Fall 2009: GENE EXPRESSION

ANSC/GENE 626   (edited by. N. Ing 8/22/09)
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The objectives of this course are:
1. To appreciate and utilize molecular biology in order to assess gene expression
2. To handle biological macromolecules with good techniques to generate high quality data
3. To critically evaluate data and use it to make decisions in experimental plans
4. To communicate experimental purposes, methods, data, and conclusions in written informal and formal formats.

Class will be held in BICH 243 in Fall semester on Thursdays (lecture from 12:00 to 12:45 p.m.) and Fridays (lab from 1;15 to 3:30 p.m.). First meeting will be in the second week of class.

9/3 and 4 Students should read the syllabus and review the protocols independently

9/10 Lecture 1: Introduction to the course, Safety rules. What is gene expression? What are the functional parts of a gene, an mRNA and a protein?

9/11 Lab 1. Introduction: Pipetting, Restriction digestion of plasmid DNA to linearize plasmid for use as a PCR positive control.

9/17 Lecture 2: Tripure reagent allows purification of three macromolecules from one tissue sample.

9/18 Lab 2. Prepare RNA from tissue

9/24 Lecture 3: mRNA Analyses: a comparison

9/25 Lab 3. Analyze RNA on Northern gels and blot: Reverse transcribe

10/1 Lecture 4: Polymerase chain reaction and uses.

10/2 Lab 4.   PCR from reverse transcription and linearized plasmid control

10/8 Lecture 5: Molecular cloning for different purposes. Plasmid vectors.

10/9 Lab 5. Analyze PCR products on gels

10/15 Lecture 6: Molecular cloning for different purposes II

10/16 Lab 6: Ligate PCR product and into plasmid vector

10/22 Lecture 7: DNA sequence: Function and analysis. Creating plasmid maps

10/23: Lab 7: Transformation of E. coli

10/29 Lecture 8: Other RNA analyses methods (Also, overnight cultures from colonies)

10/30 Lab 8: Plasmid minipreps

11/5 Lecture 9: Restriction enzymes and analysis of plasmid DNA

11/6 Lab 9: Restriction digest of minipreps and gel analysis

11/12 Lecture 10: Acrylamide gel electrophoresis for small RNA and protein analyses and Transcription in cells and out

11/13 Lab 10: In vitro transcription and analysis of the Northern blot probe

11/19 Lecture 11: Expression of recombinant proteins (Also, perform Northern blot hybridization)

11/20 Lab 11: Northern blot washing and development

11/26 and 27 HAPPY THANKSGIVING!!!

12/3 Review of results

Students can print out the class protocols on my website: http://animalscience.tamu.edu/ning/lab/courses.htm. Protocols for the Friday lab should be read by the student before the lecture on Thurs and brought to the lecture and lab classes. Students will be evaluated on the lecture material via pop quizzes, homework problems and, if the instructor deems it necessary, exams (25%). In the lab, grades will be given on preparation and participation (25%), daily lab notebook records of activities (25%) and a written laboratory report in journal paper style (25%) due the Thursday after the last class at 5 PM. A suggested text (An Introduction to Genetic Engineering 2nd ed. by Desmond S.T. Nicholl) has many relevant sections especially for the beginning material. [*Technique Videos viewed in class may be checked out from Kleberg 410/407 for 1 h during the day or overnight after 4PM (returned by 9AM).]

N.Ing LAB RULES

A. GENERAL

1. Everyone is individually responsible for the experiments. Come prepared by reading protocols and required reading in advance!! Activities will be started immediately, while explanations and discussion sessions will occur as time permits. COME PREPARED to ASK QUESTIONS, especially during discussions.

2. Equipment in this and neighboring labs is shared. Know or ask how to use it. Obey user rules, such as signing logs. Leave all equipment in good working order. If there are problems, tell someone so we can fix them!

3. Leave the lab better than you found it. Wash your own glassware, clean up your work area, write the names of reagents that are running out on the "to be ordered" list, etc.

4. **Lab notebooks** are bound volumes, are kept in black ball point pen with numbered, dated pages. They are designated only to that purpose, are labeled on the cover with researcher name, dates of inquiry and laboratory class, and are only removed from the lab with instructor permission. In them, each person describes their activities and observations each day. Each page should be dated. Each data photo or X-ray film should be labeled with initials, date, and identification of gel type, lane contents and dye migration and affixed to a page in the notebook. Record things chronologically. Start a new project on a new page with a description of its purpose. Complete protocols from the class don't have to be written each time in the notebook. Instead, write out the relevant information and refer to the handouts for specifics. For this, you may consider the handouts you receive in lab as references. Use them as such and refer to specific pages (dates) within them. Record your activities and observations clearly, using complete and understandable sentences. Write down the composition of your reagents, including buffers and buffer recipes. A stranger should be able to pick up your notebook and understand why you did an experiment, how you did it, and what results you got. Indeed, they should be able to duplicate your experiments.

KEEP UP WITH NOTEBOOK ENTRIES EVERY DAY…otherwise, data will be lost!

5. All reagents and samples saved must be labeled with black sharpie stating the date; your initials, and WHAT IT IS. Professional lab workers use simple sample numbers, such as 1 – 10 that is unique for the date. The full description of the sample is then put in the notebook. Items not labeled sufficiently are worthless and will be discarded.

6. Store things in appropriate places! For plasmids and reactions and buffers, store at –20C in storage box provided to your group unless otherwise noted. Note storage places in your notebook.

N.Ing LAB RULES (cont'd)

B. SAFETY IS THE #1 PRIORITY

1. The only safety activity not strictly enforced is wearing safety glasses: this is a good idea but is not mandatory. Wearing a lab coat is mandatory and wearing gloves will become a habit (see below).

2. Working with open flames and hazardous chemicals have strict safety protocols - ask for them and follow them.

3. Working with radioactivity is a privilege, not a right. Workers must monitor for contamination, before, during, and after the procedure. Radiation safety training is required. WE RUN A CLEAN LAB.

4. Because we work with HAZARDOUS SUBSTANCES, there is NO EATING, DRINKING, SMOKING, or APPLYING MAKE-UP in the lab.

5. Garbage must be disposed of properly. Glass and sharps, biohazard, chemical, and radioactive waste must be separated from the rest.

C. GOOD LAB TECHNIQUES

1. ICE IS NICE! Get it in BioBio Room 229. Work on it unless otherwise directed. It slows degradation of macromolecules.

2. Many reagents settle on storage, so mix them! All frozen solutions need to be thawed and mixed before using.

3. ENZYMES DO OUR WORK. They are stable as glycerol solutions at -20?C. Keep them in the freezer as much as possible. Only remove them in -20oC blocks. DO NOT WARM ENZYME STOCKS! When pipetting small amounts of viscous solutions like enzymes, check loaded pipet tip and evacuated one to assure that enzyme got into the reaction. After addition, mix reaction solution gently but thoroughly: can pipet total volume up and down OR vortex gently and flash spin in microfuge to return reaction to the tube bottom.

ADA Statement, Copyrights, and Plagiarism

The Americans with Disabilities Act (ADA) is a federal antidiscrimination statute that provides comprehensive civil rights protection for persons with disabilities. Among other things this legislation requires that all students with disabilities be guaranteed a learning environment that provides for reasonable accommodation of their disabilities. If you believe you have a disability requiring an accommodation, please contact the Dept. of Student Life, Services for Students with Disabilities in Room 126 of the Koldus Bldg. or call 845-1637.

Copyrights
The handouts used in this course are copyrighted. By handouts", I mean all materials generated for this class, which include but are not limited to syllabi, quizzes, exams, lab problems, in-class materials, review sheets, and additional problem sets. Because these materials are copyrighted, you do not have the right to copy the handouts unless I expressly grant permission.

Plagiarism
As commonly defined, plagiarism consists of passing off as one’s own ideas, words, writings, etc., which belong to another. In accordance with this definition, you are committing plagiarism if you copy the work of another person and turn it in as you own, even if you should have the permission of that person. Plagiarism is one of the worst academic sins, for the plagiarist destroys the trust among colleagues, without which research cannot safely be communicated.

If you have any questions regarding plagiarism, please consult the latest issue of the Texas A&M University Student Rules, under the section "Scholastic Dishonesty."

BIO 243 Lab Safety Precautions

1. ABSOLUTELY no food or drink in the lab. This includes gum.

2. Always wear gloves when working with hazardous compounds: ethidium bromide, radioactive compounds, acrylamide and organic compounds. Wear gloves to protect reactions from contamination from you (RNase, DNA) when working with RNA and PCR.

3. Always wear UV safety glasses when using UV illumination.

4. Use special care when working with open flames. Don't forget to turn off gas after use.

5. Clean up spills immediately. Notify instructor if hazardous compounds are spilled.

6. Discard organic solutions in appropriate waste bottles in fume hoods.

7. Discard Ethidium bromide waste in correct container.

8. All culture medium and labware used for bacteria needs to be autoclaved or add Chlorox to 5 or 10 % before disposal and washing.

9. After using radioactive materials, wash the work area and clean spills immediately. Dispose of gloves in radioactive waste as soon as you are finished.

10. All sharps (including broken glass, needles and razor blades) should be disposed in clearly marked containers, not in the general trash.

11. If you have questions about anything, ASK!

12. Lab coats are required!

I have read and will follow the above safety rules.

Signed ______________________________
Date_____________________________________

LAB REPORTS -
by Linda Guarino and Nancy Ing

TITLE - This is the most important part of a manuscript. A reader begins here, and will also finish here if the title does not promise a subject of interest to him/her. A good overall rule is to use the fewest possible words that adequately describe the contents of the paper. But, do not sacrifice words for specific information. For example 'DNA cloning' is a short title, but it is too general. A popular trend in recent years is to publish papers where the title is a complete sentence that summarizes the major conclusion of the manuscript. Personally, I prefer titles that describe the work, not the results.

ABSTRACT - An abstract is a mini version of the paper. It should provide a brief (less than 250 words) summary of the major points of the manuscript. The abstract should state the objectives, describe the methodology used, summarize the results, and state the principle conclusions. The abstract should be written in the past tense, because it refers to work done.

INTRODUCTION - First of all, state the nature and scope of the problem investigated. Review the pertinent literature (NOT NECESSARY FOR THIS CLASS). Describe the method of the investigation. State the principle results. State the principle conclusions suggested by the results. The first two parts should be in present tense, while comments relating to the present study should be in past tense.

METHODS - The methods section should expand upon the description of the methodology that was presented in the abstract. The order of presentation is usually chronological (methods used in initial stages of the study are presented first). However, sometimes it makes more sense to group similar methods into sections, even though they were not used at the same time. Due to space limitations in journals, methods are not usually described in detail if they have previously been published. If a scientist uses a protocol that is identical to one previously described, he/she would state 'The DNA was prepared according the procedure previously described (reference). If there were minor differences, he/she would state 'according to the procedure of (ref.) with minor modifications' and then describe the modifications. In this class, you may assume that the class protocol has been published. Therefore you don't need to give the details, but you need to describe the general strategy. For example, you should say 'the DNA was purified by the alkaline lysis procedure as previously described' not 'the DNA was purified as previously described'. In addition to the class protocol, you could also reference the Cloning manual or the Promega manual. The methods section should be written in past tense.

RESULTS - The results section is a presentation of the data. It should not repeat the methods given in the previous section. Each figure should be referred to here. The results section should be written in past tense.

DISCUSSION - The discussion should put the results into perspective. Discuss the results without recapitulating the results section. Show how your results and interpretations agree. State your conclusions clearly, and summarize the evidence for each conclusion. Selection of correct tense is more difficult in the discussion than in the other sections. Your own work should be described in past tense. If reference is made to published work, it should be in present tense.

REFERENCES - Only need to cite the class protocols and any other sources of material...no need for literature review so these are very few.

FIGURES and FIGURE LEGENDS - Present the important data in figure form, raw data if possible for this class. Figures should have complete legends - so that they can be understood without reading the rest of the paper. Figure legends begin with a title for the figure. Then the legend should completely describe what the figure shows. Therefore, the figure legends may contain information that is redundant with information in the Methods and Results sections. Figures and figure legends may be nested in the paper or placed at the back.

Many students have asked about length. The best rule that I can give you is that it should be long enough to convince me that you have learned something. However, I have a short attention span, and if your paper is very long and verbose, I may lose interest before I decide whether or not you have learned anything.

Suggested reading:

Day, R. A. 1988 How to write and publish a scientific paper, 3rd ed. Oryx Press, Phoenix.

9/11/09 ***GENE EXPRESSION LAB 1***

A. **Practice pipeting:** The better you are at pipeting, the better your results will be!

{Can do 1, then 2-6 then 7 or 1, then 7 then 2-6.}

1. Read instructions for Use of Micropipettors (below).
2. Tare a 1.5 ml test tube on the balance.
4. Weigh 1000 ul of water two times.
5. Repeat with Isopropanol.
6. Determine the densities of these liquids.
7. Measure the volumes of the unknown samples provided in the 1.5ml test tubes labeled "A", "B", and "C".
8. Check your results with an instructor.

Use of Micropipettors

1. Choose the correct pipet. For volumes:
   1-20 µl P20
   20-200 P200
   200 - 1000 P1000

2. Set the desired volume by holding the pipetman in one hand and turning the volume adjustment knob until the correct volume shows on the indicator. For best precision, always approach the desired volume by dialing downward (at least one-third revolution) from a larger volume setting.

3. Attach a new tip to the shaft of the pipet. Press tip on firmly to ensure airtight seal. Chose the correct tip.
   P20 yellow tip
   P200 yellow tip
   P1000 blue tip

4. Depress plunger to first positive stop. Hold pipetman vertically and immerse disposable tip into sample liquid 2mm.

5. Allow the push button to return slowly to the up position. Never permit it to snap up.

6. Wait 1 or 2 seconds to ensure that the full volume of the sample is drawn into the tip.

7. Withdraw tip from the sample liquid. Wipe the sides of the tip on the sides of tube to remove any remaining liquid.

8. To dispense the sample, place the tip end against the side wall of the receiving vessel and depress the plunger slowly to the first stop. Then depress the plunger to the second stop to expel any residual liquid in the tip.

9. With the plunger fully depressed, withdraw pipetman from the vessel. Then allow the plunger to return to the top position.

10. Discard tip by depressing the tip ejector button. A fresh tip should be used for each sample.

B. Restriction digestion of plasmid DNA for use as a positive control for PCR.

NOTE: Restriction Enzyme digestions usually contain 1-2 ug DNA and 5-12 units of enzyme in 20 ul. Working buffer strength is always 1X. Use the buffer supplied with the enzyme (optimized) if it is a single enzyme cut. If using two enzymes, use a buffer compatible to both that gives at least 75% effectiveness! There is a table in the Promega book that will help you choose.

1.. Plan a 20 ul restriction digest for the unknown plasmid containing a cDNA that you were given. Plasmid concentration is also given. From that calculate the volume of plasmid you will add to the reaction to get 0.5 ug:

____ ul H2O (to make reaction 20 ul final volume)

____ ul (0.5 ug) plasmid DNA
2 ul (which??????) 10X Buffer
1 ul Xba I restriction enzyme
20 ul TOTAL volume

NOTES ON USING ENZYMES: These are the expensive workhorses of molecular biology. Your success depends on their function. Therefore, they should be handled with care: KEEP THEM AT -20^{o}C at all times by setting up the entire reaction prior to retrieving them from the freezer. Keep them in the -20^{o}C blocks while pipeting. They are in glycerol, so are viscous. Visually check that enzyme is picked up in the pipet tip and delivered into each tube. Ensure their purity by using new pipette tips each time!!!! After addition, mix the enzyme into the reaction by gently pipeting the whole reaction up and down, or by vortexing gently and flash microfuging.

2. Check calculations for the reaction with the instructor, then set up the digestion.
3. Incubate at 37oC for 1 h.
4. Store reactions at -20oC.

GENE EXPRESSION LAB 2 ning 9/18/09

RNA ISOLATION FROM MAMMALIAN TISSUE

TODAY you will extract RNA from tissues for analysis on a Northern blot and generating cDNAs for cloning. The amount of RNA obtained is measured by sample absorbance at 260nm. To assess RNA quality, the RNA preparation is analyzed on a denaturing gel. This is transferred to a membrane ("Northern blotting") for hybridization with probes for specific mRNAs.

A. Preparing Materials for RNA Work

Avoiding degradation of RNA by RNase

You need to read about RNase, a ubiquitous enzyme that efficiently destroys RNA. Primarily, this will serve to make you paranoid and do neurotic things, like wear gloves all the time. Although working with RNA is similar to working with DNA, many RNA experiments fail miserably because of RNase, so know this enemy!

In biochemistry, RNase is the model of an enzyme that will not die: not in an autoclave or even after dehydration (by alcohol, etc.). As soon as it returns to a water environment between room temperature (R.T.) and 37oC, it chews again. It is an enzyme of all living things and is important in keeping RNA turnover high so cells aren't overwhelmed by too much RNA and so new expression of genes tightly regulates cell function.

The best way to beat RNase is to avoid it. Work with the cleanest reagents and lab-ware. Things that aren't handled by or contaminated by living things are generally RNase-free; e.g. paper towels. Test tubes and pipettes don't have to be sterile but should be used from freshly opened packages. Then protect packages from dust and fingers by resealing packages and storing in cabinets. Glassware is reserved similarly: wrapped and stored away from general use. Equipment like Pipettemen and Gel apparati for RNA are reserved for this use and are NOT USED WITH RNase!

Solutions are made with water of the highest purity. Dry chemicals are shaken out of containers: residual amounts are discarded. Nothing dirty is introduced into chemical stocks: solutions or powders.

All solutions are treated with 0.1% diethyl pyrocarbonate (DEPC). This oily liquid is added. The solution is shaken vigorously until foamy (aerobic workout). The solution is incubated 37oC or room temperature overnight to allow the DEPC to covalently attack RNase. The solution is then autoclaved to destroy DEPC (which also attacks RNA) and to sterilize to prevent growth of undesirables. Exceptions to this solution preparation protocol are 20% SDS (nothing grows in this) and Tris solutions (which DEPC attacks, too). NOTE: DEPC treatment can only correct a low level of RNase contamination! You must start clean!!!

Make 2 li DEPC-H2O and 1 li of 20XSSC per person
1. In 1 liter bottle add nanopure H2O to 1 liter level for DEPC-H2O. For 20X SSC, add 175 g NaCl and 88 g Na citrate to a 1 li bottle and dissolve in nanopure H2O.
2. Add 1ml DEPC per liter.
3. Shake till foamy for 10 sec.
4. Put in 37oC incubator O/N to allow DEPC to work optimally.

These will be autobclaved for 40 min to destroy DEPC before use.
[Autoclave the DEPC-H2O to destroy DEPC and prevent any growth in solutions that might introduce RNase.NOTE on Autoclaving: Need 35 to 40 min. sterilization time for 1 liter. 20 min. for 500ml. Use "liquid" cycle and keep caps loose]

B. RNA Extraction

NOTE 1: Tripure has phenol and guanidine salts in it...both are caustic and burn skin!!! Be careful! Wear safety glasses!!!
NOTE 2: CHCl3 (Chloroform) dissolves things like styrofoam and polystyrene - use glass   graduated pipets and polypropylene 15 & 50 ml tubes.

You know how to fight RNase to keep materials clean. GUESS WHAT! RNase is in all living systems including the one from which you'll purify RNA. So all RNA preparers begin with the realization that their worst enzyme enemy is present in the sample. In the cell, RNA is compartmentalized away from RNase so many tissues are OK for harvesting for RNA if kept cool 2-6 h after collection (of course, faster may be better). But freezing breaks intra-cellular membranes, mixing RNA with RNase. Therefore, fresh tissues are kept cool while mincing and weighing, then are put in a 1.5 ml polypropylene tube snap frozen in liquid N2. They are stored at -80oC. They may store well for 6 months but usually not for 1-2 years. This is dependent on them avoiding thawing, too. So the TWO MAIN POINTS about tissue collection are to SNAP FREEZE and KEEP at -80oC until use within 1 year. NOTE: You can't snap freeze things much bigger than 0.5 cm3. I mince to about 5 mm or less. Tissues vary with RNase content and amount of connective tissue present, so RNA yields vary in quality and amount. RNA extraction from cultured cells results in very high quality RNA, usually.

RNA Extraction from tissue with Roche TriPure reagent (contains phenol! see NOTE 1 below)
Each student will do 2 RNA preps (one from pig endometrium, one from horse testis).
Label all tubes needed NOW!

1. Homogenize 0.25 – 0.5 mg tissue (frozen or fresh) in 5 ml room temperature ("RT") Tripure solution in a 15 ml polypropylene tube. Use three 15 sec bursts at 70% power. Rinse probe in tripure (do a mock homogenization with Tripure and no tissue) between dissimilar samples.
2. Incubate RT 5min. During this time, transfer the contents equally into 4 - 1.5 ml tubes.
3. Add 250 ul chloroform to each 1.5 ml tube using a P-1000. Mix by vortexing or shaking vigorously 15 sec.

4. Incubate RT 5 min.
5. Centrifuge 15 min at 10,000 rpm at RT or 4oC in a microfuge.
6. Transfer upper phase to four clean 1.5 ml tubes with transfer pipet. AVOID THE INTERPHASE!!!! Discard lower phase and interface in phenol waste container.
7. Precipitate RNA by adding an equal volume of isopropanol. Mix by inverting tube. Incubate RT 5 min
8. Centrifuge at 10,000 rpm for 10 min at RT.
9. Wash pellet in 75% EtOH (make 10 ml with 100% EtOH and DEPC H20). This means to discard the supernatant, add the supernatant volume of wash (75% EtOH), vortex, microfuge 5 min, and discard supernatant. The purpose is to wash salts out of the RNA pellet, which should not dissolve during the procedure.
10. Air dry pellet briefly after spin. You can wipe the sides of the tubes with Kimwipes, but stay away from the pellets! Do not dry totally or you will not be able to solubilize RNA easily!!!

11. Dissolve the four similar pellets each in 25 ul 1 mM Na citrate Buffer/pH 6.4 or TE buffer (10 mM Tris, 1 mM EDTA pH8).
(Heat in 70oC block and vortex hard and repeatedly over 15 minutes.) Pool so that you have a 100 ul sample for each RNA prep.

{***RNA STORAGE***: Store at 4oC during sample use (this class). For storage over 1 week, can store at -80oC. For longer storage, add 3 volumes of 100% ethanol and store at -80oC.}

C. OPTIONAL: Analyzing Extracted RNA by Absorbances

Absorbance measures of DNA & RNA at 260 nm are used to estimate concentrations of nucleic acids. An Absorbance of 1.0 for solution of double-stranded (ds) DNA has =50 ug/ml while RNA has A260 =40 ug/ml and single-stranded (ss) DNA A260 =37 ug/ml. An unknown sample of RNA can be measured for A260 and [RNA] = A260 X dilution factor X 40

The ratio of A260/A280 is an indication of the purity of the nucleic acid. The ratio for pure aqueous DNA is 1.8 while for RNA it is 2.0. Protein, phenol, EtOH and other things often lower these ratios because they absorb at A280.

2. Add 3 ml of DEPC-H2O to four 4 ml UV transparent disposable cuvettes.

3. AT very top of cuvette, label one "Blank" and the others after the two RNA samples and a positive control: 10mg/ml salmon sperm DNA.

4. Add 12 ul aliquots of samples to @ tube except "Blank". Mix by inverting after blocking top with Parafilm.

5. Blank the spectrophotometer to read 0 absorbance at A 260 nm with the "Blank" cuvette. Then repeat with dilute samples in cuvettes. Repeat the procedure for measuring the A280 of samples.

6. Estimate [RNA] (ug/ml) = A260* diln. Factor* 40
= A260 * 250 * 40
Therefore: [RNA] (mg/ml) = A260 * 10 (= ug/ul, too!)

7. Pipet 32 ug RNA into a clean 1.5 ml tube for each RNA prep. We want 32 ug aliquots of RNA for running replicate 8 ug samples on a Northern gel. Bring volume is to 15 ul with DEPC-H2O. Store at -80oC.

NOTES:

1. If concentration is too dilute to get 32 ug in 15 ul, see the instructor. You may be instructed to precipitate the RNA from the larger volume by adding 3 volumes of 100% EtOH and 0.1 volumes of 3 M NaAc/pH 5.2. Vortex and store at -80oC.

2. A good way to store RNA without degradation for years is to add 3 vol 100% EtOH and store at -80oC

You can watch ***VIDEO on mRNA ISOLATION.***

GENE EXPRESSION LAB 3 ning 9/25/09

RNA ANALYSIS ON NORTHERN GELS/BLOTS

[See NorthernMax (Ambion) protocol **]**

TODAY we will electrophorese RNA from tissue and controls on a Northern gel that will be blotted to a nylon membrane for probing with an antisense complementary RNA (cRNA) probe.

Be clean **–** clean and protect apparati from Rnase contamination- work on clean diapers! Use all DEPC reagents, gloved hands and aerosol barrier tips.

I. Your gels have already been poured this morning for you as described below: **2 people per gel: one student load the top row, one the bottom row of wells. Top and bottom of gels should contain two replicate sets of samples.**

1. Rinse beaker/flask well with house-distilled water. Melt agarose (0.8 g) in 72 ml DEPC-H2O in an RNASE-Free glass bottle or beaker. (Bring to a boil in microwave oven and mix by swirling: repeat 2 to 3 times).
2. Cool to 70oC.
3. In a fume hood, add 8 ml 10X Denaturing Gel buffer (formaldehyde and MOPS/pH 7.0, NaAc, and EDTA) and pour into RNase-free gel mold with the ends taped. Use two thin combs.

II. You need to prepare your samples A to C:

A. Add 45 ul of Northern Sample Loading buffer (+ EtBr) to 7.5 ul of your RNA prep from the last class.
B. Get a 5 ul aliquot of positive control RNA from the instructor. Add 15 ul Northern Sample Loading buffer (+ EtBr)

C. Get a plasmid DNA template for in vitro transcription of the cRNA probe from the instructor (for actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or 18S). It will serve as a positive control for electrophoresis, transfer, hybridization, and development of signals. Make a 10 ng sample in 20 ul of Northern Sample Loading buffer (+ EtBr).

All samples should be heated at 68oC for 10 min. and then chilled on ice

III. Run gel.

Clean a gel rig with RNAseZAP! And fill it with 1 li of 1X Gel Running Buffer. Remove tape from gel and submerge it before removing the combs.

A.. Load samples A to C onto the gel under 1X Gel Running Buffer (dilute the 10X stock from the kit with your DEPC-H2O bottle, cover the gel with buffer to about 0.5 cm depth). We won't use RNA markers…we can use the internal ribosomal RNAs (rRNAs) as internal markers in samples A and B.
B. Run at 100 volts until dye front reaches bottom or sufficient separation occurs (1 to 1.5 h); can peek at gel progress with hand-held short wave UV lamp. Prepare materials for blotting during this time!!!!!!!!!!!!
C. Take photo on UV box alongside a fluorescent ruler.

IV. Northern transfer to Brightstar nylon membrane
1. Cut wicks (2 pieces 14 cm X > 30 cm), blotting papers (3 pieces 11 X 14 cm), and nylon membrane (1 piece of 11 X 14 cm) wet in transfer buffer as directed and pictured in the diagram. Also need paper towels cut in half to be bout 14 X 11 cm….need a 4 inch tall stack of these. (This is different than that in the NorthernMax kit instructions)

2. Assemble an upward capillary transfer as instructed in the diagram. You can use the rig you ran the gel in to do the transfer to nylon. Allow the transfer to continue with 500 ml of 10X SSC until the next lab.

QUESTION to be answered in your notebook: What are the compositions of your northern gel, running buffer and sample buffer used today?

REVERSE TRANSCRIPTION:

Today each student will also reverse transcribe their RNA preps with random primers to make cDNA, which is capable of being amplified by PCR. Each student should set up three reverse transcription reactions: two will be the real reactions, one for each RNA prep containing enzyme (#1 and #2). The last will be a mock reaction lacking enzyme (#3). Can use either RNA prep for #3.

Label two sterile 500 ul tubes. From the Superscript II Reverse Transcriptase protocol:
1. For each 20 ul RT reaction, combine in a tube:

Random primer	0.25 ug/ul	1 ul
1ng – 5ug of total RNA		X? ul
dNTP mix (10mM each)		1 ul
Sterile, distilled water		to 12 ul total

2. Heat tubes to 65 degrees C for 5 minutes, quick chill on ice, and flash spin, and add to each;
4 ul 5X First Strand buffer (with kit)
2 ul 0.1M DTT (with kit)
1 ul Rnasin (Promega)
3. Mix contents of the tubes gently. Incubate at 25 degrees C (room temp.) for 2 minutes.
4. Add 1 ul of Superscript II enzyme to reaction #1 and mix by pipetting up and down. To reaction #2, add 1 ul H2O and mix similarly.
5. Incubate tubes at 25 degrees (room temp.) for 10 minutes. Then incubate reactions at 42 degrees C for 50 minutes.

6. Inactivate by heating at 70 degrees C for 15 minutes. Store in -20ºC.

GENE EXPRESSION Lab 4 ning 10/2/09

Northern Blot crosslinking and PCR amplification of a cDNA

A.   PCR AMPLIFICATION OF A cDNA

Today each student will amplify a cDNA fragment from a specific mRNA (unknown, will be identified by sequence analysis later) among the reverse transcribed cDNAs in the reverse transcription reaction from last week. One negative control is water (no DNA template). This control is a must for all PCR runs. Another negative control is the mock reverse transcription reaction that lacked enzyme. A positive control is the linearized plasmid containing the cDNA from Lab 1.

For PCR you should be clean! This means do not contaminate the reactions with your DNA (on your hands and on pipettors). Wear gloves and use aerosol barrier tips to optimize cleanliness.

Label 4 thin-wall 200ul PCR tubes around the necks of the tubes. Primers will be provided at concentrations of 70 ng/ul
Each student will set up 4 - 50 ul reactions, so make a Master Mix of common reagents for 5 reactions in a 500 ul tube:

	In one reaction:	In Master Mix for 5 reactions:
H20	40.75 ul	203.75 ul
10X Taq Buffer	5 ul	25 ul
10 mM dNTPs	1 ul	5 ul
Primer 1	1 ul	5 ul
Primer 2	1 ul	5 ul
Taq enzyme	0.25 ul	1.25 ul
TOTAL	49 ul	

Pipet 49 ul of Master Mix into each labeled PCR tube.
Then add the unique DNA template (1 ul) that will be amplified from during the PCR:
1. Water (no DNA template)
2. Reverse transcription reaction
3. Mock reverse transcription reaction
4. Linearized plasmid DNA containing the cDNA – Made in the first lab.

Place the tubes in the PCR machine and run a program for 35 cycles of:
94ºC denaturation for 30 sec,
45ºC annealing for 1 min, and
72ºC extension for 1 min.

The machines will run the PCR then the tubes will be stored at 4ºC until the next lab.

B. Northern Blot crosslinking

1. Remove all papers from the Northern transfer but keep the gel and blot/filter together.

2. IMPORTANT: Mark well positions on the blot with a sharp pencil or a black Sharpie marker. Also write initials and date. Put these marks on filter's back.

NOTE: Keep the blot RNA-side-up during subsequent handling and keep it in a clean ***labeled*** container.

3. Rinse agarose particles from filter in 2X SSC with vigorous agitation for 30 sec.

4. Put blot on plastic wrap and view it on a UV box. On the side of the blot, mark positions of 28S and 18S rRNAs if those bands were visible in your tissue RNA lanes.

5. Look at the gel on the UV box. Did all of the RNA transfer out?

6. Place wet blot on top of Whatman paper saturated with 2X SSC - all on top of plastic wrap.

7. UV crosslink RNA to nylon (use Stratalinker in energy mode: 120,000 ujoules).

8. Store the blot between clean paper towels in a labeled Ziploc bag in the -20oC.

7. Clean, dry and put away your labware used for blot transfer.

DISCUSS: DNA sequence and structure using human nucleotide models

1. Make a six base DNA single strand (random sequence)…Identify 5' and 3' ends. What type of bonds exist between bases?
2. What is the chance that a specific six base sequence will occur?
3. Make a complementary DNA strand hybridized to the one in #1. How are the strands oriented to each other? What type of chemical bonds exist between the DNA strands?
4. Reverse the 5' to 3' direction of the complementary strand. What bonds (if any) did you have to break to do this? How is this strand related to the initial strand?
5. Make an EcoR I restriction enzyme site. What bonds does the enzyme cut? What makes the ends "sticky"? Do the ends have 5' or 3' overhangs or one of each?

Now restore the restriction enzyme site integrity with T4 DNA ligase.

GENE EXPRESSION Lab 5 ning 10/9/09

Analyze PCR products on agarose gels

MAKE a 1% agarose gel for DNA analysis: See appendix. Need 1 gel per 2 students.

PREPARING SAMPLES AND RUNNING THE GEL

1. In clean tubes, one for each PCR reaction, combine 20 ul of PCR product with 2ul of 10X DNA Loading buffer containing two tracking dyes (bromphenol blue and xylene cyanol). Mix well.
2. Submerge the gel under 900 mls of 1X TAE buffer in the running chamber.
3. Load the 20 ul PCR samples plus dye alongside a sample of 10ul (1 ug) Lambda HindIII EcoR I DNA markers in 5 lanes of your gel. Record your loading order in your notebook!
4. Run the gel at 100 V for 45 min.
5. After the gel run, visualize bands with short wave UV light and take a picture with the camera imaging system. (Wear goggles!)

ANALYSES

1. By comparing the bands in PCR sample lanes with Lambda standards, one can describe
  a. The molecular size of the PCR product and
  b. The amount of DNA in PCR product.

For (a), compare <u>migration distance</u> to that of Lambda standard fragments - estimate bp size.

For (b), compare <u>brightness</u> of bands to those of Lambda fragments - estimate ng. Divide by ul of original DNA sample to get its [DNA]. This is often more reliable DNA quantitation than A260 measures!!!

NOTE: On the periphery of the picture (not on the data itself) MARK DYE POSITIONS ON GEL PHOTOGRAPHS. Label all photos completely! Name, date, identity of gel and lane components!!!

GENE EXPRESSION LAB 6 **10/15/09**

Ligate PCR product (cDNA insert) to T-overhang pBluescript II KS+ (plasmid vector)

Fresh PCR product (cDNA) was generated last night to use in this TA cloning procedure. Gel analysis revealed that the PCR product was in high concentration and of expected size. **TASK:** Estimate the concentration and size from the photograph of that gel and put that in your notebook.

The cDNA band was strong and no other DNAs were in the PCR reaction so THERE IS NO NEED TO GEL PURIFY THE cDNA.

THEREFORE, the ligation procedure has been modified to give you the best success for clone generation.

Each student will make 3 ligation reactions: All three have 2 ul vector (T overhang pBluescript II KS+). One ligation reaction will use "maximal" levels of insert cDNA (3 ul, "MAX"), one ligation reaction will use "minimal" levels (1 ul, "MIN"), and one ligation reaction will lack insert cDNA ("negative control").

1. THAW THE 3 TUBES CONTAINING THE VECTOR ON ICE.
SET UP THE LIGATION REACTIONS ON ICE -----IN THE TUBES CONTAINING THE VECTOR. Label these tubes as your ligation reactions.
2. Thaw the 10X Ligation Buffer on ice (it has ATP in it which is heat labile).
3. Add 1 ul of 10X Ligation Buffer to each ligation reaction tube.
4. Add the appropriate amount of PCR product to each ligation reaction tube (see below).
5. Add the appropriate amount of H2O to each ligation reaction tube (see below).
6. Add 1 ul T4 DNA Ligase to each tube.

tube	Vector DNA	Insert DNA	H2O
MAX	2 ul plasmid vector	3 ul cDNA insert	3 ul
MIN	2 ul plasmid vector	1 ul cDNA insert	5 ul
Negative Control	2 ul plasmid vector	none	6 ul

Combine and mix components gently but well and keep on ice. Incubate 15°C overnight.

Homework - DNA sequence work described in "Lecture 7". With the raw sequence data provided for the clone you are making, use the NCBI website to analyze the DNA. Have this complete before the next lab so you can enter your answers to the four questions in your notebook at that time. We will review DNA sequencing in next Thurs. class, as well.

For ***GENE EXPRESSION "LECTURE" 7 ning 10/22/09***

WHAT IS THE GENE PRODUCT ENCODED IN YOUR cDNA?

Sequence analysis and similarity searches in GenBank

In this lab we'll identify the cDNA in your plasmid and the protein product it encodes. You only need one cDNA sequence per plasmid clone, and probably only 50 - 100 bases of that to identify the cDNA…choose your best chromatogram of the sequencing. The sequence reads from left to right as 5’ to 3’ and will begin about 50 bases beyond the primer binding site. The first landmarks I look for on a sequencing chromatogram are the cloning sites. Since you know the vector sequence, you can follow that as well. You should identify the PCR primers in the sequence (NOTE: the complement will be present for the primer furthest from the sequencing primer). Make an electronic file of the cDNA sequence between the PCR primers. You can use either the sense or antisense strand. Make sure you enter the sequence in the 5’ to 3’ direction. If you do not, the reverse sequence actually bears no relation to the correct one except for having the same overall nucleotide composition. With the cDNA sequence, search for similar sequences in GenBank with the BLAST software at the Genbank website (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html). Choose "BLAST Sequence similarity searching." Choose "Basic BLAST search." The program is "blastn" and database to be searched is "nr" the nonredundant nucleotide database. Enter the sequences of the cDNA in "FASTA Format": the first line can contain a name of the sequence and other information preceded by a ">" . Hit "enter" and start entering sequence only on the next line. If you are working during the weekday, sometimes the search takes a while. You can e-mail the results to yourself to save yourself from a long wait. Or you can wait and get the results from the website. Sample BLAST results are:

```
<b>BLASTN 2.0.3 [Nov-14-1997]</b>
<b>Reference:</b> Altschul, Stephen F., Thomas L. Madden, Alejandro A.
Sch&auml;ffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman
(1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs", Nucleic Acids Res. 25:3389-3402.

Query=
(63 letters)

Database: Non-redundant GenBank+EMBL+DDBJ+PDB sequences
323,678 sequences; 623,222,891 total letters

Searching done

Score E

Sequences producing significant alignments: (bits) Value

emb|X06624|OCPRR3 Human progesterone receptor mRNA 3'-flank 109 3e-23
emb|X51730|HSPREC Human mRNA and promoter DNA for progesterone r... 48 9e-05
gb|U91328|HSU91328 Human hereditary haemochromatosis region, his... 36 0.36
gb|U09532|HFU09532 Heterodontus francisci clone HFB15 T cell rec... 34 1.4
dbj|AB004541|AB004541 Dugesia japonica mRNA for serotonin recept... 34 1.4
emb|X59371|SCURA1 Yeast URA1 gene for dihydroorotic acid dehydro... 34 1.4
emb|X75951|SC6ORF S.cerevisiae URA1, SAC1, RSD1 and TRP3 genes a... 34 1.4
emb|Z28215|SCYKL215C S.cerevisiae chromosome XI reading frame OR... 34 1.4
```

```
>emb|X06624|OCPRR3 Human progesterone receptor mRNA 3'-flank
Length = 3566
Score = 109 bits (55), Expect = 3e-23
Identities = 62/63 (98%), Positives = 62/63 (98%), Gaps = 1/63 (1%)

Query:     1 attattgaagtaagctatgtcttacccatactatttcatacccatttaagtgaggatttt 60
             |||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||
Sbjct:   273 attattgaagtaagctatgtcttacccatactatttcata-ccatttaagtgaggatttt 331

>emb|X51730|HSPREC Human mRNA and promoter DNA for progesterone receptor
Length = 5003
Score = 48.1 bits (24), Expect = 9e-05
Identities = 49/56 (87%), Positives = 49/56 (87%), Gaps = 1/56 (1%)

Query:     8 aagtaagctatgtcttacccatactatttcatacccatttaagtgaggatttttaa 63
             |||||| |||| ||||| ||||| ||||||||| |||| || ||||||||||||||
Sbjct:  4512 aagtaaactatatcttatccatattatttcata-ccatgtaggtgaggatttttaa 4566



>gb|U91328|HSU91328 Human hereditary haemochromatosis region, histone 2A-like protein gene,
hereditary haemochromatosis (HLA-H) gene, RoRet gene, and
sodium phosphate transporter (NPT3) gene, complete cds
Length = 246282
Score = 36.2 bits (18), Expect = 0.36
Identities = 21/22 (95%), Positives = 21/22 (95%)

Query:        1 attattgaagtaagctatgtct 22
                ||||||||||||||| ||||||
Sbjct:   217521 attattgaagtaagcaatgtct 217500
```

If the alignments don't print out like they look on the screen, select the whole thing and put it in a small proportional font. The above is Courier font at 8 pt.

JUDGING THE BLAST RESULTS:

1. To determine if the sequences listed are really related, look at the E score. It should be less than 0.05 to have a 95% confidence interval that the sequences are truly related.

2. The best "hits" should also be logical: You sequenced a cDNA clone so we are looking for a hit on a known mRNA or an exon of a gene. Therefore, note the sequence positions of the Subject sequence that your entered sequence (Query) aligns to. On the GenBank report Features, make sure the region of the Subject sequence relates to an mRNA sequence. Also, look at where the cDNA aligns to (sequence position numbers) in the GenBank report.

To retrieve the ***GenBank report*** with highest similarity to yours, click on its highlighted accession number (letter and five numerals) OR:

1. Go to website: http://www.ncbi.nlm.nih.gov/

2. In "ENTREZ", choose "Nucleotides"
3. Enter the accession number in the Search box then click on "Search".

4. Choose "Retrieve one document"

5. Check the box beside the sequence desired then "Display".

5. Save to your computer or diskette and print out the file. It should look like the example below after changing font to Courier 8pt.

```
LOCUS HSPREC 5003 bp RNA PRI 12-SEP-1993
DEFINITION Human mRNA and promoter DNA for progesterone receptor.
ACCESSION X51730
NID g35651
KEYWORDS hormone receptor; progesterone receptor.
SOURCE human.
ORGANISM Homo sapiens
Eukaryotae; mitochondrial eukaryotes; Metazoa; Chordata;
Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 5003)
AUTHORS Kastner,P.
TITLE Direct Submission
JOURNAL Submitted (16-FEB-1990) Kastner P., LGME/CNRS - Ul84/INSERM, 11 rue
Humann, 67085 Strasbourg Cedex, France


REFERENCE 2 (bases 1 to 5003)
AUTHORS Kastner,P., Krust,A., Turcotte,B., Stropp,U., Tora,L.,
Gronemeyer,H. and Chambon,P.
TITLE Two distinct estrogen-regulated promoters generate transcripts
encoding the two functionally different human progesterone receptor
forms A and B
JOURNAL EMBO J. 9 (5), 1603-1614 (1990)
MEDLINE 90228361
COMMENT See also <M15716>.
Bases 1-711 were derived from genomic DNA.
FEATURES Location/Qualifiers
source 1..5003
/organism="Homo sapiens"
/db_xref="taxon:9606"
/cell_type="T47D"
/clone_lib="lambda-gt11"
/chromosome="11"
/map="q22"
misc_feature 712
/note="beginning of mRNA sequence"
CDS 1455..4256
/note="progesterone receptor (AA 1-933)"
/codon_start=1
/db_xref="PID:g35652"
/db_xref="SWISS-PROT:P06401"
/translation="MTELKAKGPRAPHVAGGPPSPEVGSPLLCRPAAGPFPGSQTSDT
LPEVSAIPISLDGLLFPRPCQGQDPSDEKTQDQQSLSDVEGAYSRAEATRGAGGSSSS
PPEKDSGLLDSVLDTLLAPSGPGQSQPSPPACEVTSSWCLFGPELPEDPPAAPATQRV
LSPLMSRSGCKVGDSSGTAAAHKVLPRGLSPARQLLLPASESPHWSGAPVKPSPQAAA
VEVEEEDSSESEESAGPLLKGKPRALGGAAAGGGAAACPPGAAAGGVALVPKEDSRFS
APRVALVEQDAPMAPGRSPLATTVMDFIHVPILPLNHALLAARTRQLLEDESYDGGAG
AASAFAPPRTSPCASSTPVAVGDFPDCAYPPDAEPKDDAYPLYSDFQPPALKIKEEEE
GAEASARSPRSYLVAGANPAAFPDFPLGPPPPLPPRATPSRPGEAAVTAAPASASVSS
ASSSGSTLECILYKAEGAPPQQGPFAPPPCKAPGASGCLLPRDGLPSTSASAAAAGAA
PALYPALGLNGLPQLGYQAAVLKEGLPQVYPPYLNYLRPDSEASQSPQYSFESLPQKI
CLICGDEASGCHYGVLTCGSCKVFFKRAMEGQHNYLCAGRNDCIVDKIRRKNCPACRL
RKCCQAGMVLGGRKFKKFNKVRVVRALDAVALPQPLGVPNESQALSQRFTFSPGQDIQ
LIPPLINLLMSIEPDVIYAGHDNTKPDTSSSLLTSLNQLGERQLLSVVKWSKSLPGFR
NLHIDDQITLIQYSWMSLMVFGLGWRSYKHVSGQMLYFAPDLILNEQRMKESSFYSLC
LTMWQIPQEFVKLQVSQEEFLCMKVLLLLNTIPLEGLRSQTQFEEMRSSYIRELIKAI
GLRQKGVVSSSQRFYQLTKLLDNLHDLVKQLHLYCLNTFIQSRALSVEFPEMMSEVIA
AQLPKILAGMVKPLLFHKK"


BASE COUNT 1233 a 1303 c 1240 g 1227 t
ORIGIN
```

```
1 ggatccattt tataagctca aagataatta cttttcagac taagaatatt tagggtaaaa
61 agtactgttc aacatctcta ctgaggatgt tatgatgtag cacactctat aagctggagc
121 taaaggaaac tttccttaaa gtgctattta ctaaaaattg gaacacattc cttaagacaa
181 atcgaagtgt ggcacacaac atccaaactt ccatcataga tacagaggtg ttaccatctc
241 ccactcccaa atttctttgt cacgctgagg atactcaaga ggagcaggac atgttggtcg
301 cagcaggaga aacttgaaag cattcacttt tatggaactc ataagggaga gaatctctta
361 tttagtatcg tccttgatac atttattatt ttaaaagata atgtagccaa atgtcttcct
421 ctgtgttaaa tctttacaaa actgaaatct taaaatggtg acaaaaattc tacttctgat
481 agaatctatt catttttcca attagatagg gcataattct taatttgcaa aacaaaacgt
541 aatatgctta tgaggttcca tcccaaagaa cctgctattg agagtagcat tcagaataac
601 gggtggaaat gccaactcca gagtttcaga tcctaccggt aattggggta gggaggggct
661 ttgggcgggg cctccctaga ggaggaggcg ttgttagaaa gctgtctggc cagtccacag
721 ctgtcactaa tcggggtaag ccttgttgta tttgcgcgtg tgggtggcat tctcaatgag
781 aactagcttc acttgtcatt tgagtgaaat ctacaacccg aggcggctag tgctcccgca
841 ctactgggat ctgagatctt cggagatgac tgtcgcccgc agtacggagc cagcagaagt
901 ccgacccttc ctgggaatgg gctgtaccga gaggtccgac tagccccagg gttttagtga
961 gggggcagtg gaactcagcg agggactgag agcttcacag catgcacgag tttgatgcca
1021 gagaaaaagt cgggagataa aggagccgcg tgtcactaaa ttgccgtcgc agccgcagcc
1081 actcaagtgc cggacttgtg agtactctgc gtctccagtc ctcggacaga agttggagaa
1141 ctctcttgga gaactccccg agttaggaga cgagatctcc taacaattac tactttttct
1201 tgcgctcccc acttgccgct cgctgggaca aacgacagcc acagttcccc tgacgacagg
1261 atggaggcca agggcaggag ctgaccagcg ccgccctccc ccgcccccga cccaggaggt
1321 ggagatcctc cggtccagcc acattcaaca cccactttct cctccctctg cccctatatt
1381 cccgaaaccc cctcctcctt ccccttttccc tcctccctgg agacgggga ggagaaaagg
1441 ggagtccagt cgtcatgact gagctgaagg caaagggtcc ccgggctccc cacgtggcgg
1501 gcggcccgcc ctcccccgag gtcggatccc cactgctgtg tcgcccagcc gcaggtccgt
1561 tcccggggag ccagacctcg gacaccttgc ctgaagtttc ggccatacct atctccctgg
1621 acgggctact cttccctcgg ccctgccagg gacaggaccc ctccgacgaa aagacgcagg...
```

So now, in the example, I know my cDNA clone is progesterone receptor mRNA (hypothetical result). I even have the translation data (amino acid sequence) in this GenBank report.

Look at the oreientation….Is the cDNA sequence the sense or antisense strand?

Draw a plasmid map of the cDNA in the plasmid vector.

HOMEWORK #4 – Answer the following questions for the sequence provided to you about a cDNA cloned into plasmid vector pCR2.1.

Q1. ***Is your cDNA the same as the GenBank entry or just related?***

Q2: ***WHAT TISSUES IS YOUR GENE PRODUCT EXPRESSED IN?*** (You can use this BLAST search or other NCBI resources)

Q3: IS YOUR GENE PRODUCT CONSERVED ACROSS SPECIES? Can you tell which species your sequence is from? ***(Note: GenBank has more human and mouse sequences in it than those of other mammals.)***

Q4: WHAT IS THE FUNCTION OF THE GENE PRODUCT? Look at the protein/gene name and titles of the references.

GENE EXPRESSION Lab 7 ning 10/23/09

Transformation of E. coli with ligated vector and insert DNAs

"Bacterial transformation" relates to the change of bacterial phenotype by introducing a plasmid containing an antibiotic resistance gene. "Competent cells" are made receptive to plasmids by making their membranes permeable to DNA with calcium treatment. Plasmids adhere to cells, enter on heat shock, and cells are selected for accepting a plasmid by antibiotic resistance selection on plates after a 1 h recovery period in broth. NOTE: All waste contaminated with E. coli must be killed with bleach or discarded in "BIOHAZARD BAGS," which are autoclaved prior to disposal.

Each student will do three transformations: one from the vector only (negative control) ligation and one from the ligation that has DNA insert too, as well as a positive control for transformation: 10 ng of circular plasmid. For the last, use any plasmid.

1. Thaw cells on ice (20 min.) Take one tube of competent cells per student.

2. Pipet 50 ul into cool 1.5 ml tubes: 1 for each ligation (vector only and vector+insert), 1 for the positive control

3. Add 1 ul ligation to cells; mix by pipetting up and down.

4. Incubate on ice 30 min. Mix every 10 min. by tapping the tube gently.

5. Heat shock 42oC, 20 sec (Be exact here!). No shaking.

6. Ice for 2 min.

7. Remove from ice and add 950 ul room temperature S.O.C. broth.

8. Agitate cultures gently, 37oC, 1 h. Tube turners or rockers or hybridization ovens are good for this. Ask if you should spread plates with Xgal and IPTG for blue-white selection.

9. Spread 400 ul on a LB + Ampicillin plate for the transformations from ligations. Spread only 50 ul for the transformation of circular plasmid.

10. Incubate 37oC O/N. Invert the plates if all the liquid goes into the LB agar on the plate. If it does not, then incubate the wet plates right side up.

GENE 626 - Lab 8 10/30/09

Plasmid Minipreps

Thursday 10/23/08 in class – **Grow overnight cultures of E. coli**

1. Count colonies on the ligation plates. Compare to the number of colonies on vector only control plate. If you have twice as many colonies in the ligation transformation plate, then 50% should contain the insert!

2. Make 6 - 10 ml overnight cultures of LB + Amp in 50 ml tubes. Label tubes 1 - 6 (initials and date).

3. Inoculate one broth culture with 1 colony from vector only transformation plate (1 culture) if available. Inoculate the other 5 or 6 broth cultures with individual colonies from Max or Min ligation plates.

4. Incubate with strong agitation (> 200 rpm) at 37oC O/N.

Friday 10/24/08 – **Alkaline Lysis Plasmid Miniprep**

NOTE 1: Phenol for DNA is buffered with Tris to pH8 for optimal partitioning. For RNA,   Phenol is water-saturated and is ~pH5. Use the correct phenol for your nucleic acid!!!
NOTE 2: ALSO, Phenol is corrosive - causes burns! Be careful! Wear safety glasses!!!
NOTE 3: CHCl3 (Chloroform) dissolves things like styrofoam and polystyrene - use glass graduated pipets and polypropylene 15 & 50 ml tubes.
In a short, mind-numbing period of tube shuffling, one can extract plasmid DNA for analysis of desirable clones

1. Spin down 1.5 ml overnight culture cells in a 1.5 ml tube 2000 rpm for 2 min - (DISCARD all materials from steps 1 -3 that are contaminated with cells in BIOHAZARD BAGS.)

2. Save the rest of the overnight culture at 4oC - IF it is a good clone, you'll want to make a glycerol stock for long term storage and a streak plate for short term use.

3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

4. Resuspend the pellet by trituration (pipetting up and down) in 150 ul of an ice-cold solution of:
  50 mM glucose
  10 mM EDTA

25 mM Tris/ HCl (pH 8.0)
4 mg/ml lysozyme (added freshly to the solution)

5. Store for 5 minutes at room temperature. The top of the tube need not be closed during this period.

6. Add 300 ul of a room temperature solution of: 0.2 N NaOH + 1% SDS
Close the top of the tube and mix the contents by inverting the tube rapidly two or three times. Do not vortex. Store the tube on ice for 5 minutes.

7. Add 225 ul of an ice-cold solution of potassium acetate (~pH 4.8). [This reagent was made up as follows: To 60 ml of 5 M potassium acetate, add 11.5 ml of glacial acetic acid and 28.5 ml of H2O. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.] Close the cap of the tube and vortex hard, put on ice, vortex again. Store on ice for 5 minutes.

8. Centrifuge for 15 minutes in an micro-centrifuge 12,000 rpm at 20oC.

9. Transfer 600 ul supernatant to a fresh tube. (Avoid all white, solid particulate matter).

10. Add an equal volume of phenol/chloroform pH8. Mix by vortexing. After centrifuging 12,000 rpm for 2 minutes in an Eppendorf centrifuge, transfer the aqueous phase to a fresh tube.

11. Add two volumes of 100% ethanol at room temperature. Mix by vortexing. Stand at room temperature for 2 minutes.

12. Centrifuge for 5 minutes in an Eppendorf centrifuge at room temperature.

13. {Wash the pellet as described in steps 13 and 14 to remove excess salt….note that the pellet should not dissolve.}Remove the supernatant. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away.

14. Add 1 ml of 70% ethanol. Vortex briefly and then centrifuge 12,000 rpm 5 min.

15. Again remove all of the supernatant. Air dry the pellet briefly (5 minutes) after wiping away residual ethanol with a Kimwipe.

16. Add 50 ul of TE (pH 8.0). Vortex and incubate at 37oC to solubilize DNA (about 5 minutes).

17. Store at -20oC until next lab session, when restriction digestion will be performed to characterize the plasmids.

GENE EXPRESSION Lab 9 ning 11/6/09

Miniprep plasmid DNA Restriction and Gel Analysis

A. To analyze the four plasmid preps from overnight cultures, we'll restrict each with Xho I and Bam HI to see if the 690 bp insert is present.
1. We will cut inserts out of the vectors for each plasmid prep to verify it. Best pipetting technique minimizes pipetting steps (and, thus, work and error). So, to set up a set of 20 ul restriction digestions, make a master mix of common components:

In single reaction	x 7 =	in master mix
6ul H2O	x 7 =	42 ul
2 ul 10X????? buffer	x 7 =	14ul
1ul 1mg/ml RNase	x 7 =	7 ul 1mg/ml RNase
0.5 ul Bam H1 (10 U/ul)	x 7 =	3.5 ul Bam H1
0.5 ul Xho I (10U/ul)	x 7 =	3.5 ul Xho 1

Use 10ul/rxn
Mix gently. Pipet 10ul into 4 tubes.

Add 10 ul of individual plasmid preps, one to each tube. Incubate 1 h at 37oC. ALSO Prepare one uncut plasmid sample: 10 ul plasmid + 10 ul TE and incubate at 37oC. After incubations, add 2 ul 10x DNA dye to each of the five tubes. Store leftover plasmid at -20oC.

2. Make a 1% agarose gel + 0.5 ug/ml EtBr in 1X TAE. Use a mid-size apparatus and two 14-well combs.

3. Add 2 ul 10X DNA dye to @ digest.

4. Load gel with uncut plasmid (1 sample) and 4 plasmid digests. Also load Lambda Hind III EcoR1 markers (1 ug) in one well of top and bottom halves of each gel.

5. Run at 120V, 1 h.

6. Photograph under UV light.

THIS WAS DONE FOR YOU. Read FYI.

Prepare the plasmid for use as a template for in vitro transcription

In order to synthesize an antisense cRNA that will hybridize to ???? mRNA, the p???? plasmid will be linearized (at the point you want the transcription product to stop) and purified (with phenol chloroform extraction and purification on low melt agarose gel electrophoresis. The best templates for in vitro transcription come from concentrated and highly purified plasmids prepared in large amounts (maxipreps). So, although some of you identified recombinant plasmids in your analyses of your minipreps in the last lab, today you will use the maxiprepped plasmid provided because it is more pure and more concentrated.

1. Restrict 5 ug Equine ??? Antisense plasmid (provided) with 2.5 ul Bam HI (10 U/ul) in a 50 ul reaction. Use Promega Buffer _____.

2. Make two agarose gels:
    A. 1.0 % regular agarose with 1 thin comb
    B. 0.8% low melting point agarose with 1 fat comb

Need 2 gels of each gel type for the entire class.

3. After restriction, extract with phenol/chloroform (1:1) pH 8. Use a gel loading tip to minimize loss of the aqueous phase.

4. Load 10 ul digest on gel A and 40 ul digest on gel B. Run gels.

5. View and photograph gel A with short wave UV light. This is an analytical gel.

6. View and cut linearized plasmid band from gel B using hand held long wave UV light. Long wave is weaker, does not show the band as well but also does not induce thymidine dimers in the DNA. Cut the band conservatively…get only the pink gel containing DNA and place it in a clean microfuge tube. Gel B is a preparative gel.

7. View the plasmid map and decide which RNA polymerase you need for the in vitro transcription reaction in the next lab.

GENE EXPRESSION Lab 10 ning 11/13/09

IN VITRO TRANSCRIPTION OF A cRNA ANTISENSE PROBE AND USE IN HYBRIDIZING A NORTHERN BLOT

[Be RNAse free…and use aerosol barrier tips! See in vitro transcription (as in Biotin-16-UTP (Roche) protocol) and NorthernMax (Ambion) protocols**]**

TODAY we will make a cRNA probe for actin or GAPDH mRNA or 18S rRNA and hybridize it to the tissue RNA (and the plasmid DNA) on the Northern blot. The lab boss gives out plasmid clones containing complementary DNAs (cDNA's, synthetic copies of fragments of mRNAs). The best probes are cRNAs (see below).

NOTE : There are 3 common types of nucleotide probes: DNA oligonucleotides (ss), cDNA (ds) and cRNA (ss). For many applications, cRNA probes are superior over:
1. end-labelled oligonucleotide probes because they are:
 a. longer (and therefore carry more label and have higher hybridization specificity)
 b. uniformly labelled throughout (so they carry more label)
2. nick-translated or random-primed cDNA probes, because they only have the desired probe strand, not the other "sense" strand that increases background.
In addition, the binding of RNA:RNA hybrids is stronger than that of DNA:DNA hybrids.

To make cRNA probes, the circular plasmid DNA is restricted or cut at a specific site with a restriction enzyme. For in vitro transcription, the plasmid and ribonucleotides are combined with a bacteriophage RNA polymerase (SP6 or T7). The polymerase enzyme binds a specific site on the plasmid and transcribes (makes RNA) using the DNA as a template. Either of the two strands of the DNA can be reproduced as cRNA: the top cDNA strand is like mRNA and, if transcribed, is called "sense" cRNA. The sense cRNA is useful as a template for translation. The bottom cDNA strand is complementary to the sense strand and hybridizes to mRNA, as does its transcription product called "antisense" cRNA. REMEMBER:
1.the cDNAs are synthetic cloned fragments of the mRNAs
and
 2. Knowing the information on the plasmid maps (See appendix) is critical to designing the probes (e.g. knowing what enzyme to linearize the plasmid with, which enzyme to transcribe with, and which strand (sense or antisense) is generated.

We'll only synthesize the antisense cRNA and it will be labeled with biotin so it will be a probe (detectable reagent) for identifying its homologous RNA in the tissue RNA samples. Ambion's "pTRI-____" constructs are foolproof: already linearized, and have all the RNA polymerase sites on the side of the cDNA so as to make only antisense transcipts. After hybridizing the probe to the RNA on the Northern blot and washing the blot, specifically bound probe will be detected with either biotin-antibody or strepavidin (which binds biotin). Whichever used will be conjugated to alkaline phosphatase enzyme which will cleave the chemiluminescent detection reagents to yield a low intensity light signal that is detected by X-ray film.

Be clean – clean and protect apparati from Rnase contamination- work on clean diapers! Use all DEPC reagents, gloved hands and aerosol barrier tips.

I. START THIS FIRST! In vitro Transcription.

Each student should set up two in vitro transcription reactions for biotin-labeling antisense cRNA probes:one for 18S rRNA and one for the p??? plasmid.

1. Thaw components at room temperature (RT) then store on ice. EXCEPTION: RNasin and RNA Polymerase, like all enzymes, stay at -20oC always! and

Melt the in vitro transcription template in low melt gel at 70oC for 10 min. Then put the tube in the 37oC block for > 5min. You will pipet the template from the tube at that location.

2. For sense cRNA add components from kit, in order, to a 0.5 ml tube at RT.

10 ul DEPC-H2O
4 ul 5X Transcription Buffer (Promega)
1 ul RNasin
2ul 100 mM DTT
2ul 10 mM rATP, rCTP, rGTP, and 3.5 mM Biotin-16-UTP
1 ul linearized DNA template
1 ul T7 RNA Polymerase

3. Mix by pipetting up and down...gently! No bubbles.
4. Flash spin in a microfuge
5. Incubate 37oC for 1h
6. Add 1 ul RNase-free DNAse
7. Incubate 15 min at 37oC

III. Assess quality of in vitro transcription product.

A. Analysis of in vitro transcribed probe on an acrylamide gel ("probe test gel", recipe in Appendix).

1. A group of four students should make one 5% polyacrylamide/ 8 M urea gel, RNAse-free, in a vertical slab gel apparatus (see Appendix for gel recipe). Add comb and allow to polymerize 20 min. ***NOTE: acrylamide and TEMED are TOXIC!!***

2. Set up gel rig with 1.5 liters of 1X TBE in DEPC water.

3. Take a 1 ul aliquot from the in vitro transcription at time 0 min and at another time at least 30 min later (record the time). Ask if RNA Century marker from Ambion are available. Combine each with 10 ul RNA Load dye containing bromphenol blue and xylene cyanol dyes. Heat 70oC for 10 min. Then load on the gel after flushing the wells free of urea leaching into them from the gel.

4. Run at 35 mamps for 30 min.

5. Stain with EtBr (agitate in 100 mls of 2.5 ug/ml EtBr in DEPC H2O, then destain in DEPC H2O) and photo on UV box.

QUESTION: Is this probe high quality? (of expected size and present in high concentration in the late sample)

B. Dot blot of serial dilutions of probe: (After 1 h transcription)

1. Make 4 serial 1:9 dilutions of each probe: Pipet l undiluted probe into a tube with 9 ul TE and mix. This is a 1:10 dilution. Repeat the dilution 3 times; so have 1:10, 1:100, 1:1000 and 1:10,000 dilutions.

2. On one 3 by 10 cm strips of Brightstar nylon membrane, mark membrane with sharp pencil or black Sharpie pen "-1", "-2", "-3", "-4" at 2 cm intervals across the width of the blot. Label rows of the blot “18S” and “unknown”. Add initials and date.

3. Dot 1 ul of appropriate probe dilutions under labels. UV Cross-link RNA to membrane as you did for the Northern blot.

4. Air dry and store in a clean place. (You'll develop the dot blot along with the Northern blot in the next lab.)

GENE EXPRESSION Lecture/Lab 11 ning 11/19 & 20/09

Thursday 11/19/09 in class

Northern blot Prehybridization & Hybridization

Since the nylon membrane likes to bind things, background sites are blocked (bound) with non-specific DNA and protein. Usually, sheared salmon sperm DNA is used in prehybe to block these sites.

1. Warm the Ambion Ultrahybe hybridization solution to 65oC. Swirl to dissolve precipitates. Cut blots down the center so student 1 and student 2 samples on the right can be probed for 18S rRNA and the left half of the blot can be probed with the unknown.

2. Wash a hybridization bottle with RNAseZAP. Insert the dry blot into the bottle so the RNA side is toward the center. Wet it with 2X SSC. Pour 2X SSC out.

3. Add 10 to 20 ml Hybridization solution and seal bottle.

4. Incubate 68oC for 30 min in the hybridization oven with bottle turning.

5. Heat cRNA probe from the last lab at 94oC for 10 min - chill on ice. Add 17 ul of heat denatured in vitro transcribed probe to the appropriate hybridization solution bottle.

8. Incubate 65oC overnight with bottle turning.

DO THE FOLLOWING IN YOUR NOTEBOOK:

1. Based on your blot photo and what you know, **draw your expected hybridization results** in your notebook. Use the 28S & 18S rRNA positions as markers. For the mouse, 28S rRNA is 4718 bases and 18S rRNA is 1847 bases.

2. I would estimate about 2 to 6 pg of a prevalent mRNA (likeGAPDH mRNA) in 5 ug of total cellular RNA sample.

QUESTION: In that sample, h**ow much RNA is in each rRNA band?**

Friday 11/20/09

Northern Blot Washing and Development

[See Biotin luminescent detection kit (Roche) and NorthernMax (Ambion) protocols**]**

After overnight hybridization, probe is maximally bound to specific sequences. It is also present on some non-specific sites. By reducing [salt], mainly in the form of SSC, hybridizations are tested for stringency. Usually temperature is increased as well so that only specific probe-binding for the target of interest remains after washing.
KEEP BLOTS WET DURING THESE PROCEDURES or you'll generate a lot of artifacts. Wash blots with vigorous shaking and seal the tupperware lids during washes so solutions don't spill onto the shakers!!! When binding reagents use gentle agitation.

1. Cut off corner of hybridization bag. Discard hybe solution.

2. Cut bag open and move blot to a clean tupperware container with a sealing lid. Can wash several blots in the same container if they are distinguishable from each other (sometimes the labeling fades with this hybridization).

3. Wash the blot in 100 mls of 42oC 2x SSC [or "low stringency wash" from Ambion] with hard shaking for 15 min at 42oC.

4. Discard wash and rewash with 100 mls of 0.1X SSC at 42oC.

REST OF STEPS AT ROOM TEMPERATURE:

5. Wash in 50 mls Washing Buffer [Maleic acid buffer (1X = 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) with 0.3% Tween-20] at RT for 5 min.

6. Repeat #5 once. Add in the probe dot blot from the previous lab at this step.

7. Transfer to a clean, freshly washed Tupperware container or tip lid and incubate the membranes for 30 min in 20 ml Block solution (1% (w/v) blocking reagent in maleic acid buffer without Tween). During gentle shaking, membranes should move independently from each other.

8. Discard Block and incubate in Strepavidin-Alkaline Phosphatase solution (1:5,000 diluted in block solution) for 30 min. Use a minimal volume (20 ml) in a small clean container like a yellow tip box lid. During gentle shaking, membranes should move independently from each other.

9. Discard the antibody solution and wash twice in Washing Buffer for 15 min each time.

10. Discard wash and equilibrate membrane in Detection Buffer [0.1 M Tris/HCl(pH9.5), 0.1 M NaCl] for 2 min.

11. Pipet 1 ml of room temperature CDP-Star Detection Reagent diluted 1:100 in detection buffer onto each blot and cover with plastic wrap. Incubate 5 min, then pour off excess reagent.

12. Wrap blot in saran wrap. To keep the blot wet and the film dry, double fold the plastic wrap and tuck all edges under the blot. As always, RNA side up!

13. Place in cassette.

14. Go to the dark room and lay a piece of X-ray film on the blot. Bend the lower right corner of the film. Close the cassette. Make sure you fold the flaps correctly so it is light tight!

15. Place cassette at 37oC or room temperature. Develop the film in 10 min in the developer machines in the dark rooms. Label films with exposure date, time and index to your notebook. Align the film with the blot and mark the positions of the well, 28S & 18S rRNA on the film. Identify and label lanes. If desired, place unexposed X-ray film on the blot and expose longer…overnight?.

Review your predicted Northern blot results in previous session. It is imperative that you know what you are looking for in order to assess and optimize the blot exposures…

GENE EXPRESSION LECTURE 12/3/09

REVIEW RESULTS

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NORTHERN BLOT ANALYSIS:

1. Qualitatively assess the blot results:
a. Is exposure optimal?

b. How many bands are evident?

c. Are they in the expected tissues?

d. What are the sizes of the hybridizing bands with respect to the rRNAs?

e. For one band, empirically determine the length of the mRNA hybridized.To do this measure migration distances for the band on the Xray film, and for 28S (4800 bases) and 18S (1800 bases) rRNAs on the EtBr stained gel photo using UV ruler as a guide. On graph paper, you can plot log base length against migration distance for the rRNAs. Find log bases from the plot, using the migration distance of the band of interest. Find antilog to get number of bases. If RNA markers are used, you can use those instead of or in addition to the 18 and 28S rRNAs.

2. Quantitate hybridizing bands

a. Can use densitometry on the X-ray film. Need a good scanner and analytical software such as BioImage IQ.

b. Many machines are being developed for direct scanning of blots. These are very powerful because they avoid the limitations of film and generate electronic data files for easy storage and publication.

APPENDIX

Use of Micropipettors

1. Choose the correct pipet. For volumes:
  1-20 ul P20
  20-200 ul P200
  200 - 1000 ul P1000

2. Set the desired volume by holding the pipetman in one hand and turning the volume adjustment knob until the correct volume shows on the indicator. For best precision, always approach the desired volume by dialing downward (at least one-third revolution) from a larger volume setting.

3. Attach a new tip to the shaft of the pipet. Press tip on firmly to ensure airtight seal. Chose the correct tip.
  P20 yellow tip
  P200 yellow tip
  P1000 blue tip

4. Depress plunger to first positive stop. Hold pipetman vertically and immerse disposable tip into sample liquid 2mm.

5. Allow the push button to return slowly to the up position. Never permit it to snap up.

6. Wait 1 or 2 seconds to ensure that the full volume of the sample is drawn into the tip.

7. Withdraw tip from the sample liquid. Wipe the sides of the tip on the sides of tube to remove any remaining liquid.

8. To dispense the sample, place the tip end against the side wall of the receiving vessel and depress the plunger slowly to the first stop. Then depress the plunger to the second stop to expel any residual liquid in the tip.

9. With the plunger fully depressed, withdraw pipetman from the vessel. Then allow the plunger to return to the top position.

10. Discard tip by depressing the tip ejector button. A fresh tip should be used for each sample.

Terribly Difficult Calculations

1. Molar solutions

1 M (mole per liter) means the solution has 1 molecular weight mass (g) per volume (liter) of soln.
A mole is a number of molecules:
6.022 x 10E23, Avogadro's number
To make 500 mls of 0.5 M NaCl (NaCl is58.55 g/mole) you need (0.5 liters)
(0.5 mole)= 0.25 mole
liter)
0.25 mole * 58.55 g/mole = 14.6 g

So: Add 14.6 g NaCl power and bring final volume to 500 ml with H2O.
2. We typically work with concentrated stock solutions. For example, our Tris/acetate/EDTA (TAE) is made as a 50X stock. We run gels in 1000 mls of 1X TAE. The way I do DILUTION PROBLEMS is:
[Stock] * y = [Desired] * Desired volume; where y is the volume of stock. To find Y needed to make 1 li of 1X TAE from a 50X stock:
50X * y = 1X * 1000 ml
y = 1X/50X * 1000 ml = 20 ml

So add 20 ml 50X TAE to a 1 liter graduated cylinder. Bring volume to 1 li w/ dH2O.

3. Note: Dilutions are applicable to problems of pipetting very small amounts. If you want to add 0.2 ul, dilute the material 1:9 and pipet 2 ul with a P-20.

4. Percentage solutions should have a (v/v) or (w/v) or (w/w) following.
a. (v/v) relates volume to volume, indicating both components are liquids: e.g. 100 mls of 75% (v/v) EtOH is made with 75 mls EtOH + 25 ml H2O
b. (w/v) indicates solid to liquid ratio: e.g., 10 mls of 10% (w/v) ammonium persulfate (APS) is made w/ 1 g of APS to 10 ml final volume with water.
c. (w/w) is rare, indicating a weight to weight relationship. To make 10 mls of a 10% (w/w) APS soln, you could weigh 1 g APS on a scale and then add water until solution weight is 10 g. (That would be 9 g = 9 mls since density of H2O is 1 g/ml).

5. Of course, these calculations can be combined. For example, to make 500 mls of 0.5 M NaCl in 1X TAE,
Combine 14.6 g NaCl with 10 mls 50X TAE. Bring volume to 500 mls with H2O.

Easy!

PROBE TEST GEL

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(short, fat sequencing gel)

5% acrylamide/urea gel

25.5g urea
19.5ml H_2O
12ml 5X TBE
7.5ml 40% (w/v) acrylamide (19:1 acryl:bis)
60ml final vol.

Heat to 37oC to dissolve urea
Cool to below RT
Filter (~optional)
Add 400 ul 10% APS (less than 1 week old, make 1ml)
50 ul TEMED

Clean gel plates with soap, rinse with H_2O extensively, then wipe with EtOH and Kimwipes. Set up plates as in Fig. 2 (except don't clamp over sponge).

Pour into 1.5mm thick vertical gel slab. Add comb.
Bubbles = Bad
Should polymerize in 15 min.

Rinse wells with 1X TBE immediately after pulling the comb and just prior to loading.

Samples in 80% (v/v) formamide loading dye
Heat 68oC 5 min for RNA. For DNA, 94oC 5 min.

Run at 25 to 35 mamps.

LIGATION IN LOW-MELT AGAROSE

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1. Run restricted vector and insert DNAs on 0.8% low-melt agarose gel in TAE buffer. (If vector is cut with a single enzyme, treat with call intestinal phosphatase (CIP) prior to gel run to prevent recircularization of the vector). Cut out bands and remove excess agarose while visualizing EtBr-staining under long wave UV light.

2. Melt DNA + agarose 70oC, 10 min.

3. For @ ligation reaction, combine H2O and DNA + agarose in 9 ul volume (usually 1 ul vector and 3 to 8 ul insert). Put at 37oC for 2-3 min. Perform a vector ligation control reaction without insert.

4. Add 11 ul ice-cold ligase mix composed of

2 ul 10X Ligase Buffer
1 ul Ligase
7 ul H2O
1 ul 10mm ATP

for each reaction. Flick tube immediately! Immediately slam on ice. Reactions will gel while ligation occurs.

5. Incubate 15oC, O/N.

6. Melt ligation 70oC, 10 min. Transform with 1 ul.

Dye Migration Related to bases in a denaturing gel (d) or base pairs in a non-denaturing gel (n):

Acrylamide %	Bromphenol Blue	Xylene Cyanol
3.5% n	100	-
5% n	65	-
5% d	35	130
6% d	26	106
8% n	45	-
8% d	19	75
10% d	12	55
12% n	20	-
20% n	12	-

e.g., in a 5% acylamide + urea gel (denaturing), Bromphenol Blue comigrates with 35 base long nucleic acids.

Making Agarose Gels for DNA

Make 1 li of 1X TAE from the 50X stock in a 1 or 2 liter graduated cylinder and mix well by inversion. Use 100 ml to make the gel and the rest to run the gel.

1%	1.5%
1g Agarose	1.5g Agarose
100ml 1X TAE	100ml 1X TAE
10ul 10mg/ml EtBr	10 ul 10mg/ml EtBr

Put components in a 500 or 300 ml beaker, cover with Saran Wrap, heat in microwave until solution boils 3 times. Allow to cool to 60oC, pour gel into mold as instructed.

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