Radioactive *In Situ* Hybridization Protocol

(HBMI Core – University of Rochester Medical Center)

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Making the Probes (Day 1)

Remove the ^{35}S from the freezer and let it thaw at room temperature for 1 hour before starting. To make the ^{35}S labeled probes, the reagents need to be mixed in the following order below. This is done in front of the safety shield and with a Geiger counter

1 probe (1X Master Mix) [Sufficient for approximately 8 slides.]

2.0μl 10X Transcription Buffer (*Roche 1465384*)
0.2μl 1M DTT
0.8μl RNAse Inhibitor (*Roche 03335399001*)
0.4μl 25mM NTP's

Mix gently and label as 'Master Mix'

In a separate tube add the following.
2.0μl Sterile deionized water **(Add first!)**
3.4μl Master Mix
4.0μl Linear DNA of interest.
9.0μl ^{35}S-UTP (*Perkin Elmer NEG/039H 1mC*i)
1.0μl RNA Polymerase **(T7, T3, SP6)** (*Roche 881767, 1031163, 810274*) **(Add last!)**

This 1X probe volume is sufficient for 8 slides. It is preferred to use more number of tubes with the 1X mix rather than using the master mix at 6X or 12X concentrations, as the transcription reaction is better facilitated at lower concentrations.

- Mix 20μl reactions and incubate at 37°C for 1 hour.

- Add an additional 1μl of RNA Polymerase, and incubate another 1 hour at 37°C.

- Add 0.5μl of DNAse I (RNAse free) (*Roche 10776785001*) and incubate 20 minutes at 37°C.

- Bring probe reactions up to 50μl volumes using deionized water or sterile TE. Label each tube with the name of the probe and date. At this point the probes can be stored at -80°C if required.

-Purify probes using mini Quick Spin RNA Columns (*Roche 11814427001*).
1) Crack the top with the opener and snap the bottom before priming the columns.
2) Then flick the columns so the matrix is flush without any air bubbles.
3) Align the flicked columns, making sure that the notch of each column faces the outer rim of the microcentrifuge.
4) Finally complete the priming process by centrifuging them at 3000 RPM for 2 min
5) Add 50μl of the probe slowly down the center (slope facing down) of the primed columns.
6) Centrifuge again at 3000 RPM for 4 minutes.

- Use the scintillation counter to measure radioactivity for 1μl of each probe (should be greater than 1 X 10^6 cpm/μl). Store probes at -80°C. Probes are normally good up to 3-4 weeks past the reference date on the ^{35}S vial.

Pre-hybridization (Day 2)

Wheaton glass dishes are used for all solutions in which slide racks are to be incubated. (*Fisher 08-813A or 08-813C*) Sterilize all dishes and graduated cylinders with RNAse Away for 1-2 min (*Fisher 1475434*) and rinse with deionized water. **Use the rack labeled 'PRE-HYB'.**

1. Bake Sections on slide warmer at 60°C for 1 hour. Allow slides to cool for 15 minutes.

2. Prepare 4% paraformaldehyde (PFA) (Sigma P6148) in 1XPBS.
   Add 60ml of 10X PBS to 540ml of Deionized water.
   Microwave for 3 min, then add stir bar.
   Use **safety mask and gloves** while weighing the PFA. **In the safety hood** add 24g of paraformaldehyde powder and stir until dissolved. Adjust pH to 7.1 and let it **cool to room temperature**.

3. Deparaffinize and rehydrate sections by placing the slides in slide racks and moving the slide rack through the following solutions.

   Xylene – 20 minutes

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   100% EtOH – 4 minutes

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   75% EtOH – 4 minutes

   50% EtOH – 4 minutes

   1X PBS – 3 minutes

   1X PBS – 3 minutes

4. 4% PFA/PBS for 15 minutes at room temperature. Save the solution for step 7.

5. 1X PBS 3 minutes and repeat with fresh 1X PBS for 3 minutes. At this step remove the frozen Proteinase K from the -20°C and thaw to room temperature.

6. Proteinase K in 1X PBS (10μg/ml) at room temperature for 15 minutes.
   Prepare this just before use.
   600ml of 1XPBS
   600μl of 10mg/ml Proteinase K.

7. 4% PFA/PBS for 10 minutes at room temperature. (Re-use from step 4.)

8. 1X PBS 3 minutes and repeat with fresh 1X PBS for 3 minutes.

9. 0.2N HCl for 10 minutes at room temperature. **Prepare this just before use.**
   590ml Deionized water
   10ml of 12N HCl (directly from stock bottle *Sigma H1758*)

10. 1X PBS 3 minutes and repeat with fresh 1X PBS for 3 minutes.

11. 0.1M Triethanolamine (pH 7.5). **Prepare this just before use.**
   590ml Deionized water
   Add 7.8ml Triethanolamine (*Sigma T1377*) to 10ml of Deionized water (taken from the 590ml container) and shake vigorously in a 50ml Falcon tube. Add to the remaining deionized water.
   pH to 7.5 with nearly 3.5ml of 12N HCl (direct from stock bottle). Test pH.
   Place slides in 0.1M Triethanolamine for 1 min then proceed to step 12.

12. Prepare acetic anhydride. **Prepare this just before use.**
   Combine 5ml of deionized water in a Falcon tube with 1.8ml acetic anhydride (*Sigma A6404*). Shake vigorously. Pour solution on top of slides in the 0.1M Triethanolamine (in the same glass jar) and incubate for 10 minutes at room temperature.

13. 1X PBS for 3 minutes and repeat with fresh 1X PBS for 3 minutes.

14. 75% EtOH for 5 minutes at room temperature.
   150ml of Deionized water
   450ml of 100% EtOH

15. 100% EtOH for 5 minutes at room temperature.

16. Air dry slides, preferably in the In-Situ room. Lay slides flat with sections facing up.

Hybridization (Day 2 continued)

1. Warm aliquot of hybridization (hyb) solution at 55°C until SDS is completely in solution.
2. Set up hybridization solution / probes in the following proportions depending on the number of slides per probe:

8 slides	4 slides	2 slides
1ml hyb solution	500μl	200μl
50μl 1M DTT	25μl	10μl
50μl ^{35}S labeled probe (>10^6 cpm/μl)	25μl	10μl

   - Mix well, cap the tubes with the green tube cap and place at 85°C in water bath for 3 minutes or until use. Mark the tubes with a Sharpie pen.
3. Add 50μl of probe per slide. Dispense directly on top of sections making sure they are completely covered with probe.
4. Unwrap the coverslips without contaminating the surface that goes over the sections.
5. Coverslip sections making sure there are no bubbles.
6. Hybridize overnight (16-18 hours) at 55°C in a humidified hybridization chamber.
   To make the hybridization chamber put 2-3 layers of Whatman paper at the bottom of a slide box and add 50% Formamide (*Sigma F7503*) and 50% deionized water.

   All contaminated gloves, paper towels, tips need to go in the solid radioactive waste container marked **^{35}S Solid waste.**

Washing (Day 3)

Prewarm the water bath to 50°C. **Use rack marked 'POST-HYB'**. Wheaton dishes will hold slide racks and the entire dish will be placed in the water bath to maintain 50°C temperature. Make sure water level is appropriate and does not enter the dishes.

1. Make 5X SSC

   150ml 20X SSC (pH 7.4)

   450ml Deionized water

   Warm 5X SSC in microwave for 3 minutes. Cool to 50°C and pour into Wheaton glass dish. Place slide rack in 5X SSC and incubate at 50°C (water bath) until all coverslips float off. Run a pair of forceps along the slides every 5 minutes.

2. Incubate in 2X SSC, 50% Formamide at 50°C for 30 minutes.

   60ml 20X SSC (pH 7.4)

   240ml Deionized water

   Microwave the water for 3 minutes first and then add 300ml formamide (*Sigma F7503*). **Do not microwave the water with formamide!**

3. Incubate in 1X TNE at 37°C for 10 minutes. (Use the 37°C water bath)

   180ml of 5M NaCl

   18ml of 1M Tris-HCl (pH 7.4-7.6)

   3.6ml of 0.5M EDTA

   Bring to 1800ml with Deionized water and divide into three 600ml aliquots for use in steps 3, 4, and 5. (Prewarm each at 37°C).

4. Incubate in 1X TNE with 10μg/ml of RNAse A at 37°C for 90 minutes. (Use the 37°C water bath)

   600ml 1X TNE prewarmed to 37°C

   Add 300μl of 20mg/ml RNAse A (*Invitrogen 12091-021*) just before use! Mix in the dish with a tip.

   At this time point it is a good idea to prepare the reagents for steps 6 -10.

5. Incubate in 1X TNE at 37°C for 10 minutes. (Use the 37°C water bath)

6. Incubate in 2X SSC at 50°C for 20 minutes. (Use 50°C water bath)
   60ml 20X SSC (pH 7.4)
   540ml Deionized water.

7. Incubate in 0.2X SSC at 50°C for 20 minutes. (Use 50°C water bath)
   6ml 20X SSC (pH 7.4)
   594 ml Deionized water

8. Incubate in 0.2X SSC at 50°C for 20 minutes. (Use 50°C water bath)

9. Incubate in 75% EtOH at room temperature for 5 minutes.

10. Incubate in 100% EtOH at room temperature for 5 minutes.

11. Air-dry slides with slides laying flat with sections face up for 20-25 min.

12. In this time, clean up the In-Situ room, especially the working area and the Wheaton Glass dishes with Radiac wash and distilled water. To decontaminate a surface, spray the area with Radiac wash and let the bubbles settle down. Discard in the radioactive liquid waste container. After that spray the same area with distilled water and discard in the same container.

13. All liquid radioactive waste **MUST** be disposed in the **^{35}S Liquid waste** container **ONLY**. All contaminated gloves, paper towels, tips need to go in the solid radioactive waste container marked **^{35}S Solid waste.**

14. After decontaminating the areas, perform the wipe tests as outlined in the room map. Inform Ashish Thomas immediately via email to record the results.

15. Lay the slides facing each other in a cassette and expose them to a Biomax M.R Film (*Kodak 8701302*) **in the dark room**. Place film directly on sections with the “notches” in the upper left corner of the film. Leave the cassette away from light, overnight for 20-24 hours. The length of time for emulsion exposure is usually 3X longer than that of film.

Emulsion / Dipping (Day 4)

1. Pre-heat hybridization oven and water bath in dark room to 47°C.

2. Aliquot 12ml of 2% glycerol in 50ml Falcon tube.
   1ml glycerol (Sigma G5516)
   49ml Deionized water

3. Heat the 12ml aliquot of emulsion and the 12ml aliquot of 2% glycerol in 47°C hybridization oven for 30-45 minutes.

4. Place dip miser (*Electron Microscopy Sciences 70510*) into 47°C water bath in the dark room, making sure that the water just covers the metal stand.

5. Make sure to prepare all the labels, tape markers, slide boxes and aluminum foil **before** turning off the lights in the dark room. Slides should be placed in boxes with dessicant (Drierite) in box, and a blank slide next to the dessicant. The slide labels should face toward the right side of the box. After dipping, all slides in the box will be developed at the same time, so organize slides according to how long they are to be exposed.

   Steps 6-12 are done in the dark room without any light exposure.

6. Immediately after removing emulsion and glycerol from 47°C hybridization oven, go to dark room and add 12ml of emulsion to the 50ml Falcon tube containing 12ml of 2% glycerol. Mix very gently, so as not to create bubbles.

7. Pour 24ml mixture into dip miser and place back into 47°C water bath. Wait for a couple minutes before dipping slides.

8. Dip each slide once for about 2 seconds into emulsion holding the labeled end of the slide. Tap the slide vertically, then invert and place in slide rack to dry (label facing down). Continue to dip all other slides.

9. When finished dipping, place slides the previously labeled slide boxes.

10. Place the lid back on the slide box, turn slide box on side (now slide labels should be facing up), and wrap three times in heavy aluminum foil. Keep slide labels facing up while wrapping the slide box in aluminum foil.

11. Store slide boxes in the same position at room temperature for 18-24 hours away from any light or radiation.

12. Then transfer slide boxes to 4°C and expose for the appropriate length of time (Approximately 3X length of film exposure).

Developing (Day 5)

1. Take slide boxes to be developed from 4°C and warm to room temperature for at least 30 minutes before developing.

2. Mix 17g of Kodak Developer (*Kodak 1464726*) with 250ml of Deionized water.
   Mix 45g of Kodak Fixer (*Kodak 1971746*) with 250ml of Deionized water.
   Allow solutions to stir for 20 minutes. Developer should be a light yellow/brown color, and the Fixer should be nearly clear. Cool each solution to slightly above 20°C.

Steps 3-5 are performed in a dark room using a safelight. Use safelight and timer to monitor exact times for developing and fixing. Developing/Fixing, washes, and counterstaining will be performed in 300ml glass dishes and appropriate slide racks (holds 10 slides) (*Fisher 08-812*).

3. Incubate slides for 2 minutes 15 seconds at room temperature in Kodak Developer

4. Dip briefly in Deionized water.

5. Incubate slides for 4 minutes at room temperature in Kodak Fixer.

6. Place in tap water until finished developing all slides.

7. After use, discard the Developer and Fixer in their appropriate waste containers.

Counter Staining

1. Dip slides through multiple changes of tap water, then scrape emulsion off the back of each slide one-by-one using a clean razor blade, and immediately place back into tap water.

2. Incubate slide rack in Toludine Blue for 5-7 minutes at room temperature.
   0.5% Toludine Blue in 10mM Sodium Acetate (pH 4.6)

3. Dip in multiple changes of tap water until remaining water is nearly clear.

4. Dip in 75% EtOH for 10 seconds.

5. Dip in 100% EtOH for 10 seconds and repeat with fresh 100% EtOH.

6. Incubate in Xylene for 1 minute and repeat with fresh Xylene. Keep slides in final Xylene while coverslipping the other slides using Permount. Lay slides flat and let them dry overnight.