Repository of Protocols Utilizing Radioactive Material

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14C-Methane Uptake and Oxidation

1. Fill serum bottle with approximately 50ml seawater. (A set of experiments may include a total of about 30 serum bottles.)
2. Cap the bottle with rubber seal held by an aluminum collar.
3. In fume hood, invert bottle and inject 500 µl of 14C-methane (approx 0.5uCi) using syringe, allowing 500 µl of seawater to escape through a second needle inserted through the rubber seal.
4. Incubate indoors under controlled light and temperature conditions. Two assays are performed.
    a. Micro-FISH
        i. Remove seal and transfer seawater sample to another bottle.
        ii. Add small amount of paraformaldehyde to sample
        iii. Fix overnight
        iv. Filter onto 0.2 um filter
        v. Store filter in 7mL vial in freezer for later radioassay
        vi. Transfer filtrate to a radioactive liquid storage container.
    b. Uptake and Respiration
        i. Remove seal and transfer seawater sample to another bottle.
        ii. Pickle the sample with NaOH
        iii. Filter a portion onto 0.2 um filter
        iv. Save filter in a 7mL vial for later radioassay
        v. Transfer filtrate to a radioactive liquid storage container.
        vi. Transfer a portion of the pickled sample from step ii. to a flask capped with a gas-tight rubber bung, fitted with cup holding a base-saturated filter.
        vii. Inject sulfuric acid through the bung. This drives all 14C-inorganic C to 14C-CO2.
        viii. Swirl and allow the 14C-CO2 to degass and collect in the base-saturated filter.
        ix. Transfer the base-saturated filter to a 7mL vial for later radioassay
        x. Save approx 1ml of degassed liquid for later radioassay and transfer the remaining degassed liquid to a radioactive liquid waste container. The pH of liquids are adjust to be between 2 and 12.5

Bacterial Production using the 3H-leucine method

May 9, 2007
Cottrell
revised by Fendt 6/6

Summary of the procedure:

Bacterial production will be measured using the 3H-leucine method. In this procedure 2 μCi of 3H-leucine is added to 1.5 ml of seawater in a sealed container, which is then incubated in a temperature-controlled water bath for 20 min – 2 h. The incubation is terminated by the addition of trichloroacetic acid, which generates a precipitate of macromolecules that is collected by centrifugation and radioassayed by scintillation counting. The supernatant containing unincorporated radiolabel is removed and then transferred to the appropriate radioactive liquid storage container. The sample tube is then air-dried and liquid scintillation cocktail is added.

Radioisotope handling:

The radioisotope stock is retrieved from the padlock-secured refrigerator, the isotope is aliquoted for the experiment and then the isotope is returned to the secured refrigerator. All isotope transfers are performed over a plastic spill tray that is lined with absorbent paper. Personnel handling radioisotopes wear gloves and lab coat that are removed before leaving the work area.

Each bacterial production experiment generates 14.3 mL of liquid by-product containing approximately 8 μCi of 3H. One pipette tip and one pair of gloves are disposed of in the dry RAM storage container. The analytical sample remains in 2 mL microcentrifuge tubes.

Monitoring for contamination:

Wipe tests are performed at the conclusion of each day of work with radioisotopes. Survey areas include the spill tray, the workbench, the floor beneath the work area and the refrigerator door handle.

Detailed procedure:

1) Add 3H-leucine to 2mL microfuge tubes (three live and one killed sample).
2) Add 75 μL of 100% TCA to the killed control
3) Add 1.5 mL of sample seawater
4) Incubate at in situ temperature in the dark for 20 min – 2 h. The longer incubation time will be best for Arctic waters.
5) Add 75 μL of ice cold 100% TCA (final conc. 5%).
6) Centrifuge for 10 min at high speed.
7) Pour the supernatant into the liquid storage container.
8) Add 1 mL of ice cold 5% TCA.
9) Invert tubes to mix the contents and repeat centrifuge step.
10) Pour the supernatant into the liquid storage container.
11) Add 1 mL of ice cold 80% ETOH
12) Invert tubes to mix the contents and repeat centrifuge step.
13) Pour the supernatant into the liquid storage container.
14) Adjust the pH of the liquid in the liquid storage container to be between 2 and 12.5
15) Air-dry tubes and store.

Light and Dark $^{14}CO_2$ uptake May 9, 2007

Cottrell

revised by Fendt 6/6

Summary of the procedure:

Uptake of $^{14}CO_2$ will be measured in samples exposed to the light and held in the dark. The isotope is added in the form of bicarbonate, which is the major form of inorganic carbon in seawater. In this procedure 50 µCi of the radiolabeled compound is added to 50 ml of seawater in a sealed container, which is then incubated in a temperature-controlled water bath for 24 h. Vacuum filtration is used to terminate the incubation and collect the cells that are subsequently radioassayed. The filtrate containing unincorporated radiolabel is transferred to the appropriate radioactive liquid storage container.

Radioisotope handling:

The radioisotope stock is retrieved from the padlock-secured refrigerator, the isotope is aliquoted for the experiment and then the isotope is returned to the secured refrigerator. All isotope transfers are performed over a plastic spill tray that is lined with absorbent paper. Personnel handling radioisotopes wear gloves and lab coat that are removed before leaving the work area.

Each $^{14}CO_2$ uptake experiment generates 100 mL of liquid by-product containing 100 µCi of the radiolabeled compound. One pipette tip and one pair of gloves are disposed of in the dry RAM storage container. The analytical sample remains on a filter that is stored in the freezer.

Monitoring for contamination:

Wipe tests are performed at the conclusion of each day of work with radioisotopes. Survey areas include the spill tray, the workbench, the floor beneath the work area and the refrigerator door handle.

Detailed procedure:

1- Dispense 50 mL of seawater into two polycarbonate bottles (one transparent and one covered with black tape).
2- Add 50 µCi of ^{14}C bicarbonate isotope
3- Incubate at in situ temperature 24 hours under artificial illumination in lab.
4- Load Hofer filtration manifold with a nitrocellulose backing filter and a 0.2-µm-pore-size polycarbonate filter.
5- Filter the sample.
6- Rinse the filter 6 times with deionized water to remove unincorporated label.
7- Store filter in a 7 mL vial. About 100 filter/vials will be generated during stay in AK.

Microautoradiography and fluorescence in situ hybridization (Micro-FISH)
May 9, 2007 Cottrell
revised by Fendt 6/6

Summary of the procedure:

Microautoradiography and fluorescence in situ hybridization (Micro-FISH) will be performed using a variety of radiolabeled compounds, including 14C bicarbonate, 14CH4, 3H-leucine and a 3H-amino acid mixture. In this procedure 1-40 µCi, depending on the radiolabeled compound, is added to 30 ml of seawater in a sealed container, which is then incubated in a temperature-controlled water bath for 2-24 h. The incubation is terminated by the addition of paraformaldehyde, which stops biological activity and preserves the cells for microautoradiography and microscopic examination. The preserved cells are collected by vacuum filtration, rinsed and stored frozen for transport back to the lab. The filtrate containing unincorporated radiolabel is transferred to the appropriate radioactive liquid storage container.

Radioisotope handling:

The radioisotope stock is retrieved from the padlock-secured refrigerator, the isotope is aliquoted for the experiment and then the isotope is returned to the secured refrigerator. All isotope transfers are performed over a plastic spill tray that is lined with absorbent paper. Personnel handling radioisotopes wear gloves and lab coat that are removed before leaving the work area.

Each Micro-FISH experiment generates 40 mL of liquid by-product containing 1-50 µCi of 3H or 14C, depending on the radiolabeled compound. One pipette tip and one pair of gloves are disposed of in the dry RAM storage container. The analytical sample remains on a filter that is stored in the freezer.

Monitoring for contamination:

Wipe tests are performed at the conclusion of each day of work with radioisotopes. Survey areas include the spill tray, the workbench, the floor beneath the work area and the refrigerator door handle.

Detailed procedure:

1- Dispense 20 mL (near shore) or 60 mL (offshore) of seawater into a polycarbonate bottle.
2- In the lab, add isotope to the bottle
    a. Amino acid mixture
        i. 0.5 nM addition
        ii. Dilute 5 µl of 1 mCi/mL stock in 45 µl of sterile, deionized water.
        iii. Add 0.25 µl of diluted isotope per ml of sample.
    b. Protein (H-3)
        i. 20 ng/ml addition
        ii. Add 0.1 µl of 0.25 mg/ml stock per ml of sample.
    c. Glucose
        i. 0.5 nM addition
        ii. Dilute 5 µl of 29 mCi/ml stock in 45 µl of sterile, deionized water.
        iii. Add 0.16 µl of diluted isotope per ml of sample.
    d. Protein- EPS (H-3)
        i. 10,000 dpm/mL addition
        ii. Add 10 µl of 1,037,000 dpm/mL EPS stock per ml of sample.
    e. ^{14}C bicarbonate
        i. 100 µCi addition
        ii. Add 100 µl of 1 mCi/ml stock.
3- In the lab, incubate organic compounds at in situ temperature for 8 - 12 hours in the dark and ^{14}C bicarbonate in the light for 24 h.
4- Transfer sample to a polyethylene bottle labeled with red tape for fixation.
5- Add 1 or 3 mL of 20% PFA.
6- Fix overnight at 4°C.
7- Filter the entire 20 ml or 60 ml sample onto one white 0.2 µm pore size PC filter
8- Store filter in a 7 mL vial in the freezer.

Protocol for monitoring phosphorylation of a protein (GST) by JNK (c-Jun N-Terminal Kinase) using radiolabeled (P_{32}) ATP

Materials needed:

Kinase reaction buffer: 40mM Tris, pH 7.5, 20mM MgCl2, 0.1%BSA
P_{32} ATP (labeled on the gamma phosphate)
Purified JNK

Purified GSTpi
Precast SDS-Page Gel
SDS sample buffer (2x) (to quench kinase reaction)
1x SDS running buffer (Tris-Glycine +0.1% SDS
Gel Drying buffer (30% Methanol, 5% glycerol)
Ziploc bags
Phosphoimager

Setting up kinase reaction:

1. Preincubate 0.5uM JNK with 2uM GST in kinase reaction buffer at 25oC for 30 min (total volume: 500uL)

To be done in the hood labeled as radioactive work area only! :

2. Add hot ATP (5uM final concentration). The activity of P-32 is _ mCi.
3. Allow the kinase reaction to proceed for 60 min
4. Take 30uL aliquots of reaction mixture and add to eppendorf tubes containing 30 ul SDS 2x sample buffer to quench the reaction at various time points (0-60 min)
5. heat samples at 80oC for 5 min
6. Load samples on an SDS page gel (one gel running box will be designated for radioactive work only and kept in the hood) and run the gel
7. Dry the gel overnight in the hood
8. Place the dry gel into a double ziploc bag and place into an additional “containment” container, such as a Tupperware container with a snap-on lid. Radiation warning tape will be put on outside of the containment.
9. Walk gel over to McKinley lab for phosphoimaging. Before leaving, area will be checked with GM meter for contamination. Gel will not be left in McKinley lab.

Safety Measures:

1. This protocol will be posted next to the hood during the experiment.
2. A Plexiglas body shield will be used during P-32 use in the hood. Plexiglas Eppendorf tube shields will be used as much as possible.
3. Body and ring badges will be worn during all times when handling P-32. Ring will be worn under the glove and turned so that the name on the ring faces the radiation source being handled.
4. Lab coat, disposable gloves, and safety glasses will be worn.
5. Bench paper will be used.
6. The GM survey meter will be turned ON and placed outside the hood (but nearby) during the experiment. Gloves will be checked occasionally with meter; items removed from the hood will be checked with the meter as they are brought out into the general lab area.
7. At the conclusion of each day of P-32 work, the GM meter will be used to scan area for contamination. Wipe tests will also be conducted and the results of the wipes documented in the radiation record book. Contamination will be immediately cleaned up.
8. All radioactive waste will be disposed in proper radioactive waste containers.
9. Any accident will be reported immediately to the RSO at x8475 (or to Public Safety x2222 after hours)

3/11/08a

Protein Labeling with 3H Amino Acids-

1. Remove eyes immediately before the experiment, isolate the lenses and place two lenses/mouse embryo in one well of 96-well plate with 199 medium. Approximately 12 embryos will be used.
2. To each well, add 3H L-amino acid mix to a final concentration of 20µCi/mL (about 2uCi/well)
3. Incubate plate for 6 hours in a humidified atmosphere of 5% CO_2 and 95% air, at 37°C
4. Remove medium from wells (and discard as radioactive waste) and wash cells with non-radioactive 199 medium 3 times (medium is discarded as radioactive waste after each rinse).
5. Add 100µL Ripa buffer to each well to lyse cells. (If this is unsuccessful in breaking open the lenses, the lenses will be transferred to a microfuge tube and homogenized with a small disposable pestle in the tube to extract protein.)
6. Spot 40µL from each well on Whatman glass microfibre filters (Cat# 1822021) (save the remainder for BCA protein determination assay Pierce Cat#23227)
7. Wash the membranes (filters) with boiling Trichloroacetic acid (TCA) 5 min and twice more with cold TCA (5 min each)(done in hood and discarded as radioactive/chemical waste)
8. Wash membranes with ice cold 95% ethanol (discarded as radioactive/chemical waste)
9. Wash membranes once with ice cold acetone (discarded as radioactive/chemical waste)
10. Let membranes dry and put into scintillation vials
11. Add 4.5mL scintillation fluid
12. Count vials

BCA assay

- Add 20µL of sample from each well to triplicate wells on 96 well plate
- Add BCA reagents
- Incubate 30 min at 37°C
- Read in plate reader

<u>Radiation Safety Measures to be Employed</u>

Wear lab coat, gloves, and safety glasses/goggles.
Work on radiation labeled worksurfaces only and use benchpaper.
Transfer radioactive material between floors using secondary containers. Use the elevator instead of the stairs.
Use three separate waste containers for the liquids generated: step 4 (aqueous), step 7 (corrosive), and steps 8/9 (flammables). Label each container with both its radioactive and chemical constituents.
Wipe tests will be conducted at the end of each work day and the results will be documented.

G-Protein Ligand Binding Assay Protocol

1) Add 200 µl of G-Protein sample (+/- receptor) to all wells wells of Multi-Screen MAFC-NOB plate (10-30 µg protein as determined by DC protein assay), 200 µl/well. Buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$.

2) Move plate behind shield to perform all subsequent manipulations. Don protective apparel – double gloves, lab coat, radiation badge, eyewear.

3) Dilute stock radiolabled ligand to make working stock at concentration of 1 – 10 nM.

4) Add 25 µl of GTP-□^{32}P at desired timepoints (1 sec – 60 min) in triplicate.

5) Between timepoints replace lid on Multi-Screen plate and incubate at 25°C (room temperature) with gentle shaking.

6) Survey hands, body, clothing, work area, pipettes, etc. using handheld survey meter.

7) Apply vacuum to plate and wash filters 3x with 200 µl/well cold 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$.

8) Blot dry on clean paper towel. Pull off underdrain carefully, and blot plate again. Allow plate to air dry for ~5 minutes.

9) Add 50 □L scintillation cocktail to each well, seal with tape and place in Wallac MicroBeta liquid scintillation top-counter for counting. Perform “quick count” 5 seconds per well, then incubate 6 hours and perform “long count” 1 minute per well.

10) Survey hands, body, clothing, work area, pipettes, etc. using handheld survey meter.

11) Wipe test all surfaces with ethanol swab, and place in scintillation vial with 3 mL scintillation cocktail. Count in Wallac MicroBeta liquid scintillation top-counter.

G-Protein Ligand Binding Assay Protocol

1) Add 200 µl of G-Protein sample (+/- receptor) to all wells wells of Multi-Screen MAFC-NOB plate (10-30 µg protein as determined by DC protein assay), 200 µl/well. Buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$.

2) Add 25 µl GTP-□S at desired concentrations (10 nM-100 µM) in triplicate.

3) Don protective apparel – double gloves, lab coat, radiation badge, eyewear.

4) Dilute stock radiolabled ligand to make working stock at concentration of 1 – 10 nM.

5) Add 25 µl of labeled GTP-□□□S into each well and into a control well for total counts. Ligand concentration should be in the range of 0.1-25 nM final.

6) Replace lid on Multi-Screen plate and incubate 1-2 hours at 25°C (room temperature) with gentle shaking.

7) Survey hands, body, clothing, work area, pipettes, etc. using handheld survey meter.

8) Apply vacuum to plate and wash filters 3x with 200 µl/well cold 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$.

9) Blot dry on clean paper towel. Pull off underdrain carefully, and blot plate again. Allow plate to air dry for ~5 minutes.

10) Add 50 □L scintillation cocktail to each well, seal with tape and place in Wallac MicroBeta liquid scintillation top-counter for counting. Perform “quick count” 5 seconds per well, then incubate 6 hours and perform “long count” 1 minute per well.

11) Survey hands, body, clothing, work area, pipettes, etc. using handheld survey meter.

12) Wipe test all surfaces with ethanol swab, and place in scintillation vial with 3 mL scintillation cocktail. Count in Wallac MicroBeta liquid scintillation top-counter.

Production of 3H glycine labeled human $VEGF_{165}$ in E. coli

Bacterial strains for $VEGF_{165}$ expression:
JM109(DE3) 20µg/ml
BL21(DE3)pLysS 34 µg/ml
Ad494(DE3)pLysS 34µg/ml

Expression and purification lasts about two weeks. A typical yield is about 3 mg to 10 mg of pure $VEGF_{165}$ per liter of bacterial culture.

1. Transformation with pRSET-$VEGF_{165}$ plasmid:
   The best way to start the protein production is to freshly transform the bacteria with pRSET-$VEGF_{165}$. Streak transformed bacteria on LB-agar plates containing ampicillin (100µg/ml) and grow overnight. Or streak bacteria from a bacterial stock, solution and grow them overnight and then pick a single colony.
2. Grow small liquid cultures: Pick single bacterial colonies from plate with transformants and expand them overnight in 3ml of **M9 minimal media with 3H glycine**
3. Large scale production of $VEGF_{165}$: Take the 3ml culture and add 1L **M9 minimal media with 3H glycine** plus ampicillin provided in a 4L flask. Grow bacteria in a bacterial shaker at 37°C (ROOM 239). Check bacterial growth by measuring optical density at 600nm. Grow the bacteria to an optical density of about 0.8 (4 to 5 hours).

Induction of VEGF protein production: Once the bacteria density has reached an optical density at 600nm of about 0.8, add isopropylthiogalactoside (IPTG) to a final concentration of 1 mM. Addition of IPTG induces production of T7 polymerase and subsequently transcription and translation of the target gene. The bacteria are then grown for an additional 2 to 4 hours at 37°C. Excessive production of the VEGF protein results in its accumulation as inclusion bodies in the bacterial cytosol. After 2 to 4 hours, collect the bacteria by centrifugation (ROOM 244), and discard the culture medium. Then freeze the bacterial pellet at -20°C (ROOM 244).

Lysis if bacterial cells: Carefully resuspend the bacterial pellet in 1/10 of the original culture volume lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA). Once the bacteria are completely suspended add lysozyme to a final concentration of 0.1 mg per mL lysate. Incubate for 20 minutes at room temperature. The lysate turns immediately very viscous because of DNA release form the bacteria. Add 20 µL of benzonase to the suspension to digest the chromosomal DNA. Stir and incubate the lysate for 3 hours to overnight at 4°C until the lysate is completely fluid (COLD ROOM 234)

Collection of insoluble material: Insoluble material, including the inclusion bodies containing the **3H glycine labeled $VEGF_{165}$**, are collected by 30 minutes centrifugation at 30,000 g. Discard the supernatant and retain the pellet.

Solubilization of the insoluble material and inclusion bodies: First wash the pellet to remove any residual bacteria. Wash the pellet with 120 mL of ice cold buffer containing 4M urea, 20 mM Tris, pH 8.0, 150 mM NaCl, 2mM EDTA. Stir and perform extraction at 4°C. The wash could take between 3 and 12 hours. Collect the insoluble material by 30 minute centrifugation at full speed. Keep the pellet and discard the supernatant.

Extract the pellet with 35 mL of buffer containing 8M urea, 20 mM Tris, pH 8.0. 150 mM NaCl, 10mM fresh dithiothreitol (DTT). Stir overnight at 4°C. The **3H glycine labeled $VEGF_{165}$** containing inclusion bodies are solubilized by the 8M urea. Collect the supernatant containing the **3H glycine labeled $VEGF_{165}$** by centrifugation at full speed for 30 minutes.

Check 10 µL aliquots of **3H glycine labeled $VEGF_{165}$** containing supernatant by 15% or 20% SDS-PAGE under non-reducing and reducing conditions and Coomassie staining. Monomeric and dimeric **3H glycine labeled $VEGF_{165}$** should be observed.

Refolding and dimerization of **3H glycine labeled $VEGF_{165}$**: **3H glycine labeled $VEGF_{165}$** should be refolded and dimerized by sequential removal of urea through sequential dialysis (COLD ROOM 234). This step is crucial and requires patience. Use large volumes of buffer for dialysis (about 2L) and leave dialysis buffer for at least one day before changing to new buffer. Perform all dialysis steps against pre-cooled buffers and dialyze at 4°C. Secondary containment trays will be used.

Dialyze overnight against 4L of 6M urea, 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA.

Then change to 4L of 4M urea buffer with 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA. Dialyze overnight.

Then change to 4L of 2M urea buffer with 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA. Dialyze overnight.

There should be no radioactivity in the second and third dialysis buffers but 1mL of buffer will be counted in the liquid scintillation counter to confirm.

Once the **3H glycine labeled $VEGF_{165}$** is in 2M urea begin a second round of folding. This is done in a redox system of oxidized glutathione/ reduced glutathione. Add to the **3H glycine labeled $VEGF_{165}$** solution oxidized glutathione to a final concentration of 0.5 mM and reduced glutathione to a final concentration of 5mM. Leave the **3H glycine labeled $VEGF_{165}$** solution in the redox buffer system for at least 48 hours at 4°C.

The final dialysis removes the urea and glutathione and allows the formation of dimeric **3H glycine labeled $VEGF_{165}$**.

To remove the urea and glutathione, dialyze for at least 24 hours against 3L of 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA.

Then change buffer again and dialyze for at least 24 hours against 20 mM Tris, pH 8.0, 150 mM NaCl, without EDTA.

Then change again to 20 mM Tris, pH 8.0, 150 mM NaCl, without EDTA dialyzing overnight.

Check dimerization by 20% SDS-PAGE under non-reducing and reducing conditions.

Purification of **3H glycine labeled $VEGF_{165}$** by column affinity chromatography on heparin agarose: **3H glycine labeled $VEGF_{165}$** has a heparin binding domain which can be used for purification. Use heparin agarose 4B grade A which contains about 5 to 7 mg of agarose per mL resin. The solution to be purified should contain about **5 to 10 mg 3H glycine labeled $VEGF_{165}$**. Use a column with a 5mL bed volume to assure purity.

Equilibrate the column with Tris buffer, pH 8.0, 150 mM NaCl. Load the **3H glycine labeled $VEGF_{165}$** solution onto the heparin-agarose column and wash with at least 50 mL of Tris buffer.

This step will be performed in room 244. Column contents will placed in radioactive waste.

Elution of **3H glycine labeled $VEGF_{165}$**: **3H glycine labeled $VEGF_{165}$** is eluted from the column with NaCl at a concentration of 0.5 to 0.6 M. Elution can be preformed with a NaCl gradient between 0 and 1 M NaCl in Tris buffer. Collect the eluate and check by 20% SDS-PAGE under non-reducing conditions. Finally to remove NaCl, collect fractions and dialyze against Tris buffer, 150mM NaCl. Store at -80°C.

Preparation of M9 minimal media with 3H glycine

To prepare 5x media, add ingredients to water in a 2-liter flask and heat with stirring until dissolved. Pour into bottles with loosened caps with loosened caps and autoclave 15 minutes at 15 lb/in^2. Cool media to <50°C before adding nutritional supplements and antibiotics (at this temperature the flask will feel hot but can be handled). Tighten caps and store concentrated media indefinitely at room temperature.

5x M9 medium, per liter
- 30g Na_2HPO_4
- 15g KH_2PO_4
- 5g NH_4Cl
- 2.5g NaCl
- 15 mg $CaCl_2$ (optional)

Before use, dilute concentrated media to 1x with sterile water and add the following sterile solutions, per liter:
- 1 ml 1M $MgSO_4 \cdot 7 H_2O$
- 10 ml 20% carbon source (sugar or glycerol)
- 0.1 ml 0.5% vitamin B1 (thiamine)
- 5 ml 20% L-amino acids to 40µg/ml
- 5 ml 20% 3H-glycine to 80µg/ml (approx. 100uCi)

Protocol for radioiodination of VEGF with ^{125}I

All steps will be carried out in the hood in room 019 McKinly Lab. All reactions are at room temperature unless stated differently.

1. Add 100 µl of TBS to a small glass test tube with 1 iodobead.
2. Add 5 mCi of $Na^{125}I$ in about 15 µl volume and incubate for 5 minutes.
3. Add 0.5 mg of VEGF in 400 µl of TBS and incubate for 10 minutes on ice.
4. Reaction mix will be applied to a disposable column containing Sephadex G25 resin and labeled VEGF will be separated from free ^{125}I.

Protocol for the use of ^{125}I-VEGF

1. Add 100 ng of ^{125}I-VEGF to 5 □l of a 8wt% low molecular weight heparin conjugated poly(ethylene glycol) (PEG-LMWH) in PBS to construct hydrogel.
2. Place this hydrogel in a transwell plate that is previously block with BSA.
3. Place 1 mL of PBS in the traswell plate and incubate for specific measrured times (1 h, 6 h, 1 d, 4 d, 8 d, 12 d, and 16 d) at 4^oC (the fridge in Dr. Cain's lab in 019 McKinly).
4. Remove PBS and replace with fresh PBS (1 mL).
5. Place the removed saline solutions in plastic gamma-counter tubes and store the tubes at -20^oC until the measurements.

Radiolabeled bacterial phospholipids

Glucose Media: Autoclave and add glucose as 0.2 um filtrate

Glucose, 2 mM
NH4Cl, 10mM
Sodium phosphate (pH 7.2), 2 mM
NSS

1. Streak out *Vibrio alginolyticus* 138-2 from the glycerol stock media on an LM plate.
2. Grow two days
3. Inoculate LM media with a colony
4. Transfer 0.1 mL to a 12 mL tube of glucose ammonium media
5. Add 1.0 mCi of ^{33}P orthophosphate
6. Grow overnight
7. Collect bacteria by centrifugation in capped tubes.
8. Wash with 1 ml NSS to remove any non-specifically bound radioactivity. Dispose of that as radioactive waste.
9. Add 1.0ml of HPLC grade chloroform and 2.0 ml of HPLC grade methanol
10. Homogenize sample with a Pasteur pipette
11. After 10 minutes at room temperature add 1.0 ml of chloroform and 1.0 ml of water, and homogenize the samples again
12. Centrifuge for 30 minutes at 2,500 x g. to produce two layers
13. Transfer the lower layer into a clean glass vial using a Pasteur pipette
14. Dry the solution by adding a spatula full of anhydrous sodium sulfate
15. Decant into a new vial, and re-extract the sodium sulfate with 1:2 Chl:MeOH to maximize recovery of 33P-labeled lipids:
16. Combine 1 ml of 1:2 Chl:MeOH with the decanted solvent and evaporate in a stream of nitrogen.
17. The evaporation will done under the fume hood that will be swipe surveyed immediately after each preparation to ensure that no aerosol contamination has occurred.
18. Use Teflon coated lids and store at -20C.

Notes:

- No vapor 33P is created by this procedure.
- The aqueous liquid waste produced from the medium and pellet wash will be properly disposed of in the designated liquid waste container
- The solid waste produced from the addition of sodium sulfate will be disposed of in the designated container for solid waste.

Micro-FISH with ^{33}P Phospholipids

I. Incubation with ^{33}P phospholipids

1. Fill the incubation bottles with 20 ml seawater.
2. Poison the killed control with 2% PFA (final concentration). Wait 15 minutes before adding the radiolabled compound to the killed control to be sure the cells are dead.
3. Add 2 microCi of the ^{33}P phospholipids.
4. Incubate at in situ temperature for 6 to 8 hours.
5. Transfer the sample to another bottle for fixation. Add 2% PFA (final concentration) and fix overnight at 4 ℃. Never add PFA to bottles you plan to reuse for incubating live samples.

II - Filtration

1. Set up the filter manifold with a 0.45 µm nitrocellulose filter supporting a 0.22 µm polycarbonate filter. Be sure to suck some deionized water through the nitrocellulose filter before laying the polycarbonate filter on top of it. Set up the appropriate amount of filters for each sample.
2. Determine the volume for filtration according to direct count. Cells should not be too close to each other to avoid false-positive cells.
3. Filter the appropriate amount of the fixed sample (try to make multiple filters for each sample.)
4. Rinse each filter three times with deionized water.
5. Store the filters in 7 mL scintillation vials in the freezer. Replicate filters can go in the same vial.

III - FISH

1. Label a 50 mL blue cap tube for the hybridization chamber.
2. Cover a glass slide with parafilm using tape on the back side of the slide to hold it in place. Label areas of the parafilm for different samples.
3. Prepare 30 µl of probe solution for each sample (1/12 or 1/16 of a 25 mm filter), using a final probe concentration of 2.5 ng/µl. Use the same concentrations for unlabeled competitor probes, i.e. unlabeled Gam42a with labeled Bet42a and vice versa.
4. Put a 30 µl drop of probe on the parafilm and place the filter piece face down on it.
5. Close the tube and incubate overnight at the appropriate temp' in the hybridization oven (temp' depend on the probe).
6. Aliquot into microtiter dishes 1 mL of warm wash buffer for each filter piece. Warm the wash buffer to proper temp' for probe in the incubator.
7. Move filters pieces to the warm wash solution (Fig. 2.)
8. Incubate at proper temp' for 15 min.
9. Rinse the filter pieces by dipping in deionized water or 80% EtOH (if using black polycarbonate filters don't wash with EtOH, color will wash out and interfere with analysis.)
10. Dry the filter pieces in the dark on blotter paper labeled to keep track of different samples.

11. Arrange the filter pieces in labeled 96-well titer plate (or 12-well plate) for the darkroom.

IV - Autoradiography

Notes:

a) All work involving photographic emulsion should be done in the dark room under a safe light.
b) All emulsions have to be kept at 4℃ when not used and warmed to 43℃ to liquefy before the experiment.

1. Dilute 43℃ emulsion with water in a black film canister.
2. After dilution let emulsion to solidify overnight at 4℃.
3. Set the emulsion in a 43℃ water bath (Fig. 6). Let it liquefy for at least 1/2 hour.
4. Set an aluminum block on ice.
5. Set the safe light on the bench close to the plate with the samples.
6. Dip a labeled glass slide in the emulsion (Fig. 7). Check the coating under the safe light, if there are a lot of bubbles don't use the emulsion, make a new dilution.
7. Working briefly under the safe light, place a filter piece with cells down on the emulsion (when preparing the filters, it is recommended to make a mark on the filter that will indicate the side with the cells, e.g. cut one of the corners).
8. Set the slide on the aluminum block (Fig. 8).
9. Allow the emulsion to gel for 15 min.
10. Transfer slides to dark boxes (Fig. 9).
11. Place dark boxes in the refrigerator for the exposure time (determined by time series).

V - Development

1. Prepare Kodak developer and fixer (not rapid fix.)
2. Place the developer, stop (water) and fix in a 14℃ water bath
3. Develop slides for 2 min, stop 10 sec in water, fix 6 min and wash in water 6 min
4. Dip in 1% glycerol (1-2 min) (Fig. 13).
5. Dry overnight in a dark vacuum desiccator.
6. Trace the outline of the filter on the back of the slide.
7. Peel the filter away from the emulsion.
8. Mount with a cover slip using an antifade mountant (4:1 mixture of Citifluor, Ted Pella and VectaShiled, Vector labs) containing 0.5 ng/µl DAPI (Fig. 14).

Sorting Radio-labeled Cells with Flow Cytometer

Goal: to count and collect marine bacteria and phytoplankton labeled with radioactive isotopes using a flow cytometer/cell sorter.

General procedure: seawater samples labeled with radioactive compounds (e.g. ^{35}S-DMSP, ^{3}H-leucine, ^{3}H-amino acids, ^{14}C-bicarbonate) are injected into a FACScaliber flow cytometer. The seawater enters a narrow pipe causing bacteria and phytoplankton to line up single file into a small liquid stream. The stream passes in front of fluorescence detectors. Cells with specific signals are removed from the stream of seawater by momentarily diverting the liquid stream into an second pipeline. Both sorted and unsorted cells and fluid are collected in external reservoirs.

This procedure has been performed safely in multiple labs in both the U.S. and Europe for the past three years. We will be following the same procedure and using the same equipment as these other labs.

Safety considerations:

1) Samples are typically 1 mL seawater with 0.05-0.01 uCi of isotope.

2) Samples are contained in plastic tubes that remained sealed until injection. Samples are also sealed during injection.

3) The fluidics system and flow cell are entirely sealed, so no radioactive aerosols are generated.

4) Only two points for contamination exist, the injection needle and the end of the tubing leading to external reservoirs for sorted and unsorted sample. However, the injection needle has a removable cover that can be cleaned. We will use extra needle covers specifically for work with radioactive samples. The external tubing can be disposed of in the solid radioactive waste.

5) The external reservoirs for sorted and unsorted samples will be held in a large plastic bin for secondary containment in case of spillage.

6) All work will be done in a single room designed only for flow cytometry.

7) Wipe tests will follow each usage.

^{3}H-Thymidine Cell Proliferation Assay

RAM: Thymidine, [methyl-^{3}H], 1 mCi/mL, ICN #012406001
Cell Growth Media: DMEM with 10% Calf Serum
PBS: Phosphate Buffer Saline (1x)

1. Prepare Samples

Seed cells in 96 well cell culture plates (1000 to 10,000 cells per well) in 100 μL cell growth media.
Incubate cells for 48 hr at 37 ^{o}C and 5 % CO_2.
In biosafety cabinet on a removable tray, remove media from wells of cell culture plate and add 100 μL serum free media (DMEM) containing 0.2 μCi [^{3}H]-Thymidine (1 μCi/mL) to each well.
Incubate cells in secondary container for 8-24 hours at 37 ^{o}C and 5 % CO_2.

2. Harvest DNA

Wash cells two times with 100 μL cold PBS.
Wash cells two times with 100 μL cold 5 % Tricholoracetic acid.
Solubilize cells by adding 500 μL 10.25 N Sodium Hydroxide.
Transfer solutions to 96 well scintillation plate or scintillation vials and count.

Waste generated:
Liquid waste: Approximately 200 mL per 96 well plate
Solid waste: Cell culture plate, pipette tips and scintillation plate or vials
Both solid and liquid waste will be cleaned with bleach to ensure nonpathogenicity and pH will be adjusted if it is higher than 12.
Wipe tests will be performed on hood, incubator, and secondary containers every day experiments are in progress and counted on departmental LSC.

Ligand Binding Assay Using Cell Lysates

Radioactive Material:

Dihydrotestosterone (5α Androstan-17β-OL-3-One), [1,2,4,5,6,7-3H(N)]- 110-150 mCi/mmole, 1mCi/mL (Perkin Elmer)

Procedure:

1) Monkey kidney COS cells will be plated in a 6-well plate and incubated at 37 °C and 8% CO_2 for 24 hours.
2) Cells will be transiently transfected using Lipofectamine and 1.5 μg total DNA per well.
3) Cells will be maintained in 10% fetal bovine serum and phenol red free DMEM for 36 hours
4) The media will be aspirated off and replaced with 2 mL of 5 nM [^{3}H]Dihydrotestosterone solution in the presence and absence of increasing concentrations of unlabeled ligands. The 5 nM solution will be prepared from the stock solution purchased from Perkin Elmer (see above). The final 5 nM solution will contain no more than 1 μCi/mL, thus each well will contain no more than 2 μCi. The cells will be incubated for 2 hours at 37 °C and 8% CO_2.
5) The media will be aspirated off into an Erlenmeyer flask. A vapor trap will be placed between the trap and the vacuum pump. The solution will be bleached prior to placement into liquid [^{3}H] waste.
6) Cells will be washed with 2 mL of 1x PBS and again solution will be aspirated off, bleached and placed into liquid [^{3}H] waste.
7) The cells will be lysed with 2% SDS, 10% glycerol and 10 mM Tris, pH 6.8.
8) The lysate will be placed into a liquid scintillation vial and transported in a secondary container to a department approved LS counter
9) After counting, the vials will disposed of in LSV [^{3}H] waste.
10) The 6-well plates will be bleached and placed in solid [^{3}H] waste.

Notes:
Typically, 2-6 samples will be run each time, at 6 different concentrations, in triplicate. Thus, 36-108 wells per assay. This will generate between 72-216 μCi per experiment.

Disposal:
The following will be contaminated and need to be disposed of:
1) 6-well plates
2) Media and wash buffers
3) Conical tubes
4) Micropipette tips
5) LSC vials

GST Pull Down Assay
(NCoR/GRIP interactions with TRb)

Purpose: Assay for protein-protein interactions in-vitro
This common laboratory experiment is based on protocols described in:
Yamamoto et al, Genes and Development, 12:3343-3356, 1998
Marimuthu et al, Molecular Endocrinology, 16(2):271-286, 2002

Part I: Preparation of Unlabeled fusion protein
a) Expression of GST-NCoR
1. Transform BL21 bacterial strain with proper plasmid (pGEX-NcoR, pGEX-GRIP1)
2. Grow a 5mL culture overnight with ampicillin and chloramphenicol
3. Seed a 1-2 liters culture the next morning
4. At OD_{600}=0.8, induce expression with 0.5mM IPTG at room temperature by shaking for 3h
5. Centrifuge in SS-34 rotor 5,000 rpm for 20 min.
6. Freeze down the bacterial pellet at -80°C for at least 30 min. Cells can be stored at –70C
7. Redisolve cells in Sonication Buffer.
   Sonication Buffer: 20mM Tris at pH=7.9, 500mM NaCl,10 mM, merceptoethanol, protease inhibitor (amount from the book)
8. Lyse by sonication
9. Centrifuge in SS-34 rotor at 10000 rpm and collect supernatant. *Lysate can be stored in –70C, however since it contains the protein, storage condition may vary depending on the stability of the protein.*

b) Resin preparation
1. Transfer 1.33ml aliquot of the resin (mix well before use) into a 15ml Falcon tube
2. Centrifuge (1 pulse at 500g)
3. Resuspend in 10 ml PBS 1x (w/o ca)
4. Centrifuge (1 pulse at 500g)
5. Resuspend in 10 ml PBS 1x (stable for once month at 4°C)

c) Affinity matrix preparation
1. Add 100ul slurry for 1 m extract
2. Agitate for 30 min on a spinning wheel at RT
3. Wash three times with 1x PBS
4. Resuspend the resin in 100 ul IPAB-gelatin buffer

Part II: Preparation of Labeled Protein by In-vitro Translation using ^{35}S-Methionine

The following protocol is based on the standard protocol reported by promega for TnT® coupled Reticulocyte expression system.
RAM: [^{35}S]-Methionine: Amersham AG 1094; Redivue L-[^{35}S]-Methionine.1000 Ci/mmol, 10 mCi/ml. Stored in +4°C in 266 BrL.
1. Reaction

In a 1.5 ml microfuge tube on ice mix:

Rabit Reticulocyte	25ul
Reaction Buffer	2ul
RNA polymerase	1ul
Amino Acid mix (-Met)	1ul
^{35}S-Methionine (>1000 Ci/mmol, 10mCi/ml	**1ul (10uCi)**
RNasin®	1ul
DNA	1ug
Water to final volume of 50 ul	
Total	50 ul

The reaction mixture is incubated for 90 min in a 37°C water bath and then placed on ice. Concentrated

protein can be stored up to 2 weeks in 4°C. Protein concentration is established by SDS-PAGE/Phosphorimaging. 15 fmol of protein (typically 5 ul) is diluted binding buffer up to 150 ul ((20 mM HEPES, pH 7.9, 150 KCl, 25 mM MgCl2, 10 Glycerol, 0.1 % Triton X-100, NP-40, and 0.01% DTT).

Part III: GST pull-down Assay

Approximately 18 samples (6 conditions in triplicate) will be performed per experiment. (Each sample contains approximately 0.5 uCi or 9 uCi per experiment.) Larger experiments may be semi-automated using a filtration manifold and filtration plates.

1. 100 ul of loaded GST affinity matrix (from part Ic) and 50 ul of ^{35}S-labeled protein (above #1, Part II) are mixed by inversion.
2. Incubate for 2 hrs at 4C.
3. The sample is loaded on a spin column and the GST beads are isolated by gentile centrifugation. The beads are wash twice with PBS-DTT buffer using spin column (Filtrates will will be collected as liquid radioactive waste)
4. Add 25 ul of SDS loading buffer directly on to pelleted resin
5. Vortex
6. Heat for 1 min at 100C
7. Analyze by SDS-PAGE/autoradiography (Part IV) or by scintillation counter (BrL 222) using scintillation cocktail.

Part IV: Confirm protein expression by SDS-PAGE/autoradiography:

1. Prepare samples for SDS-PAGE: 2-10 ul aliquots are placed in separate tubes containing 10ul of loading dye. The samples are heated to 90°C in a heat block for 5 min to denature and are then added to a 12% SDS polyacrylamide gel. The gel runs for 1-3 hours.
2. Disassemble Gel box and fix gel. The gel is removed from the box and washed with an aqueous solution of 5% acetic acid 30% methanol.

(Based on past experience, this solution should not contain radioactivity, however, the solution will be retained and tested for activity before disposal.)The running buffer (which should contain the un reacted ^{35}S-Methionine) will be collected as liquid waste. (Running buffer contains 0.025M Tris, 0.2M glycine and 1g/L SDS in water)Dry the gel. The gel is transferred onto a filter paper covered with plastic wrap and dried in a gel drier.

3. Image the gel. The dried gel will be placed in a phosphorscreen cassette and exposed for 6-24 hours. The gel will be removed before the phosophorscreen in transported to the Core Facility in McKinley for scanning.
4. Sample storage: Radioactive samples will be stored at +4°C for up to 2 weeks before discarding in solid waste.

^{51}Chromium Release Assay

Hazards:
Radioactive ^{51}Chromium

All radioactive work must be done in approved and posted radioactive use areas.
Protection:
Lab Coat
Gloves
Dosimeter ring and badge
Lead Shielding
Geiger Counter for monitoring radiation exposure
Absorbent paper or blue pad
Waste:
Solid waste should be disposed of in container for short half-life isotopes. Included in solid waste is bench paper, pipette tips, dried plates, kimble tubes, and similar materials.

Liquid waste should be stored in vacuum trap. Trap level should be monitored regularly.
Spill Clean-Up:

Decontaminate area with Lift-Away

Target Cells

1. Count cells and spin down appropriate number to approximately 10^6 cells/200 ml RP-10 in microcentrifuge tube.
2. Add 100 mCu ^{51}Cr/10^6 cells. Increase volume of ^{51}Cr as it decays.
3. Record date, amount of isotope used, activity used, and amount remaining on inventory forms. Complete Radioactive Waste Inventory forms for both liquid and solid waste.
4. Add peptide if appropriate. (i.e. antagonism assay)
5. Incubate cells for 1 hour at 37° in appropriately labeled incubator.
6. Wash cells 3X in RP-10. Aspirate liquid waste into reservoir in 7-155.
7. Resuspend in appropriate volume RP-10 for assay. Target cells are typically used at 10^4 per well and 50 ml per well.
8. Treat all pipettes and other materials that contact radiolabeled cells as radioactive and discard appropriately.

Effector Cells

1. Count appropriate effector cell(s).
2. Resuspend correct number in RP-10. Effector cells are typically used at $3x10^4$ per well in 100 ml per well.

Peptide(s)

1. Peptides are typically diluted in 50 ml PBS per well.
2. If serially diluting, be sure to change yellow tips between wells.

Controls

Spontaneous Release:
1. 50 ml Target cells
2. 150 ml PBS
3. Always done in triplicate.

Detergent Release:
1. 50 ml Target cells
2. 100 ml Triton-X detergent
3. 50 ml PBS
4. Always done in triplicate

Preparation of radiolabeled nucleic acid probes-(32P)

1. Preparation of radiolabeled nucleic acid probes for either DNA or RNA probes takes place in a designated and shielded water bath at 37 degrees Celsius. This usually requires 50 µCi of α-32P-dNTP (3000 Ci/mmol for DNA probes and 800 Ci/mmol for RNA probes).
    a. Enzyme, primer (if required), buffer and radionuclide (most often 32P-dCTP or 32P-CTP) are incubated for 15-30 minutes.
    b. The reaction is stopped by chelating the metal ions from the reaction by adding EDTA behind a Plexiglas shield.
    c. The material is the purified by spin column using a RAM designated microcentrifuge with appropriate shielding.
2. An aliquot will be removed behind appropriate shielding for Cherenkov counting in a scintillation counter.

Blot Hybridization-(32P)

Northern, Southern and dot blots of DNA or RNA requires essentially the same steps as outlined below for the hybridization:

1. Filters are wet in 2-5X standard saline citrate in the hybridization vessel /tubes(Note that these tubes are sealable and glass. As such they provide a great deal of shielding in and of themselves.) then are transferred to hybridization solution (Buffered dextran sulfate solution with yeast tRNA and sheared Salmon DNA carrier) for pre-hybridization at the desired temperature (42-72 degrees Celsius).
2. Hybridization occurs in a RAM designated hybridization cabinet designed to capture and contain and spillage. (The cabinet is stainless steel lined with a glass front door. No counts have been detected from hybridizations in progress through this apparatus using a Geiger counter for Beta emissions, construction also makes clean-up very facile)
3. The quantified radioactive nucleic acid probe is generally added at 1-2 X 10^6 dpm per ml to the hybridization buffer. The probes are denatured behind shielding by heating to 94 degrees Celsius for 3-5 minutes in an Eppendorf tube. Care is taken to allow ample room in the tube so that sample expansion does not pop the top. Filters are incubated with the probe overnight.
4. washing. The hybridization solution with probe is decanted into the appropriate waste container. All washes, usually containing 1% SDS with decreasing concentrations of SSC, will also be collected. No wash material will be disposed of through the sinks.
5. Washed filters, now with minimal residual isotope, are sealed in plastic bags and exposed to x-ray film at –80 degrees Celsius. Students are warned of the dangers of low-temperature burns. Cassettes are separated by 1/2-inch Plexiglas sheets.
6. Filters are discarded after use in the solid RAM waste container.

General considerations:

Students will be advised to follow good laboratory practices including the use of protective clothing (coats), eye protection, double gloving, and appropriate shielding, time and distance consideration for the isotope in use. All students will be advised of the use of RAM in the lab and made aware of its storage and usage areas. All equipment for these experimental procedures that comes into contact with RAM or RAM waste will be appropriately designated with radioactive material labels. As with general GLP there is no food or drink to be consumed in the lab and no make-up applied in the lab. All lab personnel will be required to complete basic radiation safety training regardless of RAM usage.

Kinase Assays-(32P)

1. The kinase in question is immunoprecipitated from the whole cell lysates using agarose-conjugated antibodies. This procedure is cold. The pellet is resuspended in minimal kinase reaction buffer and approximately 10-20 µCi of γ-32P-ATP is added behind appropriate Plexiglas shielding.
2. The reaction is incubated at 37 degrees Celsius for 10-30 minutes. The pellet is washed with cold reaction buffer to remove excess 32P-ATP. Microcentrifuges are RAM designated and behind shielding.
3. The pellet is the solubilized in Laemmli SDS-PAGE gel electrophoresis buffer and denatures by incubating at 94 degrees Celsius for 3-5 minutes.
4. The sample is resolved by electrophoresis in a RAM designated gel system; the gel dried down onto filter paper in a RAM designated gel dryer, covered in saran wrap and exposed to X-ray film.
5. The gels are discarded into the appropriate waste container.

General considerations:
Students will be advised to follow good laboratory practices including the use of protective clothing (coats), eye protection, double gloving, and appropriate shielding, time and distance consideration for the isotope in use. All students will be advised of the use of RAM in the lab and made aware of its storage and usage areas. All equipment for these experimental procedures that comes into contact with RAM or RAM waste will be appropriately designated with radioactive material labels. As with general GLP there is no food or drink to be consumed in the lab and no make-up applied in the lab. All lab personnel will be required to complete basic radiation safety training regardless of RAM usage.

Mitotic labeling-3H

1. To determine the growth rate of cells in culture, 1 µCi/ml of 3H-thymidine will be added to growth media in the presence and absence of test compounds. All cells will be handled on adsorbent blotters to capture any RAM spillage in a RAM designated class II biosafety cabinet.
2. The cells are grown in a RAM designated incubator. Students will be advised as to the potential for aerosolization and the risks of exposure as well as precautionary measures. All lab personnel are required to wear gloves, sleeves and face masks as part of the tissue culture procedures currently in place regardless or RAM usage.
3. Cell DNA is trichloroacetic acid precipitated, solubilized in NaOH and neutralized with concentrated HCl and transferred to scintillation vials where they are mixed with aqueous scintillation fluid.
4. The samples are then counted in a Beta counter to determine incorporation. Vials are disposed of in the appropriate RAM waste.

General considerations:
Students will be advised to follow good laboratory practices including the use of protective clothing (coats), eye protection, double gloving, and appropriate shielding, time and distance consideration for the isotope in use. All students will be advised of the use of RAM in the lab and made aware of its storage and usage areas. All equipment for these experimental procedures that comes into contact with RAM or RAM waste will be appropriately designated with radioactive material labels. As with general GLP there is no food or drink to be consumed in the lab and no make-up applied in the lab. All lab personnel will be required to complete basic radiation safety training regardless of RAM usage.

Metabolic Cell Labeling of Protein-35S-translabel (Met/Cys mix)

1. To study protein biosynthesis and degradation, cells will be incubated with 35S-translabel (Met/Cys mix). All cells will be handled on adsorbent blotters to capture any RAM spillage in a RAM designated class II biosafety cabinet.
2. The cells are grown in a RAM designated incubator. Students will be advised as to the potential for aerosolization and the risks of exposure as well as precautionary measures. All lab personnel are required to wear gloves, sleeves and face masks as part of the tissue culture procedures currently in place regardless or RAM usage.
3. At the designated time-points, labeled cell protein will be isolated by standard cell lysis conditions (RIPA: tris, SDS, desoxycholate, NP-40 etc.) using the appropriate precautions associated with RAM labeled samples, blot paper under work surface, work in designated areas, etc…
4. The protein sample may be either electrophoretically resolved, or immunoprecipitated followed by gel electrophoresis. The precautions and waste disposal are described above.

General considerations:

Students will be advised to follow good laboratory practices including the use of protective clothing (coats), eye protection, double gloving, and appropriate shielding, time and distance consideration for the isotope in use. All students will be advised of the use of RAM in the lab and made aware of its storage and usage areas. All equipment for these experimental procedures that comes into contact with RAM or RAM waste will be appropriately designated with radioactive material labels. As with general GLP there is no food or drink to be consumed in the lab and no make-up applied in the lab. All lab personnel will be required to complete basic radiation safety training regardless of RAM usage.

Xylosyltransferase activity assay-C14

1. To study the enzymatic xylosyltransferase activity the enzyme to be assayed will be present in buffered aqueous solution obtained from a variety of potential sources. These include lysates released from cultured cells, cell culture media, mouse plasma, or *Drosophila* hemolymph. Cells do not present a human biohazard.
2. The reactions will be carried out in up to 10 test tubes per experiment. In aqueous buffered solutions reactions will be performed by adding the source of enzyme described in 1, a peptide acceptor and C14 xylose (maximum 0.05mCi total). Reactions will be incubated for designated times (less than a few hours) and terminated by trichloroacetic acid precipitation. Reactions will be performed using the appropriate precautions associated with RAM labeled samples, blot paper under work surface, work in designated areas, etc…
3. Trichloroacetic acid precipitated reactions will be neutralized with concentrated 1M tris base, collected on nitrocellulose filters, filters will be extensively washed with aqueous solutions to remove unincorporated isotope and filters will be transferred to scintillation vials where they are mixed with aqueous “biodegradable” scintillation fluid.
4. The samples are then counted in a Beta counter to determine incorporation. Vials are disposed of in the appropriate RAM waste.

General considerations:

Students will be advised to follow good laboratory practices including the use of protective clothing (coats), eye protection, double gloving, and appropriate shielding, time and distance consideration for the isotope in use. All students will be advised of the use of RAM in the lab and made aware of its storage and usage areas. All equipment for these experimental procedures that comes into contact with RAM or RAM waste will be appropriately designated with radioactive material labels. As with general GLP there is no food or drink to be consumed in the lab and no make-up applied in the lab. All lab personnel will be required to complete basic radiation safety training regardless of RAM usage.

Pulse Chase Experiment using S-35 Trans label 11/4/2008

Labeling Step

1. 100 mm dish of sub confluent cells will yield enough TCA precipitable counts for several immunoprecipitations. Six-well plates may be used instead of 100mm dishes. One dish or well will be used for each time point desired. No more than six time points are likely to be conducted at one time.
2. Remove media from cells and rinse cells two times with PBS/methionine- cysteine free media.
3. Add 4 ml of PBS/methionine- cysteine free media and 0.5% BSA per 100mm dish.
4. Incubate 30-45 minutes at 37° C.
5. Add 100mCi/ml S-35 Trans label. This is 100uCi per dish or well.
6. Incubate at 37° C for 1 to 4 hours or overnight.
7. Add 4ml of complete media (with cysteine and methionine) and 0.5% BSA to chase for 4 to 6 hours.
8. Collect media from labeled cells into centrifuge tube. Add protease inhibitor cocktail to 10x final concentration. Mix and freeze at -20° C.
9. Gently rinse plate 2 times with 4 ml of PBS to remove all media.
10. Add 2 ml of 0.5% SDS in PBS and 1x protease inhibitor cocktail to cells.
11. Incubate several minutes until cells lyse.
12. Collect cell lysate through 23 G needle and aspirate 3-4 times to shear DNA. Transfer from plates to centrifuge tubes.
13. Rinse plates with 2ml of PBS-TD. After one minute, add this to cell lysate.
14. Again add protease inhibitors to the cell lysate tube to 1x concentration and store at -20° C.
15. Perform similar collections at different time points during the chase (30, 60, 90 mins).

TCA Precipitation Step

1. Stop the reaction by adding 20 µl ice cold 10% TCA. Mix thoroughly and incubate 10 min on ice to precipitate protein.
2. Pipette sample onto whatman GF-C glass fiber filter held in a vacuum manifold. Allow the sample to pass through the filter. Rinse the tube with 500-µl ice cold 5 % TCA. Add this wash to the appropriate filter.
3. Wash each filter with 5 ml ice cold 5% TCA solution four times. Wash four times with 10 ml of 95% ethanol. Allow each filter to dry for few minutes.
4. Place dry filters in 20 ml scintillation vial for counting.

Safety considerations:

1. To prevent hand injury, the 23G needle will not be recapped. The needle and any other sharps will be placed into a red sharps container (or other rigid container) marked with radiation warning tape and the word, “S-35”.
2. A flask-type vacuum trap will be situated between the filtering apparatus and the vacuum pump to protect the pump from contamination. There will also be a Millipore filter in the vacuum line between the trap and the vacuum pump to prevent the passing of aerosols.
3. The 23G needle aspiration will generate micro-aerosols and will therefore be performed in the tissue culture hood.
4. The TCA solution and ethanol waste generated in the TCA precipitation step are chemically hazardous as well as radioactive and will, therefore, be stored as “mixed waste” in a separate radioactive liquid waste container. These wastes will NOT be placed into the standard radioactive liquid waste container used for radioactive media, buffers, and other radioactive liquids that are not chemically hazardous. The mixed waste container shall be stored in secondary containment and be labeled as appropriate for its chemical contents (orange chemical waste label) as well as its radioactive contents.
5. Benchpaper (with the absorbent side UP) will be used to line the radioactive lab work benches.

^{14}C Primary Production Protocol

Isotope Preparation:

Sodium Bicarbonate, (^{14}C) from MP Biomedicals (formerly ICN) 1-800-854-0530
Catalog # 17441H (Sterile Aqueous solution)

Working Stock = 1mCi ^{14}C per 15 ml Milli-Q H_2O

The Milli-Q should be GF/F filtered and pH = 10 before stock is added. I use 1N NaOH to adjust the pH of the Milli-Q.

Incubations:

250 ml sample bottles are filled to neck with sample.

> We use 250 ml Wheaton glass lab media bottles w/ teflon-lined cap (you may have to contact the distributor and ask for non-graduated clear bottles).

100 ul of working stock is added to each sample bottle, bottle capped and shaken.

Each sample is placed in appropriate light sock and placed in ambient temperature flow-through deck incubator for appropriate incubation duration.

We use an 8 bottle light series: 100, 58, 34, 20, 11, 4, 1 % and dark

> Light socks are made from fiberglass window screening that can be bought at most hardware stores. The socks that we use are 12 inches long by 8 inches wide (they will actually hold 2 samples if needed to). We use clothes pins to seal the incubation socks.
>
> We have determined that 1 layer of screening provides 58% light, 2 layers equals 34%, 3 layers equals 20%, 4 layers equals 11%, 6 layers equals 4%, and 8 layers equals 1% light transmission. The screening may vary between manufacturers, so I would recommend that you calibrate your own light socks.

Filtrations:

At appropriate time point(s), a subsample is taken from each sample.

Samples are filtered through a GF/F filter on an approved isotope filtration rack.

Our time points are at 4-5 hours (gross) and 24 hours (net).

> Each filtration is accompanied by a FSW rinse (of similar salinity) of the filtration column, a FSW rinse of filter pad, and a dilute HCl (1%) rinse.
>
> Each sample is placed in a (numbered) 7 ml glass scintillation vial containing 5 ml of scintillation cocktail (we use Ecoscint Scintillation Solution #LS-271 from National Diagnostics, 800-526-3867).
>
> At the first time point, a 500 ul sample is taken from the dark bottle and placed in a scintillation vial containing 5 ml of cocktail for total activity. My total activity samples are about 15,000 dpm/ml.

Clean-up:

After the last time point has been taken, the remaining sample is poured into an approved liquid rad carboy for return to the lab. Each sample bottle receives 2 fresh water rinses (of about 100 ml each) that are also placed in the liquid rad carboy. The sample bottles are now ready to re-use.

^{35}S Labelled Riboprobe Protocol

Before starting:

1. Cut the appropriate plasmid with the correct restriction enzyme and clean the digest up with a Quiaquick PCR purification column. Elute the DNA from the column in 50µl DI.
2. Take the ^{35}S UTP out of the freezer 15 minutes ahead of time to allow to thaw. Mix well before using.
3. Thaw reagents in ribiprobe kit.
4. Make a 120µM mixture of UTP by adding 1µl of 10mM UTP into 83µl DI.
5. Make nucleotide mix by adding 3µl of ATP, GTP, CTP, and DI water.

For transcription make the following reaction mixture:

1. 12µl linearized vector
2. 9µl 5X transcription buffer
3. 5µl nucleotide mixture
4. 1µl 120µM UTP
5. 2.5µl DTT
6. 10µl ^{35}S UTP
7. 1µl RNAsin
8. 2µl RNA polymerase (SP6 or T7)
9. 2.5µl DI water

45µl total

-Incubate for 1-2 hours at 37°C.
-Add 5µl RQ1 DNAse and incubate for 15 minutes.
-Add 65µl DI to bring to a total of 100µl.
-Remove 2µl and add to 98µl DI for a total count and vortex. Spot 2µl of the 100µl mixture onto a 0.22µM Nucleopore filter and place in a liquid scintillation vial labeled TC.
-Purify the remainder using an RNEasy column as follows:

-Add 350µl RLT Buffer
-Add 250µl Ethanol and mix well
-Apply the sample to an RNEasy column and spin for 1 min. at 14K rpm.
-Discard the collection tube and place the column in a new collection tube.
-Wash the column with 500µl RPE Buffer and spin for 1 min, at 14K rpm.
-Discard the collection tube and replace with a new collection tube.
-Wash the column with 500µl RPE Buffer and spin for 1 min. at 14K rpm.
-Discard the collection tube and replace with a new collection tube.
-Spin column dry at 14 K rpm for 2 min. and discard the collection tube.
-Elute the RNA into a fresh collection tube with 50µl DI water.
-Spin for 2 min. at 14K rpm.
-Leave the collection tube attached to the column and load 50µl elution buffer (10mM Tris pH 8.0, provided by Quiagen) onto the column and spin for 2 minutes at 14K rpm.

-Remove 2µl and add to 98µl DI for the probe count and vortex. Spot 2µl of this 100µl mixture onto a 0.22µM Nucleopore filter and place in a liquid scintillation vial labeled PRC.
-For a precipitable count add 2µl of the dilution made for the probe count and add to 400µl ice-cold CT-DNA (0.1mg/ml) in a 15 ml centrifuge tube. Add 5 ml 10% ice-cold TCA and precipitate at -20°C for 30 min. Filter the precipitate onto a 0.22µm Nucleopore filter and rinse the filter with 5 ml ice-cold TCA. Add this filter to a liquid scintillation vial labeled PCC.
-For storage of the probe, add the remaining 98µl to 10ml ribohybe buffer and store at 4°C.

Determination of % incorporation:

-Add 0.5 ml of ethylene glycol to each vial and dissolve the filters.
-Add 10 ml liquid scintillation counting fluid to the vials and mix by shaking.
-Count TC, PRC, and PCC in scintillation counter.
-%incorporation= $[CPM_{prc}/CPM_{tc}]$ X 100 –OR-
-%incorporation= $[CPM_{ppc}/CPM_{tc}]$ X 100
-The two calculations should come out to be the approx. the same.

Random Primed DNA Labeling Protocol

1. Denature the DNA by heating for 10 minutes at 100°C and subsequent cooling on ice.
2. Add the following to a microfuge tube on ice and make up to a final volume of 20 µl:
   -25 ng denatured DNA, corresponding to 2µl control DNA (solution 1)
   -3µl dATP, dGTP, dTTP mixture (prepared by making a 1+1+1 mixture of solutions 2, 4, and 5)
   -2µl reaction mixture (solution 6)
   -5µl 50µCi [32P] dCTP, 3000Ci/mmol, aqueous solution, make up to 19µl with sterile DI water (control reaction: add 7µl sterile DI).
   -1µl Klenow enzyme

3. Incubate for 30 min at 37°C.
4. Stop the reaction by adding 2µl 0.2M EDTA (pH 8.0) and/or by heating to 65°C for 10 min.
5. When using labeled DNA for hybridization, removal of non-incorporated deoxyribonucleoside triphosphates is not necessary but can be performed by chromatography on a Quick Spin Column, Sephadex G-50 (Fine) or by repeated ethanol precipitation, if required.

The Randon Primed DNA Labeling Kit includes:

1. Control DNA: One vial 1 containing 20µl λ DNA, 12.5µg/ml.
2. dATP: One vial 2 containing 50µl 0.5mM 2'-deoxyadenosine-5'-triphosphate in Tris buffer.
3. dCTP: One vial 3 containing 50µl 0.5mM 2'-deoxycytidine-5'-triphosphate in Tris buffer.
4. dGTP: One vial 4 containing 50µl 0.5mM 2'-deoxyguanosine-5'-triphosphate in Tris buffer.
5. dTTP: Once vial 5 containing 50µl 0.5mM thymidine-5'-triphosphate in Tris buffer.
6. Reaction mixture: One vial 6 containing 100µl hexanucleotide mixture in 10X concentrated reaction buffer.
7. Klenow enzyme: One vial 7 containing 50µl Klenow enzyme labeling grade, 2 units/µl in glycerol, 50% (v/v).

Hybridizing Membranes Using Radio-Labeled Probes and Washing of Filters After Probing

1. Wet membrane in 0.4 M Tris-HCl for 5 minutes at room temperature.
2. Place filter in either a hybridization bag, or canister and add 10-20 ml hybridization buffer.
3. Seal bag or canister and pre-hyb the membrane for 15 min. at 45°C for DNA and 55°C for RNA in a hybridization oven.
4. After the pre-hyb is complete, remove the hybridization buffer.
5. Add the radio-labeled probe to the hybrid. bag or canister with the membrane.
6. Hybridize overnight at 42°C for DNA and 55°C for RNA in the hybridization oven.
7. The next morning remove the probe to a 15 ml centrifuge tube, label appropriately, and store at 4°C.
8. Remove the membrane from the bag or canister to a radioactive plastic container.
9. Wash the membrane 3 times with PSE for 1 hour at 65°C.
10. Wash the membrane 3 time with PES for 30 min. at 65°C.
11. Place the membrane of gel-blot paper and allow to dry.
12. Wrap the membrane in saran-wrap and either expose to x-ray film or quantify using a molecular imager.

Bacterial $^{33}P_i$ uptake from phytoplankton lysate experiment:

1 mCi of ^{33}P (as orthophosphate, P_i) will be diluted in 10 ml Milli-Q water to make the working stock (100 µCi ml^{-1}).

Day 1: Standard $^{33}P_i$ uptake experiment-

1. Isolate 1 liter of phytoplankton alone (no bacteria or viruses) in a clear, plastic 1 liter bottle. At time zero, add 300 µl stock $^{33}P_i$ (30 µCi total) to whole sample. Shake sample.
2. Filter 10 ml sample onto a 1.0 µm filter and read the $^{33}P_i$ on the filter (P_i incorporated by phytos) and in the filtrate (P_i not yet incorporated).
3. Samples are read in 7 ml glass scintillation vials with Ecoscint Scintillation Solution (National Diagnostics). Filters are placed in vials, 5 ml of Scintillation fluid is added to the vial, the vial is vortexed, and the sample read. For filtrate, 1 ml of filtrate is added to 4 ml of scint fluid in the tube, mixed and read on the scint counter.
4. Repeat as necessary over time (15, 30 and 60 minutes, every hour after that). When the phytos have incorporated all the $^{33}P_i$, (again, if this is possible- in the very P-limited Gulf of Mexico, turnover time for the P_i pool measured with $^{32}P_i$ was 30 minutes-3.5 hours) all the radiation will be on the filter.

All liquid waste will go into one container (separate from ^{14}C waste), including 2 filtered seawater rinses used to clean the incubation bottle at the end of the experiment. Solid waste will be stored in a separate container.

<u>Bacterial uptake experiment (start one on day 2)</u>:

5 Time points: 0, 6, 12, 18 and 24 hours (more up to 48 hours if time permits)

1. Whole phytos only incubation with $^{33}P_i$: as above, add 300 µl of $^{33}P_i$ working stock to 1 l of isolated phytoplankton, incubate X hours (until all $^{33}P_i$ is taken up by phytos, if possible, as determined above), and filter 10 ml onto 0.2 µm Nuclepore polycarbonate filter. Count $^{33}P_i$ in on filter (fraction 1) and in filtrate (fraction 2). Do three of these in 1 l bottles.

Fraction 1: $^{33}P_i$ in phytoplankton
Fraction 2: $^{33}P_i$ not taken up by phytoplankton

2. t= 0 time point.
   In one incubation, add nothing. This will be the negative control and will tell us the release of $^{33}P_i$ into the dissolved fraction (fraction 5 below) through leakage from phytoplankton and grazing by protozoa.
   In second incubation, add viruses and bacteria from concentrate at ambient concentration. This will be the experiment where we can follow flow of $^{33}P_i$ from phytos to bacteria and dissolved pool (hooray!).
   In third incubation, add higher concentration of viruses and ambient concentrations of bacteria. This will allow us to quantify viral impact on P cycling through exaggeration of their role (higher concentrations of viruses compared to phytos and bacteria).

At each time point:

a. Filter 10 ml onto a 1.0 µm Nuclepore filter (fraction 3)
b. Filter 10 ml onto a 0.2 µm Nuclepore filter (fraction 4).
c. Filtrate is >0.2 µm (viruses and dissolved pool, filtrate 5).

Fraction 3: $^{33}P_i$ still in phytoplankton
Fraction 4: $^{33}P_i$ in bacteria
Fraction 5: $^{33}P_i$ in dissolved pool- either released from phytos and not taken up yet by bacteria, or never taken up by phytos, or incorporated into viral DNA

It is likely that this experiment will be repeated at a different station later in the cruise.

As with the phytoplankton uptake experiment on day 1, all liquid waste at the end of the experiment will go into a designated container, as well as 2 filtered seawater washes. Solid waste will be disposed of in a separate, designated container.

EMSA Protocol

A. Primer design

Design complementary 20-nucleotide primers to gene of interest, adding restriction enzme half sites at each end (usually BamHI)

B. Primer annealing reaction:

5.0 µL upstrand oligo (1 µg/µL)
5.0 µL downstrand oligo (1 µg/µL)
90.0 µL 0.25M NaCl

1. Heat reaction at 100ºC for 10 minutes using heat block
2. Turn off heat block, and allow annealing reaction to cool to room temperature
3. Digest annealed oligos with restriction enzyme, as indicated in manufacturer protocols.

C. Primer labelling

Set up reaction and purify probe according to NEBlot protocol (attached)

D. Making acrylamide gel

1. Clean a 20 x 20 cm plain glass plate and a 20 x 20 notched glass plate with 70% ethanol. Add spacers at sides and bottom and clip together at equidistant spacing using 4 large clips.
2. Mix 38.75 mL MQ, 5 mL of 5X TBE, 6.25 mL of 40% acrylamide stock solution (lab 2 fridge), 500 ul of 10% Ammonium persulphate (lab 2 fridge) in 50 mL Falcon tube.
3. When ready to pour, add 37 ul of TEMED (gel will begin polymerising at this point). Invert several times and carefully pour between plates, avoiding the formation of bubbles. Insert a 14-well comb at top between plates. Let sit at room temperature for about an hour.
4. Moisten blue roll with 0.5X TBE and wrap around gel(s). Wrap with cling film and store in cold room for use the following day.
5. Make up 2 L of 0.5X TBE as follows: 200 mL of 5X TBE, dilute up to 2 L with H_20. Store in cold room for use the following day.

Make up 10X Binding buffer:
1 mL: 100 µL 1M Tris, pH 7.5
250 µL 2M KCl
50 µL 1M $MgCl_2$
10 µL 1M DTT
20 µL 500mM EDTA
500 µL glycerol (50%)
70 µL H_20

E. Binding reaction

1. Have gel(s) ready the night before, storing in cold room. Using fine line marker, mark bottoms of wells of gel(s) and number to assist in loading. Remove end spacer with forceps.
2. Secure gel(s) into gel apparatus. Test seal by pouring 0.5X TBE buffer (also made night before and stored in cold room) into top chamber only to start. If no leak found, continue pouring buffer into bottom compartment up to fill line. Using a 1000 ul pipettor set at 500 ul, pipette buffer up and down in each well to rinse out. Prerun gel(s) at 100V for at least ½ hour.
3. Meanwhile, pull out from -80 proper number of cell extract tubes for run to be done (one tube will be enough for a 14-well gel). [See cell extraction protocol for details of preparation]
4. Set up binding reaction:
   2.0 µL 10X Binding buffer
   1.0 µL poly dIC (1 mg/mL)
   1.0 µL cold competitor or H_20
   variable volume extracts (1-2 µg)
   bring volume to 9 µL with H_20

5. Incubate 15 minutes at 15ºC (cold room)

6. Add 1 ul of labelled probe

7. Incubate 15 minutes at 15ºC (cold room)

F. Gel loading

1. After gel has pre-run and incubation period is finished, carry materials needed from hot room into cold room.

2. Again using 1000 ul pipettor, rinse out wells with buffer.

3. Using fine-point 20 ul gel tips, carefully load gel wells with incubation mixture. Run at 200V for **exactly 2 hours** using timer on power supply. Any longer could result in free probe ending up in running buffer and a **big contamination mess to clean up.**

G. Gel drying

1. Remove gel plate(s) from apparatus and carefully carry into hot room.

2. Remove side spacers with forceps. Using handle of forceps, slowly separate glass plates to expose one side of gel (make note of which side it is relative to where you started loading)

3. Place a sheet of Whatman paper on center of gel suface and carefully smooth outward toward edges. Place a second sheet on top of first and carefully invert gel and paper so that second glass plate is now on top. Carefully remove second glass plate.

4. Cover gel surface with cling film, avoiding wrinkling as much as possible. Label Whatman so that directionality of gel is clear.

5. Place one sheet of blue roll flat on gel dryer mat. Lay gel and Whatman assembly on top, cover carefully with another sheet of blue roll. Cover all this with gel dryer sheet and cover. Turn on vacuum pump, check for wrinkling of top blue roll and immediately remedy if needed.

6. Check cycle (top), temp(80C) and time (1 hour), then press start. When paper looks completely flat (no bulge from gel), gel is completely dry.

H. Photographing of gel

1. When gel is dry, lift up one corner of gel dryer sheet and then turn off vacuum and gel dryer, if still running. Discard blue roll and extra sheet of Whatman.

2. Tape Whatman containing gel inside a lead-lined cassette. Label outside of cassette with gel number and date. Carry back to lab.

3. Carry cassette(s) into dark room with box of blue-sensitive film. Turn out white light and turn on photo-safe red light. Cut large sheet of film in half and place half on gel, securing with tape. Make sure cassette is securely closed before turning off red light and leaving dark room.

4. Carry cassette(s) to Flint -80 freezer and incubate in freezer at least 18 hours overnight.

5. The next day, allow cassette to warm up (about an hour). Bring into dark room, turning off white light and turning on photo-safe red light. Remove film from cassette, carefully removing all traces of tape, and feed short end first into developer. Make sure to label developed film with date, film number and cell extract used.

Gel Mobility Shift Assay for transcription factor binding

Gel Preparations

1. **Assembly for gel casting**: Take clean plates and the spacers. Snuggly tighten them to make casting module. Fit it on the stand without the rubber spacer on bottom. Add some water to make sure water is not leaking out at the bottom. Keep the comb handy. Drain off the water by inverting the gel cast. Few droplets sticking to glass plates don't interfere with gel.
2. **Gel solution preparation and casting**:
    - Water distilled 50 ml
    - 30:2 acralamide: bis 8 ml
    - 10X TBE 1.5 ml
    - 10% APS 600 ul (0.0600 gm in 600 ul)
    - Temed 60 ul
    - Add temed at last. Mix well. Now gel is ready to pour.

    Take 25ml pipette and try to pour the gel on the side of cast near spacers. So it trickles on the side and fills from bottom without any air bubbles trapped inside. Fill it up to the top. Take comb and insert from one side keeping angle so that each tooth of comb can be inserted one by one in Gel without trapping any air bubble. The gel should polymerize in half and hour and ready to use. Make sure its not leaking from anywhere when you leave it to polymerize.

Oligoneucleotide Labeling

We want our final labeled product to be 4.8 pm/ul so dilute initial single stranded oligoneculeotide at 480 pm/ul. Now we need to anneal them.

- 100 ul of annealing buffer (10mm Tris-HCl, Ph 7.5, 20mm NaCl)
- 1 ul or each primer (480 pm of each of them)

1. Heat it for 95C for 5 min on heat block. Leave the block at RT afterwards.
2. Store at ¨C20* freezer once cooled down.
3. Labeling with ^{32}P
    - 2 ul of oligo
    - 1 ul of PNK buffer
    - 4 ul of water
    - 1 ul T4- Kinase
    - 2 ul of ^{32}P ATP
4. Incubate at 37* for 30 minutes
5. Add 40 ul of distilled water
6. Now we want to remove unlabelled oligos. We use Quick Spin Column (TE) for Radiolabelled DNA (Roche) for it.
7. Keep column at room temp for half an hour. Remove the cap first and then bottom
8. Seal to avoid generating suction. Let the supernatant drain off with gravity.
9. Add 50 ul of your reaction.
10. Spin them around 1000xg (2200 RPM for the machine in tissue culture room) for 4 minutes with tubes provided with column to collect labeled product.
11. Make final volume of 100 ul by adding water.

Binding reaction

Now we want to initiate reaction between labeled oligos and nuclear extract of the cell line of your interest.

- 5X GS-A buffer
- Poly dIdC 1ul
- Nuclear extract 10 ug
- Water to make final volume

1. These ingredients go in each tube. Incubate on them ice for 10 min.
2. Add cold competitor in designated tubes (2 ul or more according to your need)
3. Incubate on ice for 10 min
4. Add hot probe 1ul in designated tubes and incubate for RT for 20 min or on ice for 45 min.
5. Add 2 ul of loading dye to stop reaction.
6. You are ready to load them on Gel.

Gel Run

1. Before we can run the samples we need to pre-run it for 30 min at 80 volts.
2. Prepare the buffer 0.5X TBE for running.
3. Load the samples one by one. Making sure not to spill in upper chamber (buffer will become radioactive otherwise)
4. Run the gel @ 120 V for 2-3 hours. When dye front is reaching lower fifth of gel. Stop the reaction. (Again if you let it long, lower chamber buffer will be radioactive).

Gel drying and imaging

1. Decant the buffer after making sure it is not radioactive in to the sink. Otherwise collect it in radioactive liquid waste container.
2. Get the gel cast out. Separate the glass plate carefully so gel sticks to either of the plate. Cut whatman size 3 papers to size of gel. Put couple on it. Gel should adhere to it and will be easy to lift off.

Now we want it to be dried. Cover it with cellophane. Dry it for ~ 40 min at 80* C. Keep the suction on and turn of the temp for 15 min. Disconnect the suction and turn off the pump. Now gel is dried and ready for imaging.

We use Biomax MR Kodak films for it. It is single sided so orientation is important. Put the gel in cassette and take individually rapped film to dark room. Open the film in dark room. Put it on the gel with notch on film on upper right hand or lower left hand. Close the cassette. You can leave it at RT for 7-8 hrs or in ¨C80 for 4-5 hrs. Take it back to dark room. Open the cassette and develop the film.

Preparation of nuclear extract

1. Wash cells with PBS two times. (Take about 10^7 cells)
2. For adherent cells wash cells and then after scrapping them spin them down. Store on dry ice or ¨C80* till all cells are ready for further steps.
3. Add 200 ul of Buffer A / 10^7 cells. Pipette up and down till no clumps remain. To prepare buffer A take 2.4875 ml of stock solution of buffer A and add 12.5 ul of DTT before use.
4. Incubate on ice for 10 min. Mix occasionally by flicking with finger.
5. Spin full speed 10 sec at RT.
6. Remove suspension. Remember this protocol leaves cytoskeletal proteins with nuclear extract. Use another protocol to get those proteins separately.
7. Add double the amount of pallet buffer C. To prepare buffer C, take 2.1375 ml of stock solution and add 12.5 ul of DTT and 350 ul of cocktail protease inhibitors. Flick the tube, don¡¯t pipette up and down.
8. Incubate 30 min on ice.
9. Spin 30 min full speed at 4* C. (cold room)
10. Transfer the supernatant. It should not be sticky. Measure the protein concentration.

Northern Blot Protocol

DNA template preparation

1. PCR DNA template using a 5' addition of (TAATACGACTCACTATAGGG for T7) to your reverse primer. Templates should be 200-1000bp in length. PCR conditions may vary (all that is needed is to produce a template for RNA synthesis)

Radioactive labeling
(use NEBlot Kit – catalog # N1500S)

2. Dilute 25 ng of purified DNA template in 33 μL RNase Free water
3. Denature by boiling in a water bath for 5 minutes
4. Incubate on ice for 5 minutes
5. Add the following reactants to the denatured template
    a. 5 μL 10X labeling buffer
    b. 6 μL dNTP mixture
    c. 5 μL P32 dCTP (3000 ci/mol, 50 μCi
    d. 1 μL DNA polymerase I – Klenow fragment
6. Incubate at 37°C for one hour
7. Stop the reaction by adding 5 μL 0.2 M EDTA (pH 8.0)
8. Purify from unincorporated nucleotides by using Sephadex G-50 spin columns (GE catalog # 27-5335-01)
9. Before using probes in blot denature probe in boiling water bath for 5 minutes
10. Quickly incubate on ice for 5 minutes. Probes may now be used directly in hybridization.

Preparation and running of RNA gels
1. Cast MOPS-formaldehyde gel
2. Add agarose to 1X MOPS and melt agarose in microwave (~30 seconds), after cooling to ~55°C add
    a. 0.6 g Agarose
    b. 47.3 mL 1X MOPS
    c. 2.7 mL 37% formaldehyde
3. Prepare fresh loading buffer
    a. 250 uL 100% formamide
    b. 83 uL 37% formaldehyde
    c. 50 uL 10X MOPS
    d. 50 uL 100% glycerol
    e. 20 uL 1.25 % bromophenolblue
    f. 47 uL DEPC water
4. Prepare samples for loading onto the gel
    a. Add 20 μL loading buffer to 1 μg RNA
    b. Denature sample mix at 65°C for 10 minutes
    c. Chill on ice for 1 minute
    d. Run gel at 70 volts for 4 hours
5. Stain gel briefly in 0.5 μg/mL ethidium bromide and examine gel on the UV box

RNA blotting and fixation
1. Rinse gels prior to blotting in 20X SSC (2 washes, 15 minutes each)
2. Blot with capillary transfer with 20X SSC overnight (use on nylon membranes, positively charged)
3. Fix the membrane by baking at 80°C for two hours.

Hybridization

1. Prewarm an appropriate volume of GE Rapid Hyb solution to hybridization temperature of 42°C
2. Prehybridize membrane with Rapid Hyb for 30 minutes with gentle agitation in an appropriate container. (membrane should move freely)
3. Remember to denature probe by boiling for 5 minutes and rapidly cooling in ice water
4. Add denatured probe to prewarmed Rapid Hyb and mix well, but avoid foaming (bubbles may lead to background)
5. Pour off prehybridization solution and add probe/hybridization mixture to membrane
6. Incubate for 6 hours to overnight at 42°C with gentle agitation
7. Wash twice for 5 minutes each in 2X SSC, 0.1% SDS at 15-25°C with agitation
8. Wash twice for 15 minutes each in 0.1X SSC, 0.1% SDS (prewarmed to wash temperature) at 68°C with agitation

Imaging

Acquire image using InstantImager Electronic Autoradiography. Image times range from 10-60 minutes.

Immunological detection
(All incubations at 15-25°C with agitation)

1. Rinse briefly for 1-5 minutes in washing buffer
2. Incubate for 30 minutes in 100 mL Blocking solution
3. Incubate for 30 minutes in 50 mL antibody solution
4. Wash twice in 100 mL washing buffer for 15 minutes each
5. Equilibrate for 2-5 minutes in 100 mL Detection buffer
6. Place membrane on a development folder (or hybridization bag) and quickly apply ~1 mL out of the dropper bottle until the membrane is evenly soaked
7. Immediately cover the membrane with the second sheet of the folder to spread the substrate evenly and without air bubbles
8. Incubate for 5 minutes at 15-25°C
9. Image the membrane on storm at 465 nm (expose for 5-20 minutes)

Stripping and reprobing of RNA blots

1. Rinse membrane thoroughly in DEPC treated double distilled water
2. Incubate twice for 60 minutes each at 80°C in stripping buffer to remove probe
3. Rinse twice in 2X SSC for 5 minutes each
4. Prehybridize and hybridize with a second probe or store membrane in a sealed bag

Northern Blotting Protocol

I. Collect cells and isolate RNA

A. <u>RNA sampling</u>

1. Aliquot samples into 15 mL RNase-free tubes according to culture OD_{600}:
    a. OD<1, 10 ml
    b. OD=1, 8 ml
    c. OD 1-2, 5 ml
    d. OD>2, 3 ml
    e. Spin tubes at 5,000 rpm (4,000 xg), 4°C for 10 min.
2. Pour off supernatant. Cell pellets can be stored at -85°C for no more than a week.

B. <u>RNA isolation</u>

3. Setup vortex adapter and pre-measure 40 mg of glass beads into 1.5 mL RNase-free tubes.
4. Resuspend cell pellets in 1 mL of RNase-free SET buffer, centrifuge at 5,000 rpm, 4°C, for 10 min. (If pellets were frozen, allow them to thaw before resuspending.)
5. While cells are spinning, supplement 1 mL RNase-free SET + 20 mg/mL lysozyme with 100 µL Proteinase K.
6. Pour off supernatant and resuspend pellets in 220 µL RNase-free SET + 20 mg/mL lysozyme, Proteinase K.
7. Incubate mixture at room temperature for 6 min.
8. Transfer mixture into the 1.5 mL RNase-free tubes containing the pre-measured glass beads and vortex on adapter for 4 min.
9. Add 1 mL of Trizol to mixture and mix by pipeting. Split sample in two and dilute with Trizol (i.e. transfer 500 µL of sample into a new RNase-free tube and then add 500 µL of Trizol to each tube, to bring the final volume to 1 mL.)
10. Add 200 µL ice-cold RNase-free chloroform and shake tubes vigorously by hand for 15 sec, and incubate at room temperature for 3 min.
11. Spin samples at no more than 12,000 rpm for 15 min at 4°C.
12. Transfer upper (aqueous) phase (<500 µL) to a new RNase-free tube. Do not touch the interface to prevent contamination.
13. Add 500 µL of 70% EtOH and mix by vortexing.
14. Pipette up to 700 µL of sample inot RNeasy Mini Spin Column. Close tube gently and centrifuge at room temperature ≥8,000 xg (≥10,000 rpm) for 15 sec. Discard flow-through.
15. Repeat step 15 for remainder of the sample.
16. Add 700 µL Buffer RW1 to RNeasy Mini Spin Column. Incubate at room temperature for 4 min. Close the tube gently and centrifuge for 15 sec at ≥10,000 rpm. Discard flow-through and collection tube.
17. Transfer the RNeasy Mini Spin Column into a new 2 mL collection tube (supplied). Pipet 500 µL Buffer RPE onto the RNeasy Spin Column. Clost the tube gently and centrifuge for 15 sec at ≥10,000 rpm. Discard flow-through.
18. Add another 500 µL Buffer RPE to RNeasy Spin Column. Close tube gently and centrifuge 2 min at ≥10,000 rpm to dry the membrane.
19. Place the RNeasy Spin Column into a new 2 mL collection tube (not supplied), and discard the old collection tube with flow-through. Centrifuge at full speed for 1 min.
20. To elute, transfer the RNeasy Spin Column into a new 1.5 mL collection tube (supplied). Pipet 50 µL RNase-free water directly onto the RNeasy membrane. Close tube gently and centrifuge for 1 min at ≥10,000 rpm to elute.
21. Repeat step 21 into a second collection tube.
22. To quantify samples, measure 1.5 µL on the NanoDrop spectrophotometer. To evaluate degradation, run sample on 1.0% agarose gel.
23. Store samples at -85°C.

II. Radioactive probe generation

1. PCR desired template (gene) from genomic DNA. PCR conditions may vary (all that is needed is to produce a template for RNA synthesis).
2. Prepare 25 ng of purified DNA template in 33 µL of RNase-free water.
3. Denature by boiling in a water bath for 5 min.
4. Incubate on ice for 5 min.
5. Add the following reagents to the denatured template (from the NEBlot Kit):
    a. 5 µL 10x labeling buffer
    b. 6 µL dNTP mixture (2 µL of dATP, dTTP, dGTP)
    c. 5 µL P^{32} dCTP (3,000 ci/mmol, 50 µCi)
    d. 1 µL DNA polymerase I – Klenow fragment (3'→5' exo^{-})
6. Incubate at 37°C for one hour.

7. Stop the reaction by adding 5 µL of 0.2 M EDTA (pH 8.0)
8. Purify from unincorporated nucleotides by using illustra G-50 spin columns.
9. Before using probes in blot, denature probe in boiling water bath for 5 min.
10. Quickly incubate on ice for 5 min. Probes may now be used directly in hybridization.

III. Preparation and running of RNA gel

1. Add agarose to 1x MOPS and melt agarose in microwave (~45 sec):
    a. 0.6 g agarose
    b. 47.3 mL 1x MOPS
2. After cooling to ~55°C, add 2.7 mL 37% formaldehyde.
3. Cast the gel in a medium gel tray.
4. Prepare fresh loading buffer:
    a. 250 µL 100% formamide
    b. 83 µL 37% formaldehyde
    c. 50 µL 10x MOPS
    d. 50µL 100% glycerol
    e. 20 µL 1.25% bromophenolblue
    f. 47 µL RNase-free water
5. Prepare samples for loading onto gel:
    a. Add 20 µL loading buffer to 1 µg RNA
    b. Denature sample mix at 65°C for 10 min
    c. Chill on ice for 1 min
6. Run the gel in 1x MOPS (running buffer), with 0.05 µg/mL ethidium bromide.
7. Run gel in 1x MOPS buffer at 70 volts for 4 hours.
8. Examine the gel under UV.

IV. RNA blotting and fixation

1. Rinse the gel twice with DEPC treated water.
2. Transfer the gel into 10 gel volumes of 20x SSC for 40 minutes.
3. Blot with capillary transfer with 20x SSC overnight (use on positively charged nylon membranes).

Note: Invert the gel before placing onto a wet blotting paper, and then place the positively charged nylon membrane. (RNA is closer to the membrane, to migrate in this way.)

4. Fix the membrane by baking at 80°C for two hours or UV cross linking. (You can use both)

V. Staining the membrane

1. Transfer the membrane to a glass tray containing methylene blue solution. Stain the membrane for just enough time to visualize the rRNAs (~ 3-5 mins).
2. Destain the membrane by washing in 0.2x SSC and 1% SDS for 15 min at room temperature.

VI. Hybridization and imaging

1. Prewarm an appropriate volume of GE Rapid Hyb solution to hybridization temperature for 65°C for DNA probes. (Refer the GE manual for other probes like RNA)
2. Prehybridize membrane with Rapid Hyb for 30 min with gentle agitation at 65°C.
3. Remember to denature probe by boiling for 5 min and rapidly cooling in ice water.
4. Add denatured probe to prewarmed Rapid Hyb and mix well, but avoid foaming (bubbles may lead to background).
5. Pour off prehybridization solution and add probe/hybridization mixture to membrane.

Note: Do not add the probe directly onto the membrane, to avoid strong back ground.

6. Incubate for 6 hours to overnight at 65°C (For DNA probe) with gentle agitation.
7. Wash twice for 5 min each in 2x SSC, 0.1% SDS at 15-25°C with agitation.
8. Wash twice for 15 min each in 0.1x SSC, 0.1% SDS (prewarmed to wash temperature) at 68°C with agitation.
9. Acquire image using …

VII. Stripping

1. Rinse membrane thoroughly in DEPC treated water.
2. Incubate twice for 60 min each at 65°C in stripping buffer to remove probe.
3. Rinse twice in 2x SSC for 5 min each.
4. Prehybridize and hybridize with a second probe or store membrane in a sealed bag. (You may want to image the membrane before prehybrization to ensure probe removal.)

VIII. Buffer

SET Buffer

Component	Final Concentration	Amount
Sucrose	25%	125 g
EDTA (0.5M, pH 8.0)	0.05M	50 mL
Tris-HCl (1M, pH 8.0)	0.05M	25 mL
Water		Up to 500 mL

Treat with DEPC and autoclave.

10x MOPS Buffer

- Dissolve 41.8 g of MOPS in 700 mL of sterile DEPC-treated H_2O. Adjust the pH to 7.0. Add 20 mL of DEPC-treated 1M sodium acetate and 20 mL of DEPC-treated 0.5 M EDTA (pH 8.0). Adjust the volume of the solution to 1 L with DEPC-treated water. Sterilize the solution by passing it through a 0.45-μm Millipore filter, and store it at room temperature protected from light. The buffer yellows with age if it is exposed to light or is autoclaved. Straw-colored buffer works well, but darker does not.

20x SSC

- Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of water. Adjust pH to 7.0. Adjust the volume to 1 L with water. Treat with DEPC and autoclave.

Methylene blue staining Buffer
(0.5M Na-acetate pH 5.2, 0.04% methylene blue):

- Dissolve 4.1 g Na acetate in 80 ml sterile distilled water. Adjust the pH to 5.2 using acetic acid, make the vol upto 100ml. Add 40mg methylene blue dye, dissolve and filter through 45um membrane filter. Store at RT.

Stripping buffer

- 50% deionized formamide
- 0.1x SSC
- 0.1% SDS

To treat with DEPC:

- Add DEPC to a final concentration of 0.1%
- Mix for 1 hour at 37°C or overnight at room temperature
- Autoclave

Single-stranded DNA sequencing:

1. Prepare the following extension reaction in a microcentrifuge tube:

750 ng	M13 template DNA
2 ul	*Bst* reaction buffer
2 ul	*Bst* nucleotide extension mix
1 ul	oligonucleotide primer (2.5 ng/ul)
0.5-1 ul	[alpha]-32-P-dATP or [alpha]-35-S-dATP
1 ul	diluted *Bst* polymerase (0.1 U/ul)
q.s.	sterile ddH2O
12 ul	

[alpha]-32-P-dATP (PB 10384) and [alpha]-35-S-dATP (SJ 1304) from Amersham.

Dilute the *Bst* polymerase (BioRad 170-3406) in *Bst* dilution buffer.

2. Incubate the reactions for 2 minutes at 67degC, and briefly centrifuge to reclaim condensation.

3. Remove 2.5 ul aliquots for each reaction into the four base-specific termination mixes (either short or long), already pipetted into a V-bottomed microtiter plate (Dynatech).

4. Incubate the reactions for 10 minutes at 67degC, and briefly centrifuge to reclaim condensation. It is possible to store the reactions at -70degC at this stage.

5. Stop the reactions by the addition of 4 ul of agarose gel loading dye and incubate for 5-7 minutes at 100degC.

For double-stranded DNA sequencing:

1. To denature the DNA and anneal the primer, incubate the following reagents in a boiling water bath for 4-5 minutes and rapidly cool the reaction by plunging into an ethanol/dry ice bath.

3 ug	plasmid DNA
5 ng	oligonucleotide primer
q.s.	sterile ddH2O
9 ul	

2. Incubate the reaction in an ice-water bath for 5 minutes, and then add the following reagents:

2 ul	*Bst* reaction buffer
2 ul	*Bst* nucleotide extension mix
0.5-1 ul	[alpha]-32-P-dATP or [alpha]-35-S-dATP
1 ul	diluted *Bst* polymerase (0.1 U/ul)
15 ul	

[alpha]-32-P-dATP (PB 10384) and [alpha]-35-S-dATP (SJ 1304) from Amersham.

Dilute the *Bst* polymerase (BioRad 170-3406) in *Bst* dilution buffer.

3. Proceed with the sequencing reaction as described above in steps 2-5 for single-stranded templates

DNA Sequencing

The sequencing reactions described below work perfectly well if you are short of cash to buy sequencing kits. It is based on the Dideoxy sequencing method of Sanger et al., 1977. However, due to the number solutions that need to be made, I recommend purch asing a sequencing kit, we use either the T7 Sequencing Kit (Pharmacia, 100 reactions) or the Sequenase 2.0 Sequencing Kit (USB via Amersham in the U.K., 100 reactions).

Reactions are performed in sterile 1.5ml microcentrifuge tubes.
Primers are synthesised on an Applied Biosystems 381A DNA synthesiser.

Approximately 3ug denatured, high quality dsDNA (i.e. prepared as described in 'Plasmid Isolation using PEG') are typically used for standard sequencing reactions.

DNA Sequencing Reactions

You will need:

Freshly made 2M NaOH
3M sodium acetate, pH 4.5
Sterile, distilled water
Absolute ethanol
70% ethanol
7 x DNA annealing buffer (280mM Tris.Cl, pH 7.5, 100mM $MgCl_2$, 350mM NaCl)
Termination mixes (40mM Tris.Cl, pH 7.5, 50mM NaCl, 10mM $MgCl_2$, 150mM dTTP, 150mM dATP, 150mM dCTP, 150mM ^{c7}deaza-dGTP and 15mM of the respective ddNTP)
5 x DNA labelling mix (10mM dGTP, 10mM dCTP, 10mM dTTP, 200mM Tris.Cl, pH 7.5, 250mM NaCl)
300mM DTT
[a-35S]-dATP (~ 1000Ci/mmol, Amersham or DuPont)
T7 DNA polymerase (Pharmacia)
Stop solution (95% deionized formamide, 20mM EDTA, pH 7.5, 0.1% each of bromophenol blue and xylene cyanol FF)

1) Denature dsDNA by the addition of 8ul DNA (approximately 3ug) to a sterile microcentrifuge tube containing 2ul freshly made 2M NaOH vortexed briefly and incubate at room temperature for 10 minutes.

2) Neutralise DNA by the addition of 3ul 3M sodium acetate, pH 4.5 and 7ul sterile, distilled H_2O and precipitate by the addition of 60ul ethanol. Recover DNA by centrifugation, at maximum speed, for 10 minutes in a microfuge. Rinse DNA briefly in 70% ethanol, air dry and re-dissolve in 10ul sterile, distilled H_2O.

3) To a microcentrifuge tube containing 10ml denatured template DNA, add 4.44ng primer (2ul of a 2.22ng/ul stock) and 2ml 7 x annealing buffer. Heat the mixture to 65°C for 2 minutes and allow to cool slowly, over a period of about 30 minutes, to roo m temperature.

4) While the annealing reaction is taking place, take 4 sterile microcentrifuge tubes per sample and label G, A, T, C, respectively. Place into each tube 2.5ul, respectively, of the corresponding termination mix. Pre-warm tubes to 37°C.

5) After completion of the annealing reaction, the labelling reaction is initiated by the addition to the annealed template/primer of 2ul 1 x labelling mix, 1ul 300mM DTT, 1ul [a-35S]-dATP (~ 1000Ci/mmol) and 3 units T7 DNA polymerase (2ul of a 1.5 units/ ul solution). The solution is pipetted briefly to mix the components and incubated at 4°C for 2-5 minutes.

6) Termination is achieved by transferring 4.5ul of the labelling reaction into each of the 4 tubes labelled G, A, T, C, respectively and incubating at 37°C for 2-5 minutes.

7) After termination, 5ul stop solution should be added to each tube, mixed by pipetting and the samples stored at -20°C for later use.

DNA sequencing gels

Gels used for DNA sequence analysis are of the wedge type. These produce a voltage gradient which decreases as DNA migrates down the gel, thus retarding the rate of migration of smaller fragments and allowing more readable sequence information to be obtai ned from one gel. DNA sequencing gels are cast between the 38 x 50cm and 38 x 47.5cm glass plates of the Bio-Rad SequiGen™ sequencing system.

You will need:

A standard detergent
2% dichlorodimethyl silane in hexane
Absolute ethanol
6% sequencing acrylamide (5.7% acrylamide, 0.3% bisacrylamide, 48% urea, 1x TBE)
25% AMPS (freshly made)
TEMED

N.B: Wear gloves while handling solutions of unpolymerised acrylamide. Unpolymerised acrylamide is a neurotoxin.

1) Clean the glass plates extensively with detergent and water, tap water, distilled water and finally ethanol. Wipe dry with a clean paper towel.

2) Siliconize the smaller of the two plates using the 4% solution of dichlorodimethylsilane in hexane. The solution should be spread evenly over the plate and allowed to dry before being repeated. Once dry, the plate should be washed with 100% ethanol and again wiped dry using a clean paper towel.

3) Gel plates are then assembled as described in the manufacturers instructions using two 0.25 - 1mm wedge spacers.

Polyacrylamide sequencing mix for use in the gels was stored at 4°C in a dark bottle.

4) 35ml of the acrylamide mix is used to first plug the bottom of the gel. Chill the acrylamide on ice and add 150ul 25% AMPS and 150ul TEMED. Mix by swirling and then poured briskly into the gel mould. The quantities of AMPS and TEMED may have to be esti mated empirically to cause setting in approx. 5 minutes.

5) Once the plug has set, 85ml of acrylamide is then used to form the main gel itself. To the acrylamide (chilled on ice beforehand), add 110ul 25% AMPS and 110ul TEMED. The solutions are mixed thoroughly, placed into a 50ml syringe and injected, carefull y, between the glass plates. In order to facilitate ease of pouring, the glass plates were inclined at an angle of approximately 10° to the horizontal in a large developing tray to prevent spills. Again, the quantities of AMPS and TEMED used may need to be varied in order to give polymerisation in approx. 30 minutes - this may be especially critical if the ambient temperature is abnormally warm.

N.B: It is critical to chill the acrylamide for the main gel in order to prevent polymerisation while the gel is being poured. You may also need to adjust the AMPS/TEMED quantities used. You should aim to have the plug set in ~5 mins and the main g el after ~30 mins.

6) Immediately after the gel is poured, a flat 0.25mm spacer (or reversed shark tooth comb) should be placed into the acrylamide on the gel top such that it intrudes into the gel by approximately 10mm. This allows the formation of a flat gel surface essen tial to the effective use of the shark tooth combs during electrophoresis. Clamp large bulldog clips across the top of the gel plates during gel polymerisation to ensure a leak-free fit of the combs. Allowed to polymerise

for 1 hour at room temperature an d then use directly or store overnight at 4°C, tightly wrapped in clingfilm to prevent dehydration of the gel.

7) Remove the gel former and pre-electrophorese the gel at 1800V to heat the gel and running buffer to the required operating temperature (55°C) prior to the loading of the samples. Running buffer is 1 x TBE.

8) Insert sharks tooth combs such that the tips protrude approximately 0.5mm into the gel surface.

9) Thoroughly wash the wells immediately prior to the loading of the samples with running buffer to remove any urea which leaches from the gel.

10) Sequencing reaction mixtures, containing loading buffer, should be boiled for 2-3 minutes to denature any secondary structure and loaded into the wells (3ul/well), in the order G, A, T, C.

11) Electrophorese at 1800V (preferably 75W constant power) until the xylene cyanol dye front is approximately 5 cm from the bottom of the gel. Monitor the gel temperature to ensure it stays at 60°C or below (preferably 50-55°C).

N.B: Allowing the gel temperature to exceed 60°C for extended periods of time will cause the hydrolysis of urea in the gel.

12) After electrophoresis is complete, combs should be removed and the small siliconized glass plate gently removed from the remaining plate. The large plate, with the gel still attached, is then immersed in a fixative solution containing 10% acetic acid, 10% methanol for approximately 15-20 minutes. This process is used to remove urea from the gel.

13) Transfer the gel to a large sheet of Whatman 3MM paper and dry on a vacuum gel drier at 85°C for 75 minutes prior to autoradiography.

Radioactive Oligo labeling of DNA

Keep minimize time working with 32P dCTP and use shield to protect.

The procedure is based on the Klenow fragment, or large subunit, of DNA polymerase I. Klewnow retains the 5' - 3' polymerase activity but lacks the 5' - 3' exonuclease activity. Since Klewnow can not begin synthesis without a primer, a synthetic set of primers (6 bp random oligomers) are added to the single stranded DNA template. Some of these primers will have homology to most regions of DNA and can thus provide a point of initiation for synthesis.

1. Mix probe stock briefly remove the desired amount of whole plasmid or insert stock (Good DNA amount in labeling DNA is 100 ng) and bring up to 31 µl with H2O in a MFT. If the stock is in low-melting agarous, boil the entire stock to melt for 1 min, then voltex prior to transfer and mix with H2O. If the DNA quantity in the stock is unknown, use 15 µl of the stock for labelling.

2. Boil the MFT containing the probes for 10 min. in water bath to denature the DNA. Place on ice slurry containing NaCl for 30 sec., then place in a rack at room temperature.

3. Immediately, add 19 µl Oligo reaction mixture to the MFT, and vortex lightly. Reaction mixture labeled "rxn mix", and placed on ice before boiling DNA at step 2.
Oligo reaction buffer :
Mixture of stock solution A, B, C in the ration of 1:2.5:1.5. Stored at -20 C.
Stock Volume 19 µl (for 1 MFT)
Oligo buffer 10.0 µl
BSA(10ng/µl) 2.0 µl
Klenow 2.0 µl
32P dCTP (3000 Ci/mmole)3 5.0 µl

4. Incubate at 37 C for 1.5 hr (or longer). At room temperature for 2 hr or longer (or overnight, if desired).

5. Stop reaction by adding 85 µl reaction stop buffer (RSB) to MFT.

6. Remove the uncorporated 32P with sephadex G-50 spin columns (which see). After adding the reaction mix to the column, wash the tube with 100 µl of STE to the column.

7. Assay the reaction by using a hand-held Geiger counter. A good reaction register between 1 and 2 kcps (thousand counts per sec) at a distance of about an inch from the phototube. If the reaction do not work, reading would be low, possibly 10-fold decease, indicating the probe would not be useful.

8. Boil for 10 min, cool on ice for 1 to 2 min. Using the same tube from the spin column, dilute the entire probe with hybridization buffer immediately (should not be sit longer than 20 min).**Solution A**

Stock

2-mercaptoethanol (bME) 18 µl
DXTPs (A, T, G) 5 µl each
Solution O 850 µl
Store at -20 C

Solution O

Conc. Stock 250 ml 100 ml
1.47 M Tris-Base 44.52 g 17.81 g
0.147 M MgCl2 7.47 g 2.99 g
Adjust pH to 8.0 with conc. HCl.

Solution B

Conc. Stock 250 ml 100 ml
2 M Hepes 119.15 g 47.66 g
Adjust pH to 6.6 with 4 N NaOH. Store at 4 C.

Solution C

Hexamer or oligo nucleotides. Add 55 µl H2O directly to the Pharmacia bottle which contains 50 units of lyophilized hexamer. Store at -20 C.

Klenow

Dilute to 1 unit Klewnow fragment by adding klewnow buffer. Stock Klenow from BRL comes as 500 units in 84 µl. Dilute to 1 unit by adding 420 µl of klenow buffer to the 500 unit/84 µl stock. Store - 20 C.

Klenow buffer (250 ml)
Conc Stock
7 mM Tris-HCl 0.211 g
7 mM MgCl2 0.355 g
50 mM NaCl 0.730 g
50 % Glycerol 125 ml
Add the above to about 80 ml H2O. Stir to dissolve. Adjust volume to 250 ml with H2O. Store at -20 C.

BSA

Stock BSA from BRL comes as 50 mg/1000 µl. Add 4 ml of H2O for a final concentration of 10 mg/ml. Store at -20 C.

RSB (Reaction Stop Buffer)

Conc. Stock Volume (ml)
10 mM 1 M Tris pH 8.0 1.0
2 mM 0.5 M EDTA 0.4
0.2 % 20 % SDA 1.0
H2O 97.6

STE (Sodium chloride and TE)

Conc. Stock Volume (ml)
10 mM 1M Tris pH 8.0 10.0
1 mM 0.5 M EDTA 2.0
100 mM 5 M NaCl 20.0
H2O 968.0
Autoclave, and store at room temperature.

Kinase end-labeling of DNA

Typical 5'-kinase labeling reactions included the DNA to be labeled, [[gamma]]-32-P-rATP, T4 polynucleotide kinase, and buffer (3). After incubation at 37degC, reactions are heat inactivated by incubation at 80degC. Portions of the reactions are mixed with gel loading dye and loaded into a well of a polyacrylamide gel and electrophoresed. The gel percentage and electrophoresis conditions varied depending on the sizes of the DNA molecules of interest. After electrophoresis, the gel is dried and exposed to x-ray film, as discussed below for radiolabeled DNA sequencing.

Protocol

1. Add the following reagents to a 0.5 ml microcentrifuge tube, in the order listed:

sterile ddH2O	q.s	
10X kinase buffer	1 ul	
DNA	x ul	
[[gamma]]-[32-P]-rATP	10 uCi	
T4 polynucleotide kinase	1 ul (3U/ul)	10 ul

[[gamma]]-[32-P]-rATP (35020) ICN and T4 polynucleotide kinase (70031) from United States Biochemicals.

2. Incubate at 37degC for 30-60 minutes.

3. Heat the reaction at 65degC for 10 minutes to inactivate the kinase.

Labeling oligonucleotides with 32P ATP

Wear gloves throughout and work in radiation area. Monitor area before and after use.

Mix the following in an eppendorf tube:

1. 0.5 microgram oligonucleotide dissolved in H2O.
2. 3 microliters 10x kinase buffer.
3. 2 microliters ^{32}P ATP from ICN (>5000 ci/mmole).
4. H20 so that the final volume is 30 microliters.

Add 25 units T4 polynucleotide kinase and incubate 60 min at 37 deg.

If desired, double amounts of oligonucleotide and all other reagents to give 60 microliters total reaction volume. Purify phosphorylated oligo on a single spin column as described below. This will double the yield of radioactive primer.

Purify labeled Oligonucleotide away from unincorporated ATP

Currently, we use mini Quick Spin Oligo Columns (#1 814 397) from Roche to purify the labeled oligonucleotide.

Prepare the column according to the manufactuer's instructions by centrifugation of the resuspended matrix for 1 min @ 1000 x g.

Insert column into a new eppendorf tube and add oligo labeling reaction, adding slowly to center of column. Centrifuge 1000 x g for 4 min.

Recover purified labeled oligo. For most applications, add 70 microliters TE to the 30 microliters recovered for a total of 100 microliters.

Quantitate radioactive incorporation by counting 1 microliter of a 1/10 diluted sample. Expect between 20 -100 million cpm total.

10x Kinase Buffer

0.5 M Tris pH 7.6
0.1 M MgCl2
50 mM DTT

Random primer generation of [32P]-labelled DNA probes

DNA probes are prepared using a modification of the method of Feinberg and Vogelstein, (1983).

You will need:

Nuclease-free BSA
[a-32P]-dCTP (Amersham or DuPont)
Klenow DNA Polymerase (New England Biolabs or Pharmacia)
500mM EDTA, pH 8
dNTP solutions (separate solutions of 100mM dATP, dGTP, dCTP and dTTP in TE, pH 7.0
Solution O (1.25M Tris.Cl, 125mM $MgCl_2$, pH 8.0)
Solution A (1ml solution O, 18ul b-mercaptoethanol, 5ul of each dNTP solution
Solution B (2M HEPES, pH 6.6)
Solution C (Random hexanucleotides at 90 OD_{260}nm/ml)
Sterile, nano-pure water

1) Digest plasmid DNA with the appropriate restriction enzyme,fractionate electrophoretically, and purify by the NaI/glass method (see Gene-Klene Protocol). Resuspend in sterile,distilled H_2O at approximately 2ng/ul.

2) Boil DNA at 100°C or 10 minutes.

3) Snap chill in an ice/water bath and hold on ice prior to use.

4) Carry out labelling reactions at room temperature by the addition, to a sterile Eppendorf tube, of the following reagents in the stated order:-

5ul OLB buffer (see below)
1ul 10mg/ml nuclease free BSA
17.5ul (25ng) DNA fragment
370KBq [a-32P]-dCTP (1ul of a 370KBq/ul stock)
0.5ul Klenow fragment of *E. coli* DNA polymerase (0.5-1 unit)
OLB buffer is made by mixing solutions A, B and C in the ratio 10: 25: 15, respectively.

5) Allow reactions to continue for at least 5 hours.

6) Terminate reactions by the addition of 1ul 0.5M EDTA, 74ul sterile, distilled H_2O and 5 minutes incubation at 100°C.

The specific activity and the percentage incorporation of 32P-dCTP into the probe should be determined by TCA precipitation of 1ml probe and scintillation counting.

Standard Southern Blot Procedure

1. Digest 10 ug high molecular weight DNA in a total volume of 45 ul with 50-100 units of restriction enzyme and 1/10th volume of the appropriate 10X RE buffer 4 hours-overnight at 37°C. Add 1 ul of RNase for the last 1 hr of digest.
2. Pour a 350 ml 0.8-1% agarose gel in TAE buffer with 17.5 ul ethidium bromide (10 mg/ml) using a comb containing 20 2 mm x 1 cm wells.
3. Add 1/10 volume (5 ul) of 10X DNA loading buffer to each sample.
4. Load gel and include 15 ul of premixed size markers (lambda HindIII/uX174 HaeIII) in both of the outside lanes.
5. Run the gel at 120 V for 5-6 hours at room temperature. Recirculate buffers at 3 hr.
6. Photograph the gel with a ruler next to the size markers and cut off the upper left hand corner of the gel. Soak the gel for 1 hour with gentle shaking in 300-500 ml of denaturation buffer at room temperature.
7. Pour off denaturation solution and rinse the gel with a small amount of renaturation solution.
8. Soak the gel for 2 x 30 minutes at room temperature in 500 cc of renaturation buffer with gentle shaking.
9. Put on disposable gloves and set up a standard blotting chamber using 10X SSC as the transfer buffer
10. Cut a piece of .45 micron nitrocellulose (Schleisher and Schuell) to exactly the size of the gel. Keep hands off of nitrocellulose. Drop the nitrocellulose onto the surface of a glass dish containing doubly distilled water and wet completely. Then submerge the nitrocellulose in the water.
11. Flip the gel over (so bottom of gel is facing up) and place onto the Whatman paper on the bottom of the blotting apparatus. Note: place 4 sheets of Whatman on top of the blotting stone.
12. Using a spatula surround the gel with pieces of parafilm which underlap the gel by 1 mm on each side.
13. Transfer the nitrocellulose to a dish containing 10X SSC for 30-60 seconds.
14. Place the nitrocellulose on top of the gel and roll out any bubbles with a piece of disposable 5 ml pipet. Cut 2 pieces of Whatman 3 MM paper to exactly the size of the gel and quickly soak in 10X SSC before placing on top of the gel. Roll out bubbles with the 5 ml pipet.
15. Cut a stack of paper towels to the size of the gel and place on top of the Whatman strips.
16. Place the top on the blotting apparatus and weight down with a 0.5 kg weight or a 500 cc bottle filled with water.
17. Blot overnight.
18. Put on gloves. Remove and discard paper towels and Whatman filters. Carefully remove the nitrocellose sheet with the gel in position and flip over onto a clean glass plate. Mark the wells with a #2 pencil and remove and discard the gel. Cut upper left hand corner of nitrocellulose.
19. Soak the nitrocellulose briefly (1-2 minutes) in 2X SSC.
20. Place nitrocellulose face up on a piece of dry Whatman paper and air dry 15-20 minutes.
21. Place the nitrocellulose between 2 pieces of precut Whatman paper and tape shut into a folder. Bake for 2 hours at 80°C in a vacuum oven.

SOLUTIONS:

Denaturation Solution: 1.5 M NaCl Store R.T.

0.5 M NaOH

Renaturation Solution: 1.0 M Tris.Cl (pH 8.0) Store R.T.

1.5 M NaCl

20X SSC: 175.3 g NaCl

88.2 g NaCitrate (trisodium salt), pH to 7

H_2O to 1 L

Ethidium Bromide: 10 mg/ml in water. Stir 6-12 hours. Store 4°C in brown bottle.

50X TAE: Trisma base. 242 g/L.

Glacial Acetic Acid: 57.1 ml/L.

O.5 M EDTA (pH 8.0): 100 ml. Store R.T.

10X Loading Buffer: 0.42 % Bromophenal blue Store at 4°C

0.42 % xylene cyanol
50% glycerol in water
Size Markers: 50 ug/ml HindIII cut mlDNA in T.E Store -20°C
50 ug/ml HaeIII cut rX174 DNA in T.E.
1/10 volume 10X loading buffer
Solutions:
1. tRNA: yeast tRNA (Sigma) in H_2O at 10 mg/ml. Store 4°C.
2. STE: 10 mM Tris (pH 7.5) Store R.T.
1 mM EDTA
100 mM NaCl
f
C. **PreHybridization Reaction**:
1. Place nitrocellulose blot in a seal-a-meal bag and seal 0.5 cm from each edge on 3 sides leaving the top open.
2. Prepare 25 ml of hybrization cocktail as follows:
Fluka Formamide 12.5 ml
20X SSC 6 ml
1 M Tris pH 7.5 0.2 ml
50X Denhardt's Solution 2.5 ml
10% SDS 0.5 ml
50% Dextran Sulfate (Oncor) 2.5 ml
Water to 25 ml
3. Boil 0.25 ml of salmon sperm DNA (10 mg/ml in TE) for 7 minutes in an eppendorf tube.
4. Quick freeze both the salmon sperm DNA on dry ice.
5. Thaw the the salmon sperm DNA at 68°C quickly.
6. Add the salmon sperm DNA to the premixed and warmed hybridization buffer and mix well.
7. Add the hybridization cocktail to the Southern blot in the seal-a-meal bag. Avoid bubbles in the hybridization bag by rolling them out with a 10 ml pipette on top of a glass plate. Make sure that the blot is uniformly wet.
8. Prehyb at 42oC for 2 hrs.
C. **[^{32}P]-Labelling of Probe**:
1. Add 25 ng of DNA to a total volume of 10 ul of H_2O in a microfuge tube.
2. Boil x 10' and place on ice. Spin down 10 sec in a microfuge
3. Immediately add:
3 ul of dATP, dGTP, dTTp mixture - Thaw rapidly and
2 ul solution 6 (reaction mixture) - keep on ice
5 ul [a32P] dCTP (3000 Ci/mM)-must have reference date within 1 wk of labelling
1 ul Klenow enzyme (5 U/ml)
4. Incubate 37°C x 45'
5. Add 2 ul 500 mM EDTA and place at 65°C for 10' to stop reaction.
6. Prepare spin column: 1) Place G-50 spin column (Boehringer) and collection tube in 15 ml polystyrene tube
2) Spin 3000 RPM x 2'
3) Discard collection tube and replace it with another in polystyrene tube
7. Add 2 ul yeast tRNA (10 mg/ml in H_2O) and 50 ul STE to hexapriming mixture and mix.
8. Load hexapriming mix onto spin column
9. Spin 3000 RPM x 4-5'
10. Discard column and place effluent in eppendorf tube.
11. Count 1 ul of effluent to determine the specific activity of the probe. The specific activity should be at

least 1 x 109 DPM/ug DNA

12. Boil probe x 10' and place on dry ice prior to use.

D. **Hybridization**

1. Add the probe to the edge of the prehybridization solution without squirting directly onto the blot by pipetting and mix probe into solution so that it is uniformly spread over the blot.
2. Place the seal-a-meal bag on a glass plate and gently roll out all bubbles with a 5 ml disposable pipet. Seal the final side of the seal-a-meal bag 0.5 cm from the top of the blot.
3. Wash the seal-a-meal bag well with running water and place in a 42o C water bath overnight.
4. In the morning remove the blot from the bag directly into 500 cc of 2X SSC/0.1% SDS. Wash with gentle shaking at room temperature for 10 minutes. Repeat the 2X SSC/0.1% SDS wash for an additional 10 minutes at room temperature with gentle shaking.
5. Rinse the blot with a small volume of 0.1X SSC/0.1% SDS at room temperature for 10 seconds and discard.
6. Add 500 cc of 0.1X SSC/0.1% SDS to the blot in a tupperware container. Seal the top and place at 65°C for 30 minutes. Repeat this 65°C wash for an additional 30 minutes.
7. Remove the blot onto clean sheet of Whatman paper and listen to it with a Geiger counter. It should not be hot.
8. Let the blot air dry until surface liquid is gone, but the blot is still damp.
9. Wrap the blot in saran wrap and tape to a clean sheet of Whatman paper which has been marked asymmetrically with dots of radioactive ink.
10. Autoradiogram using an intensifying screen at -70°C for 4-24 hours.

E. **Stripping Southern Blot on Nitrocellulose**:

Treat blot with:

1. 0.5 N NaOH x 10 min. at R.T. (250 ml)
2. 0.5 N NaOH x 10 min. at R.T.
3. 1 M Tris pH 7.6 x 10 min. at R.T. x 2

Dry blot slightly on Whatman, immediately place into hybridization cocktail.

Regular Northern blot membranes probing

Prehybridization

Heat the prehybridization solution in 65 C water bath

prehybridization solution: 1% BSA (Bovine Serum Albumin fraction V)
0.25 mM Na2HPO4 (pH 7.2 adjusted with H3PO4)
1 mM EDTA
7% SDS
1% BSA

Place the nylon membrane in a conical tube
Add 15 mL of prehybridization solution
Incubate at 55 C for over 2h or ON

Prepare the probe:

Random primer Labeling Kit (USB product # 78430)

25 ng in 33uL of template DNA
5 μL 10X primer mix (nanomers)
H_2O adjust vol to 38 uL

Mix gently and spin-down
Denature DNA and primers by heating for 5 min at 100 C
Place tubes on ice for 2 min

Add
5 μL 10 X nucleotide mix (-dCTP), coming within the kit
5 μL [α-^{32}P]-dCTP (at 3000 Ci/mmol)
2 μL Sequenase DNA Polymerase (coming within the kit)

Mix by gently pipetting
Incubate the labeling reaction at 37 C for 10 min
Terminate the labeling reaction by adding 2 μL 0.5 M EDTA

Cleaning the probe (this is done to remove the unincorporated ^{32}P)

Use 800 μL Sephadex G-50 (for random primers) hydrated in 1M Tris-HCl pH 8 in spin column
Spin at 3000 rpm for 2 min
Add 50 μL of H_2O to the reaction tube
Add probe to top sephadex bed
Spin 4 min at 3000 rpm

Hybridization, washing and exposition

Add the cleaned and labeled probe directly to prehybridization solution used for the prehybridization

Hybridize at 55C over 8 h
Wash the membrane with the washing solution (0.2X SSC, 0.1% SDS) twice.
Wrap the membrane with plastic bag and exposure on phosphor-imager
After 1-2 days, scan the image.

(small RNAs) Northern blot membranes probing

Prehybridization

Heat the ULTRAhyb-Oligo Hybridization Buffer ® in 65 C water bath

Place the nylon membrane in a conical tube
Add 15 mL of ULTRAhyb-Oligo Hybridization Buffer®
Incubate at 42 C for over 2h or ON

Prepare the probe:

OptiKinase™ for 5' end labeling (USB product # 78334X)

2.5 µL 10 µM oligo template
2 µL OptiKinase™ Reaction Buffer (10X)
1.5 µL OptiKinase
2 µL [γ-32P]ATP (at 3000 Ci/mmol)
H_2O adjust vol to 20 µL

Mix by gently pipetting
Incubate the labeling reaction at 37 C for 60 min

Cleaning the probe (this is done to remove the unincorporated ^{32}P)

Use 800 µL Sephadex G-25 (for oligos) hydrated in 1M Tris-HCl pH 8
in spin column
Spin at 3000 rpm for 2 min
Add 60 µL of H_2O to the reaction tube
Add probe to top sephadex bed
Spin 4 min at 3000 rpm

Hybridization, washing and exposition

Add the cleaned and labeled probe directly to ULTRAhyb-Oligo Hybridization Buffer®
used for the prehybridization

Hybridize at 42C over 8 h
Wash the membrane with the washing solution (0.2X SSC, 0.1% SDS) twice.
Wrap the membrane with plastic bag and exposure on phosphor-imager
After 1-2 days, scan the image.

L-arginine Uptake Protocol

Materials

Material	Vendor	Product #
HEPES Buffer	Fisher Scientific	ICN1688449
1x Phosphate Buffered Saline (PBS)	Fisher Scientific	BP24384
1 µCi L-[3H] arginine monohydrochloride	Perkin Elmer	NET1123001MC
L-arginine monohydrochloride	Sigma Aldrich	A5131
0.5 % SDS	Bio-Rad	#176-2240
0.5 Normal NaOH	Fisher Scientific	AC12426-0010
Pico Pro Vial-4mL (scintillation vial)	Perkin Elmer	6000252

Preparation of Aortic Rings

1. Anesthetize animals with an intraperitoneal injection of ketamine (40-100 mg/kg) and xylazine (5-10mg/kg).
2. Sacrifice animal by exsanguination via removal of the heart.
3. Immediately dissect aorta and place into ice cold HEPES buffer
4. Clean aortic segment of all surrounding fat and connective tissue, then cut into 3-4 mm long ring segments.

Incubation with L-[^{3}H] arginine

5. Incubate/shake rings for 10 minutes in HEPES buffer at pH 7.4, 37°C.
6. Add 2 mL of solution contining L-[^{3}H] arginine and L-arginine, in a final concentration of 1mM, to rings and incubate for 1 minute.
7. Remove buffer and quickly wash rings 4x with ice cold PBS.

Preparation of Lysate and Scintillation Counting

8. Solubilize rings in 1ml of 0.5% sodium dodecyl sulfate (SDS) in 0.5 N NaOH.
9. Load 700 µl of lysate into scintillation tubes.
10. Count [^{3}H] radioactivity via liquid scintillation counting using Tri-Carb 2900 TR Scintillation Counter.

Note:

- The remaining lysate will used for protein content determination by the Bradford method (See Bradford Protocol)
- To correct for nonspecific uptake, additional studies will performed in which aortic segments are incubated in 10mM unlabeled L-arginine in HEPES buffer, and associated radioactivity subtracted.

Bacterial Incorporation of C-14 Sodium Bicarbonate and its Conversion to 14CO2.

Growth Conditions

- Transfer individual colonies of C.acetobutylicum or C.ljungdahlii into 10ml of CGM (Wiesenborn et al., 1988) or ATCC 1754 respectively to start primary cultures.

- Add 2µl of 1mCi/ml ^{14}C Sodium Bicarbonate (PerkinElmer #NEC086H001MC) to 1ml of sterile medium. Ascetically transfer the 1ml medium containing 2µCi of sodium bicarbonate to 150ml serum bottle, containing 30ml of appropriate media.

- Transfer a 10% inoculum from exponentially growing primary culture to the 30ml medium containing 2µCi of ^{14}C sodium carbonate (ref).

- Cells are grown to exponential phase under anaerobic condition with CO_2 as a sole source of carbon, before harvesting for further analysis. All cultures will be grown at 37°C in the shaking incubator inside the fume hood and within the area approved for the use of radioactivity. 14C incorporated into biomass or converted into gaseous CO_2 will be determined as described below.

Radioisotopic Tracer Experiments

I. Trapping radiolabelled gaseous CO_2:

- Sparge the 30ml culture by injecting sterile nitrogen through a pipetting needle into the sealed 150 ml serum bottle and providing an outlet with a shorter 1inch pipetting needle. This strips any dissolved CO_2 in the medium that is unincorporated.
- The free CO_2 in the head space along with CO_2 stripped by nitrogen gas is passed through a sealed serum bottle (150ml) containing 40ml of 4N NaOH.
- This is done by connecting a rubber tubing to the outlet of the pipetting needle and passing the gas mixture (CO2 + N2) through 4N NaOH by a similar setup (fig1).
- This traps both 14C radiolabelled and unlabeled CO_2 in 4N NaOH. Excess "non-radioactive" gas from the serum bottle containing 4N NaOH is vented into the fume hood as shown in fig1. Amount of radiolabelled CO_2 trapped in the alkaline solution is analyzed by liquid scintillation counting as described below.

II. Harvesting cells to measure carbon 14 incorporation:

- 30ml cell suspension is transferred to 50ml polypropylene tube. Whole cells are harvested by centrifugation at 14,000g for 18 min at 4°C.
- Transfer the supernatant to a fresh 50ml polypropylene tube for TCA treatment.
- Re-suspend the cell pellet in 10ml 100mM Na-K phosphate buffer (pH 3). Centrifuge at 14,000g for 18min at 4°C. Discard the supernatant in appropriate waste container.
- To collect lysed cells from the first centrifugation step, treat the cell-free supernatant with trichloroacetic acid (TCA) to a final concentration of 10% w/v.
- Centrifuge the TCA treated cell-free supernatant at 14k g for 18min and discard the supernatant in appropriate waste container.
- The obtained sediment is combined with whole cells harvested earlier, which is termed "biomass" or "cell constituents".
- The cell constituents are re-suspended in 10ml of solution containing a final concentration of 0.1M sodium dodecyl sulfate (SDS) and 0.2N NaOH.
- 5ml of the re-suspended solution is aliquoted into 1.7ml tubes and heated at 90°C for 2hrs for dissolving. Incorporation of radiolabelled carbon into biomass is analyzed by liquid scintillation counting.

Liquid Scintillation counting:

0.5 Milliliter samples of SDS/NaOH-solubilized biomass material and alkali-trapped carbonate solution is added to 7 ml of safety solve scintillating fluid (RPI corp.) and mixed to form a homogeneous solution. All samples are counted in Beckman coulter LS6500 scintillation counter.

Cellular acetylation of proteins/compounds by ^{14}C labeled pyruvate or acetate

Summary of the procedure

This protocol is used to measure the level of cellular acetylation level of a specific protein or compound using ^{14}C labeled pyruvate (or ^{14}C acetate). Cells will be lysed and the protein/compound isolated by immunoprecipitation and counted. LNCap or HEK293T cells will be treated with 1-50µCi of the ^{14}C pyruvate (or ^{14}C acetate) and ligand and incubated for 10' to 3 h. The protein/compound is immunoprecipitated and acetylation level measured by scintillation counting.
We anticipate running 6-24 samples per experiment (6-1200 uCi).

Radioisotope handling:

All areas of work and equipment will be clearly labeled for RAM use prior to the start of the experiment including the biosafety cabinet, cell incubator and workbench and refrigerator.
The radioisotope stock is secured in refrigerator. All isotope transfers are performed over a spill tray and lined with absorbent paper. Personnel handling isotopes glove and lab coat are removed before leaving the work area.

Detailed procedure:

1. Prepare samples

i) Seed LNCap Cells in 6 well cell culture plates with 2ml growth media

ii) Incubate cells for 24 hours at 37°C and 5% CO_2

iii) Treat the cells with 1-50µCi of the ^{14}C pyruvate and compound with concentration raging from 1µM to 60µM

iv) The cells are placed in a secondary container and transferred to CO_2 incubator for up to 3 hours.

Cells are removed from the incubator in room BRL 231 and transferred in a secondary container to the RAM area in room BRL 265.

2. Prepare the cell lysate

i) Wash cells two times with 1ml cold PBS

ii) Detach the cells with 1mL of the lysis buffer and collect the lysate in 1.5 mL eppendorf tube

iii) Centrifuge the tubes and collect the supernatant

3. Immunoprecipitation of the compound

i) To the supernatant in a 1.5 mL eppendorf tube, add 4µL of antibody stock

ii) Incubate the supernatant for 1 h at 4°C

iii)To this supernatant, add 20µL of streptavidin beads, (product # 3419S from Cell Signaling)
iv)The tubes are placed in a secondary container and placed on the rocker platform at 4°C overnight.
v)Spin down the beads and wash with cold PBS buffer three times

4. Radioautography
i) Suspend the beads in Laemmli sample buffer and boil for 5 minutes (heat block) (this step may not be necessary).
ii) Detection of radioactive level of the compound by using the scintillation counter in room BRL 230

Waste generated:
Liquid waste: Approximately 60mL per 6 well plate
Solid waste: cell culture plate, pipette tips, tubes and scintillation plate or vials.
Liquid waste generated from cell lines will be decontaminated with bleach and prior to discarding as liquid RAM waste.

Each ^{14}C pyruvate acetylation experiment generates 2ml of liquid by-product containing
1-50µCi of the radiolabeled compound. One pipette tip and one pair of gloves are disposed of in the dry RAM storage container.

Monitoring for contamination:
Wipe tests are performed in the end of each day of work. Survey areas include the spill tray, the biosafety cabinet, the incubator, the workbench, the floor beneath the work areas (biosafety cabinet and work bench) the refrigerator, refrigerator door handle, the centrifuge, rocker platform, heat block and secondary container.

Materials
^{14}C pyruvate and ^{14}C acetate are from American Radiolabeled Chemicals, Inc
For 1-^{14}C acetate, the product number could be one of the following ARC 0101A, ARC0101D, ARC0101 and ARC0101B
For 2-^{14}C or 3-^{14}C pyruvate, the product number could be one of the following ARC 0222 and ARC 0220

Lysis buffer:
PBS
5% glycerol
0.05% triton-100
1 tablet protease inhibitor per 10 mL