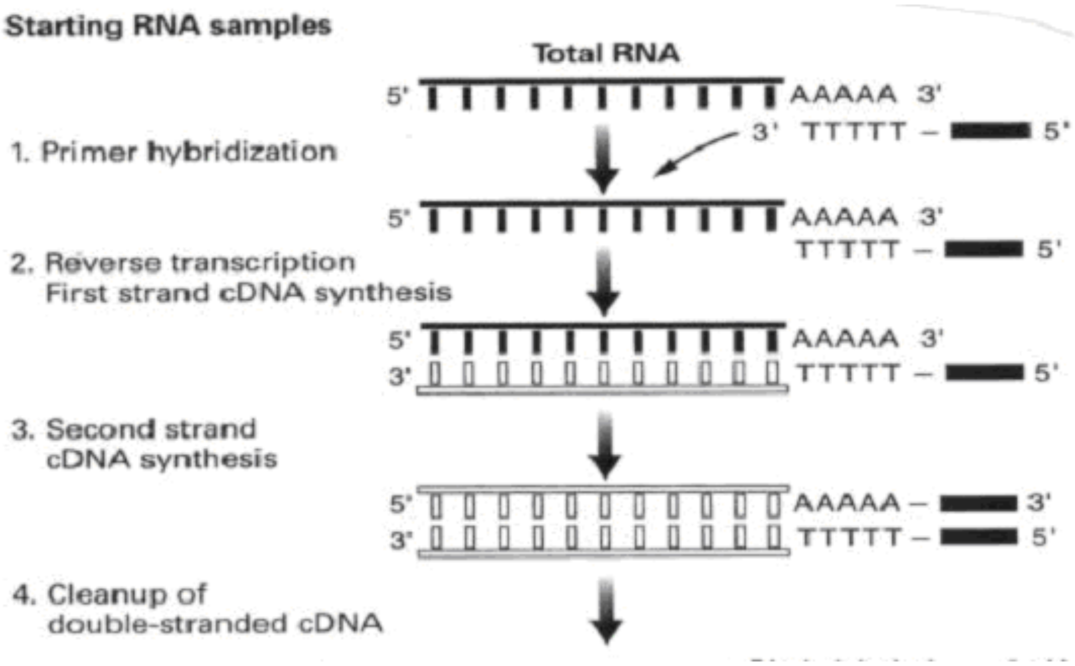
$$5' - \underbrace{\text{GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG}}_{\text{T7}}\text{-}\underbrace{\text{TTTTTTTTTTTTTTTTTTTTTTTT}}_{\text{d(T)}_{24}\text{-Binds to Poly-A of mRNA}} - 3'$$

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 nicks in the phosphodiester bonds (between the phosphate and the ribose sugar) of the bound mRNA strand (not the cDNA stand already made) creating exposed 3'OH groups. DNA polymerase I enzyme uses these 3' OH groups as a priming site; displacing the RNA in the strand and creating another cDNA strand using the first cDNA strand as a template. It continues in this fashion until it reaches another nicked site, where it stops. The DNA polymerase I can only displace RNA, not DNA. After cDNA has been synthesized from all the nicks, the 3'OH nicks are reconnected or ligated. This is accomplished using DNA ligase enzyme. After the two hour synthesis process, all the RNA has been replaced with cDNA and ligated to form one double stranded cDNA molecule. However, because the DNA polymerase does not synthesize completely to the end of the strand, a T4 DNA polymerase is added. This enzyme ensures the full synthesis of the T7 promoter site from the original T7 oligo d(T) primer used in the first synthesis reaction. The final product is a double stranded cDNA molecule which includes the original mRNA sequence (in DNA form) on the second strand plus the full T7 RNA polymerase binding site at the 3' end of the molecule—the dark bar shown on step 3 on page 20.

Synthesis of cDNA:

The starting material for your target preparation will be high quality, fully intact, total RNA from *A. lyrata* that you extracted on Day1. The minimum concentration to start with is 100-270 ng/µl. After isolation and purification of the plant RNA, the concentration is determined by absorbance at 260 nm (A_{260}) on a spectrophotometer (1.0 A = 40 ng/µl RNA). The A_{260}/A_{280} 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 Day 2 class at UVM) and running on an agarose gel (EGEL) prior to starting the assay.

Overview of cDNA Synthesis Reactions



Day 2

Microarray Target Preparation- cDNA synthesis

First Strand cDNA Synthesis

Note: You will be measuring and transferring very small volumes. Centrifuge all reagent tubes before starting as some have only 1 µl in them. When pipetting , look closely at your pipet tip to ensure you have picked up the sample and look at the tube to which you are delivering it to ensure it has been added.

____1. Label a 0.5 ml PCR tube with your identification information. From the concentration of your RNA determined on Day 1, calculate the volume of the sample needed to contain 3.0 µg. Transfer this amount to the tube. Add DEPC water to bring the volume to 10.0 µl.

Example: RNA concentration of your sample = 800 ng/µl or .8ug/µl.
3.0 µg/.8 µg/µl = 3.75 µl. Transfer this amount to the tube.
10.0 µl – 3.75 µl = 6.25µl = volume of DEPC water to add to tube.

- RNA concentration of your sample: ________________µg/µl
- 3.0 / (your RNA concentration) = ________________µl. This is the volume of your sample you will add to the tube.
- 10.0 – (the volume of your RNA sample) = ___________µl. This is the volume of DEPC water to add to the tube.

____2. Add 2.0 µl T7 oligo d(T)24 (@50 µM) reagent to the tube. Make sure you see the reagent transfer. Vortex the tube and centrifuge at full speed for 5 seconds. Place the tube in a thermocycler set at 70°C for 10 minutes.

____3. While the 70°C incubation is in progress prepare the following master mix in a new tube. Add the following reagents **IN ORDER** to make the first strand master mix. Vortex and centrifuge at full speed for 5 second and place the tube on ice.

First strand master mix

First Strand Buffer 5X	4 µl
0.1M DTT	2 µl
10mM dNTP	1 µl
Superscript II	1µl

Use a P2 micropipette to measure volumes of 2 µl or less.

____4. After the 70℃ incubation in step 2 is finished, add the first strand master mix made in step 3 to the tube and incubate in a thermocycler at 42℃ for 60 minutes. When first strand synthesis incubation is finished, place the tube on ice. During this incubation, prepare the second strand master mix.

Second Strand cDNA Synthesis

____1. Make the following master mix in a new tube. Keep it on ice. Centrifuge all reagent tubes before using to ensure the contents are at the bottom of the tube. Add the reagents in the order listed.

Second strand master mix

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

Use a P2 micropipette to measure volumes of 2 µl or less

____2. Vortex the tube and centrifuge for 5 seconds at full speed.

____3. When the 42℃ incubation is finished, transfer all 130 µl of the second strand master mix to the first strand synthesis tube. Vortex and centrifuge for 5 seconds at room temperature. Incubate the tube for 2 hours at 16°C in a thermocycler.

The next two steps may be performed by the instructor

____4. At the end of the 2 hour incubation and while the sample is still at 16℃, add 2µl T4 DNA polymerase reagent to the tube. Mix the tube well by inversion. Incubate at 16℃ for 5 minutes. 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 µl 0.5 M EDTA to the tube to stop the T4 DNA polymerase reaction. Store the tube at -20℃.

Day 3

Technical Overview: Cleaning the cDNA:

The double stranded cDNA generated on Day 2 must be cleaned and purified before proceeding to the next step. This is done using a solution of phenol-chloroform-isoamyl alcohol (PCI) at a alkaline pH to denature proteins and trap them in the organic phase of the reagent. cDNA remains in the aqueous phase and the two phases are separated using a phase lock gel system. The phase lock gel tube is centrifuged causing the gel in the tube to migrate between the aqueous phase (on the top and containing the cDNA) and the organic phase (on the bottom and containing the denatured proteins). Recovery of cDNA is easy using this method. The pH of the PCI solution is maintained by Tris buffer at pH 8.3 to ensure the cDNA will dissolve in the aqueous layer.

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

The In vitro Transcription (IVT) Reaction:

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. T7 RNA polymerase enzyme binds to this site and generates cRNA molecules. In this arrangement the original mRNA sequence is the template strand and the cRNA generated is antisense relative to this sequence. Two unique nucleotides are used in the reaction. A select proportion of the C and U ribonucleotides have molecules of biotin attached. A and G nucleotides are not biotinyated. As a result, the cRNA generated has random amounts of biotin label incorporated into its structure. The biotin will be the beginning of the signal generation process used on the gene chips based on a strepavidin-phycoerytherin reaction. During the IVT reaction the T7RNA polymerase binds to the T7 promoter site and generates a molecule of cRNA. During the 16 hour incubation time for the IVT, a single enzyme can generate many thousand cRNA molecules from the cDNA template. The net result is a total increase in RNA at the end of the reaction. For a typical IVT reaction, 1.5 to 2.5 µg/µl yields are expected. 10 µg of cRNA is carried forward to Day 4 for fragmentation and hybridization.

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. The Tris layer floats on top of the PCI layer. 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

Note: PCI tends to leak out of the pipet tips so work quickly when measuring it or transferring it from one container to another.

Day 3

Precipitating the cDNA:

____1. The total volume of the cDNA synthesis step from Day 2 is 162 µl. The PCI step requires equal volumes of aqueous and organic mixtures. Centrifuge a Phase Lock Gel tube at full speed for one minute to insure the gel is at the bottom of the tube. DO NOT VORTEX PHASE LOCK TUBES.

____2. Add 162 µl of the **bottom layer** from the Phenol Chloroform Isoamyl Alcohol (PCI) to the contents of the cDNA synthesis reaction tube from Day 2 and vortex for 5 seconds to mix the contents.

Note: PCI tends to leak out of the pipet so work quickly and hold the cap tightly as leaking can occur during the vortex step.

____3. Transfer all of the cDNA-PCI mixture to the phase lock gel tube using a micropipet. DO NOT VORTEX the phase lock gel tube.

____4. Centrifuge at full speed for 2 minutes.

____5. Label a new 1.7 microcentrifuge tube. Use a micropipette to transfer the top layer from the phase lock gel tube to the tube. Try to collect as much of the layer as possible.

____6. Add the following to the 1.7 ml microcentrifuge tube and vortex.

Ethanol (100%)	405 µl
NH4OAc (7.5M)	80 µl
Pellet Paint	1 µl

____7. Place the tube in the centrifuge with the hinge of the tube facing out and centrifuge at full speed for 20 minutes at room temperature.

____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 and on the side of the tube under the hinge. Place on ice and immediately proceed to next step.

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 P1000 micropipet, carefully remove the liquid from the tube. Tip the tube to remove of as much liquid as possible, but don't lose the pellet. Remember that the pellet is your sample!

____2. Add 500µl ice cold 80% ethanol (store in -20C^{o} freezer) to the tube. Gently cap the tube and invert it slowly several times. Watch your pellet very closely. Place the 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.

____3. Using a P1000 micropipet, 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. To do this, remove as much as possible using a P1000 micropipet. Centrifuge the tube at full speed for 5 seconds and use a P20 micropipet to remove the last few microliters of liquid. The goal is to remove as much ethanol as possible without disturbing the pellet.

____6. Place the tube on its side with the cap open in a drying box for 10 minutes to evaporate the remaining ethanol. The dried pellet is easily lost once it is dry. Be **very** careful to handle the tube gently. When done, close the cap gently. Visualize the dried pellet to confirm it is present in the tube.

____7. Resuspend the pellet in 22 µl of DEPC water and place the tube on ice.

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

____1. The ENZO bioarray kit contains all the reagents needed to prepare biotin labeled cRNA from cDNA.

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 an RNase-free area free from air currents.

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

The Instructor will perform this task.

NOTE: Prepare sufficient master mix for the number of samples plus one. This will ensure enough for pipetting purposes.

____2. Label a 0.5 ml PCR tube. Combine the following in the tube and pipet up and down several times to mix. Spin in a centrifuge at full speed for 5 seconds to get all reagents to the bottom of the tube.

cDNA mixture	22 µl Enzo master mix [from above] 18 µl
Total Volume	40 µl

____3. Incubate the tube at 37°C for 16 hours in the thermocycler. When the reaction is complete, store the sample at -20°C.

Day 4

Cleaning and fragmenting the biotinylated cRNA

The biotinylated cRNA generated on day 3 must be cleaned and purified from the enzymes and reagents used in the IVT reaction. Removing the unused biotinylated NTPs, buffers and proteins is necessary as they interfere with quantitative measures. Proteins and NTP's absorb at 260 and/or 280nm and will affect the quantitation of the cRNA. They also interfere with hybridization of the cRNA targets on the gene chip.

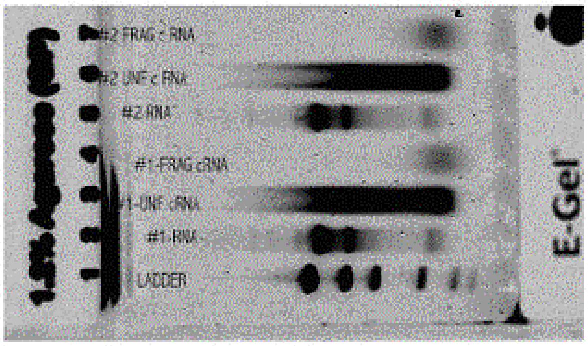
You will use an RNeasy spin column to clean the IVT product similar to that used on Day 1. The cRNA is washed and eluted from the spin column with DEPC water. The cRNA is analyzed with a spectrophotometer and the concentration and purity determined. An A_{260}/A_{280} ratio of 1.8-2.1 indicates a clean cRNA sample that can be used for subsequent steps.

The cleaned cRNA is too large to hybridize onto the gene chip. It is fragmented into randomly short segments using a non-enzymatic, heat catalyzed magnesium salt reaction. The final product is assessed on an agarose gel.

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 :

____1. Remove the Day 3 tubes from the freezer and let thaw. Transfer the entire cRNA sample to a new1.7 ml microcentrifuge tube. Add 60 µl of DEPC water and 350 µl BME-RLT and vortex for 5 seconds.

____2. Add 250 µl 100% ethanol and vortex again.

____3. Label an RNeasy spin column and transfer the entire cRNA sample to the column. As before, be careful not to touch the tip of the pipet to the silica membrane. Place the spin column in a 2 ml collection tube, close the lid and centrifuge for 15 seconds at full speed.

____4. Remove the spin column from the collection tube. Pipet the pass through liquid (the liquid in the collection tube) back onto the spin column and centrifuge again for 15 seconds. Discard the collection tube (with the flow through fluid) and place the spin column into a new 2 ml collection tube.

____5. Pipette 500 µl of RPE buffer onto the spin column. Close the tube and centrifuge for 15 seconds at full speed. Discard collection tube and the pass through liquid. Place the spin column into a new collection tube.

____6. Add another 500 µl RPE buffer to the spin column. Close the tube and centrifuge for 15 seconds at full speed. Discard the collection tube and pass through liquid.

____7. Transfer the spin column into a new collection tube and at full speed for 1 minute.

____8. Label a new 1.7 ml microcentrifuge tube with your identification information. Transfer the spin column to this tube.

____9. Pipet 30 µl DEPC water onto the RNeasy silica-gel membrane and wait 1 minute. Make sure the DEPC water contacts the entire membrane. Be careful not to touch the membrane with the pipet tip. Close the tube and centrifuge for 1 minute, at full speed.

____10. Carefully transfer the 30 µl that is recovered in the 1.7 ml tube back onto the center of the silica membrane of the same spin column. Place the column back in to the same 1.7 ml collection tube and incubate for 1 minute at room temperature. Centrifuge for 1 minute at full speed. This double extraction ensures that the maximal amount of cRNA is eluted from the membrane.

____11. Label a new 1.7 microcentrifuge tube with your identifying information. The old tube lid may have been contaminated in the last few procedures. Transfer the cRNA this tube.

____12. Determine the cRNA concentration as on Day 1. Prepare a sample for analysis by adding 2 µl of your sample to 48 µl DEPC water. Determine the concentration and the A_{260}/A_{280} ratio .

Absorbance $A_{260}$of sample:_________________

A_{260}/A_{280} ratio:_________________

Concentration of RNA in sample:_______________ µg/µl

Total yield of RNA = 30 µl x concentration = ___________________µg.

Record these data on the page 5 data sheet.

Calculating the Adjusted cRNA Concentration:

The final concentration of cRNA must be adjusted because the original amount of RNA you started with on Day 1 has been carried along in the experiment. Over 98 percent of that RNA was not mRNA but rRNA and tRNA. The amount of RNA measured above also includes these RNAs in the sample and must be adjusted to remove them from the final cRNA concentration.

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

$$\text{adjusted cRNA concentration} = \frac{[A\text{x}B]\text{-}C}{B}$$

Calculation example:

- The amount of RNA started with on Day 1 = 3 µg (C in the formula)
- Concentration of RNA from step 12 above = 1.8 µg/µl (A in the formula)
- Volume of elute = 30 µl (B in the formula)

Total yield from step 12 above = 1.8 µg/µl x 30 µl = 54.0 µg (A x B in the formula)

Example math:

- The adjusted cRNA concentration = (54.0 µg – 3 µg)/ 30 µl = 1.7 µg/µl

The cRNA concentration in the sample is 1.7 µg/µl and this value is used in future calculations.

Record these data on the page 5 data sheet.

Fragmenting the cRNA for Target Preparation

The cRNA molecules generated in the IVT reaction are too large to hybridize on the gene chip. In this step they are fragmented into randomly sized pieces of 35 to 250 bases in length. The cRNA is fragmented using a metal-induced hydrolysis reaction, referred to a hammerhead cleavage. Both the unfragmented and fragmented cRNA are visualized on an agarose gel. This is important because you need to ensure the unfragmented cRNA has not degraded during the handling steps leading up to this point.

Affymetrix recommends 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 0.6 µg/µl. The fragmentation buffer contains a basic solution of TRIS acetate buffer, magnesium acetate, and potassium acetate, which causes a metal induced RNA hydrolysis at high temperature. See the diagram on the next page.

____1. You will fragment 10.0 µg of cRNA that will be used for gene chip analysis. To determine the volume of your sample needed in this step, divide 10.0 by the concentration of your adjusted cRNA sample .

Example: your adjusted cRNA concentration = 1.7 µg/µl.

10 µg/ 1.7 µg/µl = 5.9

Use 5.9 µl of your sample in the next step. (Record this value on the page 5 data sheet.)

The fragmentation reaction requires 16 µl of volume for the sample. The additional volume is made up with DEPC water. To determine how much DEPC water to use, subtract the volume of cRNA used from 16.

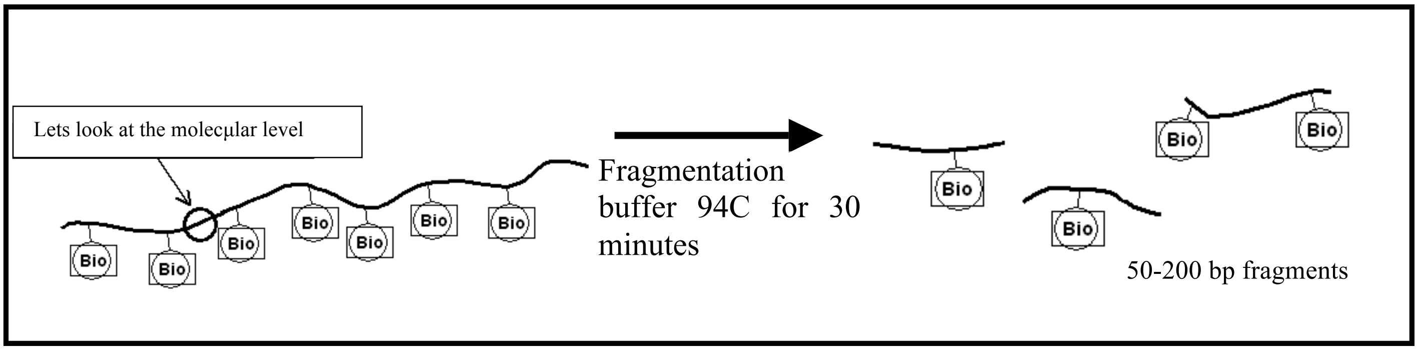
16.0 µl – 5.9 µl = 10.1 µl of DEPC water used.

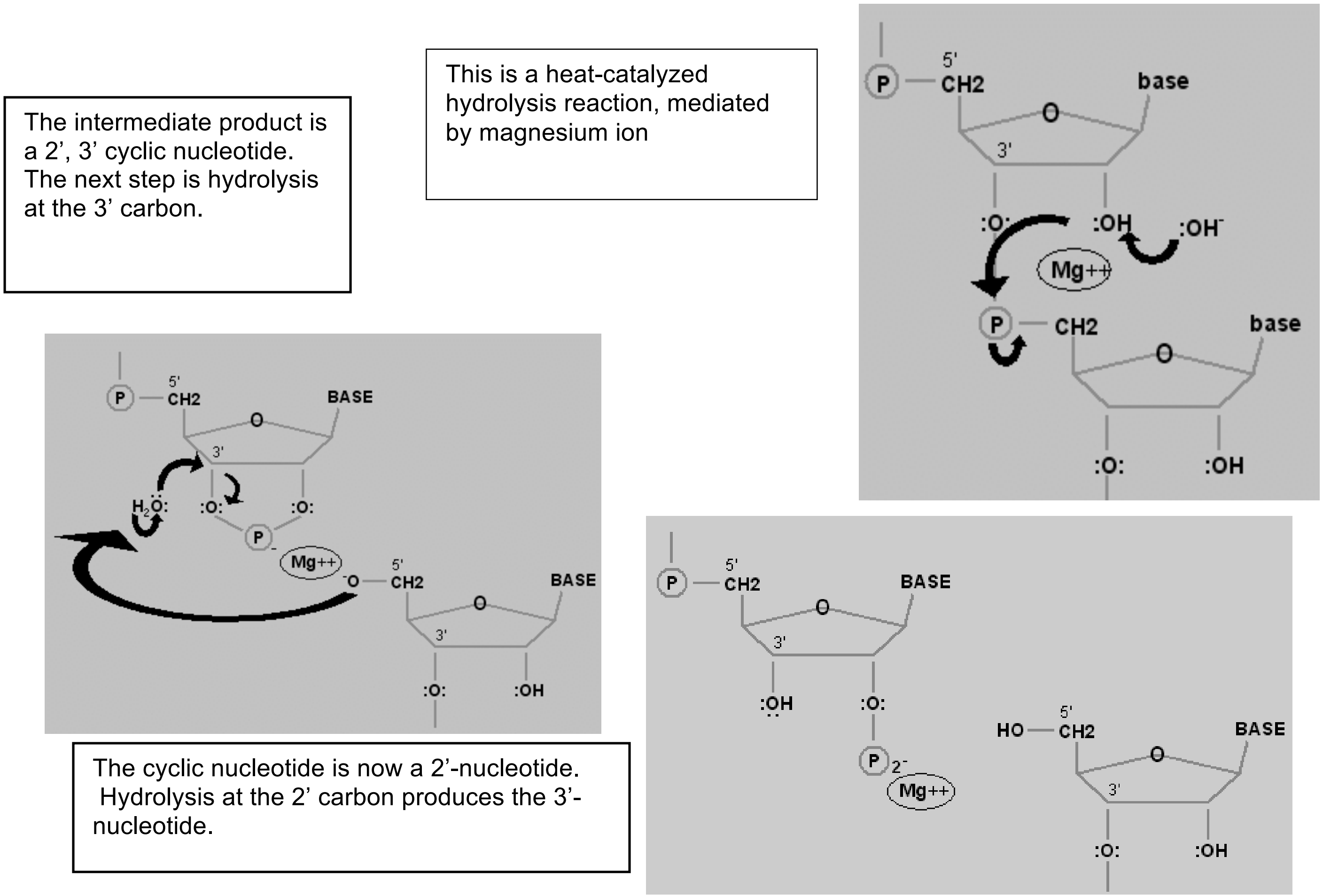
cRNA volume = ____________________µl
DEPC water volume = ____________________µl

____2. Label a 0.5 ml PCR tube with your identification information. Transfer the amount of cRNA determined in step 1 above to the tube. (In the example this is 5.9 µl). Add DEPC water to bring the volume to 16.0 µl. (This is 10.1 µl in the example). Add 4.0 µl 5x fragmentation buffer to the tube. The total volume in the tube should be 20.0 µl.

____3. Vortex the tube and centrifuge for 10 seconds. Incubate the tube at 94°C for 30 minutes in a thermocycler. Put on ice following the incubation.

Fragmentation: Hammerhead Cleavage





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

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

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

Agarose gel assessment of unfragmented and fragments cRNA samples:

Use an agarose gel to assess the quality of your unfragmented and fragmented cRNA.

____1. Set up the E-gel electrophoresis apparatus. 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 µl of water to each well on the E-gel.

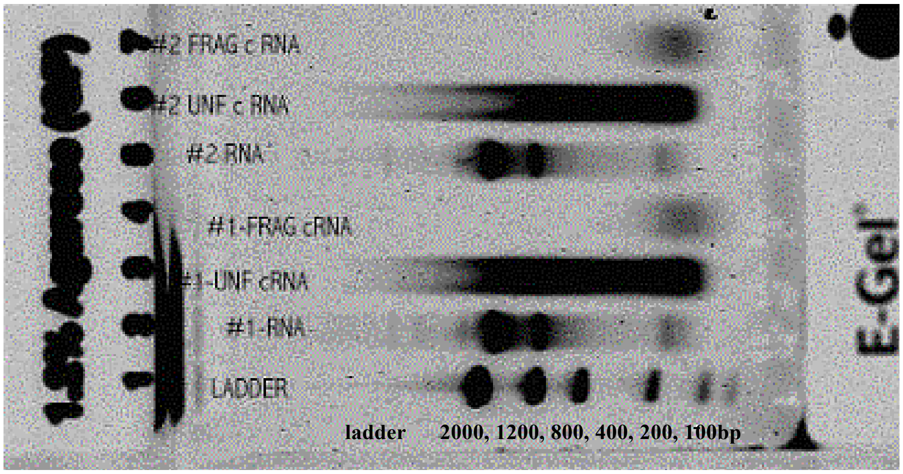
____3. Add 2µl of each sample to each well and pipet up and down to mix well. Run the fragmented and unfragmented cRNA of each sample next to each other. Record the identity of each sample in each lane.

____4. Add 4 µl of a DNA ladder (size standards) to one lane of the gel

____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 as the cRNA molecules are of various lengths.



To Be Performed at the University of Vermont

These steps will be performed at the University of Vermont Microarray Core Lab and will be shown as a powerpoint presentation during your class visit.

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 biotinlylated 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.

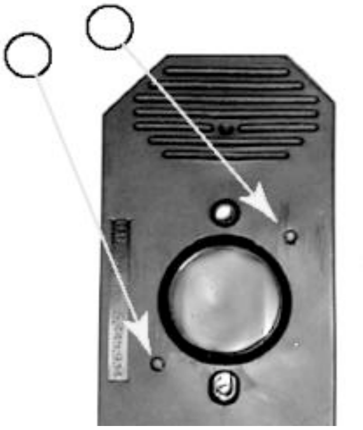
____1. Prepare the following:

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

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

Hybridization to the Arabidopsis ATH1 Genome Array

After the hybridization mixture has been made, it is denatured at 94℃ 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.

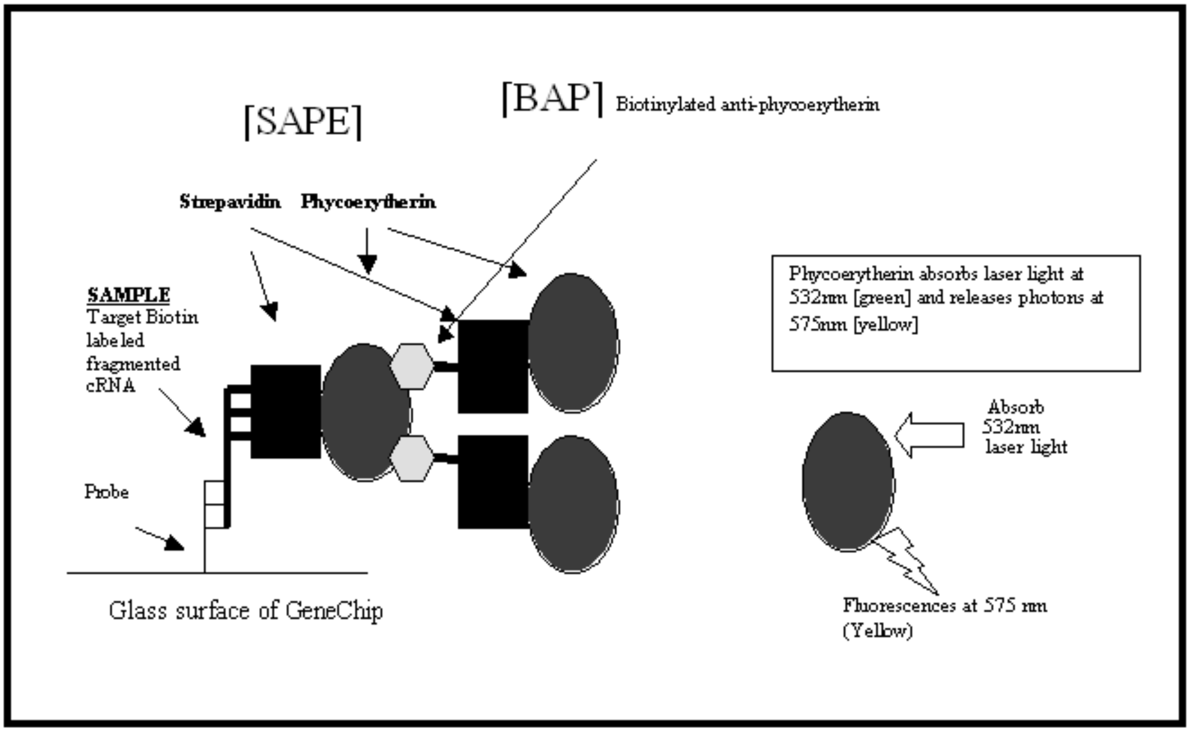


Staining the Arabidopsis ATH1 Genome Array

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.

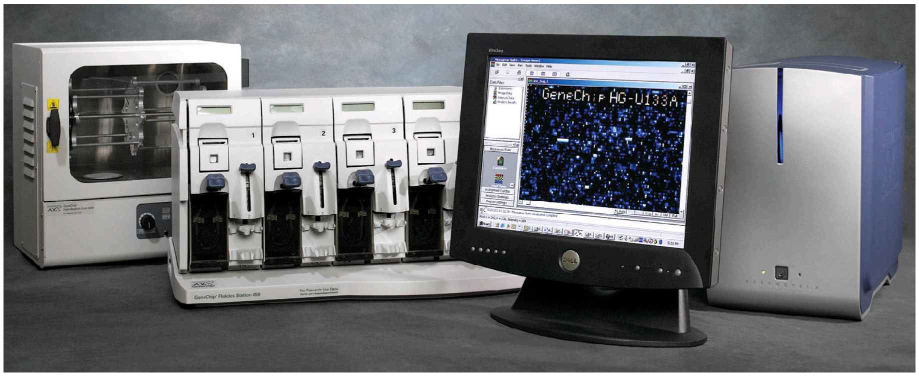
A schematic of the staining chemistry



Scanning the Arabidopsis ATH1 Genome Array

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 µm beam is scanned back and forth across the glass surface at a pixel resolution of approximately 2.5 µm. Fluorescence is detected using a high sensitivity photomultiplier tube [PMT]. This enables high resolution detection of fluorescence from the probe set feature [pixel] that is only 11 µm square. There are approximately 1 million probe areas on each chip!

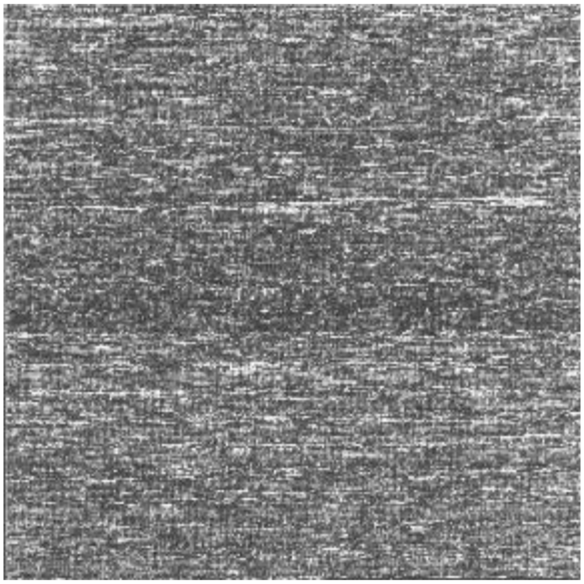
GeneChip

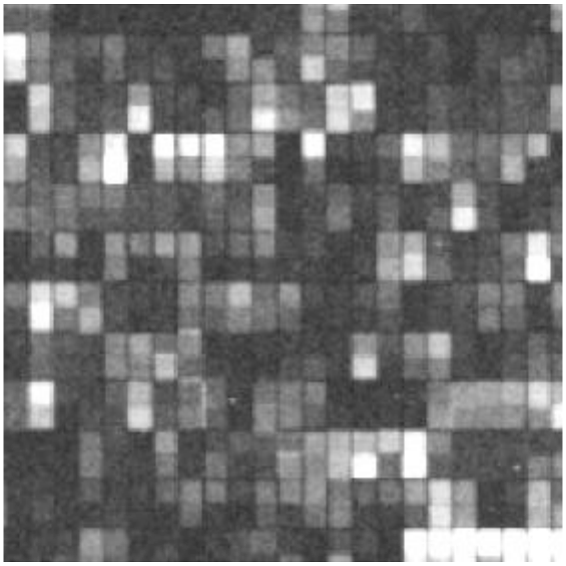


The Affymetrix GeneChip System

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. Once your chips are scanned we will review the quality control specifications and then proceed to analyzing the data.