

RNA Preparation and Northern Blot Protocol

RNA Preparation

- 1) Set up for tissue homogenization:
 - Label three 5mL flacon (2063) tubes for each sample
 - Label one 8ml Starstedt tube for each sample
 - Get a bucket of ice and liquid nitrogen for your samples
 - Get seven 50 ml conical tubes for washing homogenizer:
 - *put 30mL of H₂O in three of the 50mL tubes
 - *put 30 ml of 100% EtOH in three of the 50mL tubes
 - *put 10 ml of RNAzol in one 50 ml tube (do this under the hood)
 - *plug in homogenizer, turn on power, and set to #5, and clean off homogenizer using your conical tubes of H₂O, EtOH, and RNAzol (in this order) **[ALWAYS make sure the probe is submerged in liquid before you turn it on]**
 - Place one set of labeled 5 ml tubes in the ice bucket and put 200λ of chloroform in each tube (do this under the hood)
 - Place Starstedt tubes in ice bucket and put 2mL of RNAzol in each tube (do this under the hood)

- 2) Homogenize the tissues: **[DO ALL OF THE FOLLOWING STEPS UNDER THE HOOD]**
 - Place tissue in corresponding Starstedt tube containing RNAzol
 - Homogenize the tissue (Do not homogenize tissue for more that 10 seconds because it will get too hot. For liver, one 10 sec bout is plenty. For heart, SKM, BAT and WAT, do a second 10 sec bout.)
 - After tissue is homogenized, pour the sample into the corresponding 5mL tube containing 200λ chloroform→ cap lid snugly→ vortex well for 15 seconds until sample looks pale blue throughout→ place in ice
 - Wash the probe between each sample using the 50mL conical tubes of H₂O, EtOH, and RNAzol (in this order), then wipe with kimwipe→ halfway through your samples start using a new 50mL tube of H₂O and EtOH
 - After all tissue samples are homogenized let the samples sit on ice for 5 minutes

- 3) Spinning samples:
 - Spin down samples in Beckman centrifuge for 15 minutes at @10,500 rpm **[make sure you always have the centrifuge balanced]**
 - While your samples are spinning, and put 1mL (equal volume) of chloroform into the next set of 5ml falcon tubes **(do this under the hood)**
 - do not prepare the chloroform tubes ahead of time as they will evaporate**
 - Take samples out of centrifuge and place on ice
 - Pipet off the aqueous phase (the clear upper liquid) and put into the corresponding 5mL tube containing 1mL of chloroform→cap lid snugly and vortex well for 15 seconds→ place on ice *(be careful not to disturb the other phases and contaminate the upper phase)* **(do this under the hood)**
 - After each sample has been transferred spin down the samples for 15 minutes at @10,500 rpm

- While your samples are spinning, take the last set of labeled 5mL tubes and put 1mL (equal volume) isopropanol into each tube
- Take samples out of centrifuge and place on ice
- Pipet off the upper aqueous phase and put into the corresponding 5mL tube containing 1mL of isopropanol → vortex well for 10 seconds → place on ice (**do this under the hood**)
- After the supernatant of each sample has been transferred, put the tubes in a tube rack and place in 4° overnight

4) Cleaning up the hood:

- Take the unused 50mL tubes of H₂O and EtOH and clean the homogenizer → make sure it is extremely clean and free of any tissue → wrap in foil and put away
- Put all chloroform and RNazol waste from spins into the bottle under the hood labeled “*organic waste*”
- All empty tubes and tips should be left in the hood to dry out for at least 24 hours → after the RNazol smell is gone, the empty tubes and tips can be thrown away in a glass/sharps disposal box

5) Pelleting RNA and resuspending:

- After overnight incubation, take samples out of the 4° and spin down in the centrifuge for 15 minutes at @ 10,500 rpm
- While samples are spinning, label a 1.5ml microfuge tube for each sample
- Take samples out of centrifuge and place on ice, discard supernatant → resuspend pellet in 1mL of 75% ethanol and transfer to new 1.5 ml microfuge tube → place on ice
- Spin down the samples in the microfuge located in the cold room for 5 minutes at @ top speed
- Take samples out of microfuge and place on ice → remove as much of the ethanol as possible and discard it → let samples air dry on ice (*do not over dry → 5-10 min max*)
- Resuspend pellets in the appropriate amount of 0.1% DEPC water

Approx amounts:

- ~**50mg liver: 80-100ul DEPC water**
- Half of mouse heart= 20-25ul DEPC water**
- Soleus= 8-11ul DEPC water**
- Half mouse Kidney= 50ul DEPC water**

6) Getting sample concentration:

- Turn on the Spectrophotometer by hitting the button marked UV → let warm up 10-15 minutes
- label two microfuge tubes for each sample and put 199λ of H₂O and 1λ of sample into each tube, also label one tube as a blank and put in 200λ of H₂O
- Use Program 7 on Spec by hitting “Program”. Use “STEP” to find Program 7, then press RS to begin.
- Clean out cuvette with H₂O and Acetone and read blank first → clean out cuvette after every sample → finish reading the rest of your samples
- When you are finished with the Spectrophotometer always turn off the UV by hitting the UV button

7) Calculating concentration:

-For Northern Blots, load 15ug per well if possible. For soleus, 8ug per well is usually plenty→

*take your A260 values for a sample and find the average

*next multiply the A260 average by 8 (if dilution was 1:200)

(1 A260=40ug RNA)

*then divide 15 by this number → this will give you the amount of RNA you should use in your dilution

*subtract the amount of RNA from 6 → this will give you the amount of water you should use in your dilution

*example:

$(A260 \text{ value} + A260 \text{ value}) / 2 = \text{average}$	$(.5653 + .5934) / 2 = .5794$
$\text{average} * 8 = X$	$.5794 * 8 = 4.64$
$15 / X = \text{RNA needed}$	$15 / 4.64 = 3.23\lambda \text{ RNA}$
$6 - \text{RNA} = \text{H2O needed}$	$6 - 3.2 = 2.77\lambda \text{ H2O}$

Gel Preparation

1) Making gel:

-Prewarm 10mls of 10X MOPS in a 50ml conical tube in the 55° water bath

-Put 73mls of water in a 250ml flask→ add 1.2g of agarose→ microwave until agarose is dissolved→ let the flask sit in the 55° water bath until you are ready to pour the gel

2) Preparing samples for gel:

-For each sample, mix together to make your “master mix”:

12.5λ DI formamide

4.0λ 37% formaldehyde solution

2.5λ 10X Mops

0.1λ 0.5mg/ml ethidium bromide

-Add 19.1λ of the master mix to a 1.5ml tube for each sample

-Add the amount of water that you calculated above followed by the amount of RNA. Pipet to mix.

-Cook the samples in a 60° dry bath for 15 minutes

-Quench the samples on ice for 2 minutes

-Add 2.5λ of blue RNA tracking dye to each sample and place back on ice

3) Pouring gel:

-Put tape around the edges of gel plate so that the liquid will not leak out when you pour it, and place comb in the plate

-Add 16.2mls of 37% formaldehyde to the prewarmed 10mls of 10X MOPS→ swirl together

-Add this mixture to the 250ml flask containing your agarose solution→ swirl together

-Pipete your liquid into the plate→ remove any bubbles that remain

-Let the gel solidify for at least 15 minutes or until solid

4) Loading the gel:

- Prepare a 1X MOPS buffer for the running buffer (*mix 100mls 10X Mops + 900mls H₂O*) → pour into gel rig
- Take the tape off of the gel and immerse the gel into the buffer
- Pull out the comb gently
- Load samples
- Let the gel run at 85V for at least 4 hours-longer if looking for large transcripts

Gel Transfer

1) Preparing for transfer:

- After the gel has run for about 4 hours, take a picture of the gel under the UV light → you should be able to see 28s and 18s → cut off the top left corner of the gel to give yourself an easy way to know which side is the top of the gel
- After taking a picture, get another square pyrex dish and put about an inch of de-ionized water in it and place the gel in the water → let the pyrex dish sit on the shaker for 10-15 minutes
- Cut a piece of Gene Screen the approximate size of your gel → hydrate the membrane in water for 2-3 minutes → soak it in 10X SSC in a square pyrex dish for 10-15 minutes prior to use
- Take an oblong pyrex dish and place a sequencing gel plate crosswise on top on the dish (*gel plates are in the drawers by radioactivity*)
- Pour about 500mls of 10X SSC into the oblong pyrex dish
- Cut a piece of Whatman paper that is about 6 inches longer than the oblong pyrex dish and about the width of the glass plate → soak the Whatman paper using the Under-Over method (drag paper under the plate through the 10X SSC and the up onto the plate until it is completely wet) → make sure that the ends are still in the 10X SSC since the Whatman paper is your wick
- Use a 10ml pipet to roll out any bubbles under the Whatman paper
- Cut two more pieces of Whatman paper that are about 1 inch bigger than your gel on each side

2) Transferring gel:

- After gel has soaked in DI H₂O, place it **face down** on the Whatman paper (this means the cut you made will now be on the upper right hand side)
- Roll the bubbles out using a 10ml pipet
- Place the soaked piece of gene screen on top of the gel → roll out the bubbles
- Place parafilm around each side of the gel so that there is parafilm touching every edge of the gel, this will avoid transfer from Whatman directly to the paper towels above the gel
- Place the 2 pieces of Whatman that you cut on top of the gel → using a 10ml pipet, wet the Whatman with the 10X SSC in your pyrex dish in order to get the transfer started → put about 3 inches of brown paper towels on top of the Whatman → put a glass plate on top of the paper towels and a small glass bottle that will act as a weight
- Let the gel transfer overnight (16-24 hours)

3) Take down gel:

- After the blot has transferred to the Gene Screen, check the blot under the UV to make sure the transfer worked well, use a pencil to mark 28s and 18s, and take a picture of your blot

- Also check the gel to make sure everything has transferred, it should be completely blank
- Place the membrane in the UV crosslinker and run through one auto crosslink cycle
- After the first crosslink cycle, turn the membrane over and run it through a second crosslink cycle
- Wrap the membrane in saran wrap until you are ready to prehyb

Labeling a probe:

- Use High Prime Kit from Roche (catalog number 1 585 584)
- Add 25ng of gel isolated (Qiagen Gel Extraction Kit) DNA fragment
- Add water to a total volume of 8ul
- Denature the DNA by heating at 100C for 3 minutes
- Incubate on ice for 2 minutes
- Quick spin

CHECK YOUR GLOVES!!!!

- Add:
 - 3ul ATG mix
 - 5ul 32PdCTP
 - 4ul High prime reaction (vial1) Do not take out of freezer as this is highly temp sensitive. Take pipet to the freezer to take your aliquot and put back right away.
 - Incubate 30 minute at 37C
 - Stop the reaction by adding 60ul STE

Put a clean empty tube in the bottom of the column to catch the flow-through
 Prepare column by pushing 80ul of STE through column. Push slowly, approx 1cc/sec
 Make sure at least some of the liquid has started to come out of the bottom of the column. If not, add a little more (50ul) and push again.
 Put a fresh tube beneath the column
 Add probe (80ul) to column and push through into the clean tube
 Check the tube at the bottom. Approx 50ul should be in the tube.
 Add an additional 80ul of STE and push through the column. Your tube should now contain approx. 100ul.
 Add 2ul of your probe to a dry scint vial and count
 Use this number to calculate the amount of probe to use for the hybridization.
 Store probe in plexiglass box in -20 with date labeled on the tube.

Prehybridization

Let Quik Hyb solution warm:

- Get a pyrex dish and put about ½ inch of H₂O into it → put your blot in the H₂O and let the dish shake while you are preparing for your prehyb
- Turn on the hyb oven and set to 65°C
- Get a hyb bottle from above the sink in room 824 → make sure that the blue cap has a white foam ring in it
- Take the Quik Hyb out of the 4° → invert it several times to mix
- Put 10mls of the Quik Hyb into your hyb bottle and swirl around a little → put bottle in oven to prewarm the solution for 3-5 minutes
- Take your blot out of the H₂O and roll it up and put it inside your hyb bottle, RNA side facing in
- Put hyb bottle into the oven and let it rotate for at least 20 minutes to prehyb

Hybridization

1) Add probe: **Make sure you are wearing gloves and a lab coat**

-YOU WILL NEED:

- Salmon Sperm DNA, tube labeled "SS", in -20

-labeled probe which should be stored in plexiglass box in -20

-RAD should ALWAYS be behind the shield!!

-Take a new 1.5ml microfuge tube and label it with your initials

-Add 30 λ of the Salmon Sperm DNA to the new 1.5ml tube \rightarrow add *3-5 million counts of your probe into the same tube, here's how you figure out how much probe to add:

***you should use 500,000cpm/ml of Quik Hyb solution**

-After you have added the SS DNA and your probe together, make sure the lid is locked, give it a quick spin, and then put it on the 100°C block in the radioactivity area for 10 minutes

-After the probe has boiled for 10 minutes, place it on ice immediately and let it sit on ice for 2 minutes, quick spin

-Pull your hyb bottle out of the oven and place it in one of the metal tube racks in radioactivity, put the rack behind a shield

-Pipet all of your probe into the hyb bottle containing your probe \rightarrow put the lid securely on the hyb bottle and swirl the solution around so that the probe and the Quik Hyb mix together well \rightarrow make sure the solution is covering the entire blot and put the hyb bottle back in the oven

-Let the blot rotate at 65°C for 2-4 hours

Washing the Blot

1) Washes:

-After 2-4 hours, pull hyb bottle out of oven

-Open up liquid radioactive waste:

*make sure you put a chux on the ground and put the lid to the liquid waste leaning up against the box on top of the chux

*unscrew the cap to the liquid waste and use the funnel to prevent any spillage

-Leave the door to the oven open and set the temperature to 25°C

-Add the first wash to the hyb bottle \rightarrow put about 20mls (bottom line of hyb bottle) of 2X SSC into the hyb bottle, put the lid back on the bottle and swirl it around to get all the Quik Hyb off of the membrane

-Pour out the 2X SSC into the liquid radioactive waste

-Add the second wash to the hyb bottle \rightarrow put about 20mls of 2X SSC into the hyb bottle, swirl it around to make sure it gets on the entire membrane, and put the bottle back in the oven and let it rotate for 10 minutes at 25°C

-Take your hyb bottle out of the oven and pour out the 2X SSC into the liquid radioactive waste

-Add the third wash to the hyb bottle \rightarrow put about 20mls of 2X SSC/1% SDS into the hyb bottle, swirl it around to make sure it gets on the entire membrane, and put it back in the oven to let it rotate for 10 minutes at 25°C

-Take your hyb bottle out of the oven and pour out the 2X SSC/1% SDS into the liquid radioactive waste

-Make sure the door to the oven is closed and turn the temperature up to 65°C

-Add your fourth and final wash to the hyb bottle \rightarrow put about 20mls of

1X SSC/1% SDS into the hyb bottle, swirl it around to make sure it gets on the entire membrane, and put it back in the oven to let it rotate for 10 minutes at 65°C (*the length of this hot wash may vary depending on the intensity of your signal and background*)
-After the last wash, dump out the 1X SSC/1% SDS into the sink and wash it down the drain—SIGN DRAIN LOG FOR RAD WASTE
-Pull the membrane out of the hyb bottle and place it between two paper towels and pat it to dry it off (*don't let the membrane get too dry as this will permanently crosslink the probe and the membrane*)

Getting a picture of your blot:

1) Preparing for film:

-Lay down a piece of saran wrap that is a little more than twice the size of the membrane → place the dried blot on one side of the saran wrap and pull the other side over the top of the blot, then fold up all the edges so that the blot is completely wrapped up
-Get a film cassette, film (use the smallest size that will fit your blot), and a black cassette bag and take all of this, along with your blot into the dark room

2) Putting film down:

-Do not TAPE your blot to the intensifying screens as the tape will ruin the screen!!
DOT Labels are OK to use.
-Make sure that all lights and computer screens are off in the dark room
-Lay down a piece of film directly over your blot (*make sure that the rough marker edge is on the top left-hand side of your blot → the same side as the cut you made in the blot*)
-Close the cassette lid and lock it → put the cassette inside the bag to insure that no light will get into the cassette
-Put a piece of tape with your initials on the outside of the bag and place the whole thing in a -80°

Exposing your film:

-Let the film expose overnight at first, but it may need a longer exposure..could consider STORM. Will NEED STORM is you need to quantify the bands.
-Develop the film the next morning in the dark room → Make sure the film is completely warmed to RT and dried before putting into the machine.
- bring more film with you just in case you need to expose the blot for a longer or shorter amount of time.
-Make sure the membrane is completely dry and at RT before putting another film down to expose.

Supplies:

RNAzol	Tel-Test	CS105
DEPC	Sigma	D5758
5ml Falcon tubes	Fisher	14-959-11A
8ml Starstedt tubes	Starstedt	60-542
Gene Screen	Perkin Elmer	NEF-972
MOPS	SIGMA	M1254
Whatman Paper #3	VWR	28458-005
Quik Hyb 1L	Stratagene	201221
Push Columns to label Probe	Stratagene	400702
8x10 film	Fisher	05-728-24
5x7 film	Fisher	05-728-26
32PdCTP	Perkin Elmer	BLU513H
High Prime Labeling Kit	Roche	1 585 584
Salmon Sperm DNA (sonicated)	Five Prime Three Prime	955155637
DI Formamide	Fisher	BP228-100
37% Formaldehyde	Fisher	F79-500
Chlorofom	Fisher	C298-500
Isopropanol	Fisher	A416-500

10x MOPS

Mops Free Acid (MW: 209.3) 41.86 grams
 Sodium Acetate (MW: 136.1) 6.8 grams
 EDTA 0.5M 20ml
 Adjust pH to 7.0 with NaOH (70mls 1M NaOH)
 QS to 1L
 Sterile filter
 Wrap with foil to store dark
 Store at 4C

FINAL

0.2M
 0.05M (or 4.1g of anhydrous)
 0.01M

20x SSC

Sodium Chloride 175.3g/L
 Sodium Citrate 88.2g/L
 pH to 7.0 with a few drops of HCL
 QS to 1L

FINAL

3M
 0.3M

STE

Sodium Chloride 0.5844gram 10mM
 EDTA 0.5M 2 mls 10mM
 Tris 1M pH 7.5 2 mls 20mM
 QS to 100mls