# Non-radioactive *In Situ* Hybridization

**Brad St. Croix (Aug 23rd, 2000)**
**version 1A**

***Part A: Digoxigenin-labeled Riboprobes***

1. PCR is used to generate DNA Templates for antisense or sense riboprobes by incorporating the following T7 promoter into the antisense or sense primer.

5′-GGATCCTAATACGACTCACTATAGGGAGA-3′

⇒In a typical reaction using a purified source of template (e.g. purified DNA, bacterial vector etc.) sufficient template can be derived from 4-50ul PCR reactions following 28 cycles. Run a small amount of PCR product on an agarose gel. If a robust PCR product is present and background bands are absent, gel purification is unnecessary.

2. Collect the PCR product into one tube, extract twice with phenol/chloroform (pH 8.0), and ethanol precipitate.

3. Resuspend pellet in 10mM Tris (8.0), measure DNA concentration, and dilute to 50ng/ul.

4. In vitro transcription. At Room Temperature (RT) mix the following reagents which can be obtained from Boehringer Mannheim, in the order shown:

| | |
|---|---|
| DEPC water | 9 ul |
| 200ng DNA template (50ng/ul) | 4 ul |
| 10X DIG RNA Labeling Mix (cat# 1277 073) | 2 ul |
| 10X transcription buffer (cat# 1465 384) | 2 ul |
| RNAse inhibitor (cat# 799017) | 1 ul |
| T7 polymerase (cat# 881 775) | 2 ul |

1. Incubate 2 hours at 37$^{o}$C.

2. Add 2ul of DNAse I, RNAse-free (cat# 776 785) and incubate at 37$^{o}$C a further 15 min.

3. Add 2ul 0.2M EDTA and place on ice.

4. Measure RNA concentration, and raise volume to 200ng/ul with DEPC water.

5. Verify RNA integrity and concentration by running 5ul (1ug) of RNA on a urea gel along side known concentrations of Marker (e.g. RNA Century-Plus size markers, Ambion cat# 7145). I mix RNA with 2X gel loading buffer from Novex (cat #LC6876) and run on a 6% TBE-U urea Novex gel (cat # EC6865).

6. If the gel verifies the RNA concentration, dilute to 50ng/ul with DEPC water, aliquot and store at –80$^{o}$C. Probes are stable at –80$^{o}$C for months to years.

***Part B: Tissue Preparation***

1. Fresh tissues are collected into any tissue culture medium (e.g. Dulbeccos Modified Eagles Medium + 1% FBS) and placed on ice.

2. Using a weigh boat rinse tissues in cold PBS, then cut sections into squares (~1.5 cm x 1.5cm) with a razor blade. Add a thin layer of OCT compound into a disposable tissue-embedding mold (e.g. Polysciences peel-a-way T-12 molds, cat #18986.) With forceps, dab tissue pieces onto Whatman paper to remove excess moisture and place section overtop of OCT. Layer OCT on top of the section, and store on ice until all samples are embedded.

3. Transfer to cryostat or –20$^{o}$C freezer. Before ice crystals form, or when they just begin to form at the surface (usually between 5-10min) snap freeze in methanol/ dry ice bath.

⇒Drying with whatman, and cooling slowly at the start helps prevents the tissue from breaking away from the OCT during sectioning. The OCT blocks can be stored for several months at –80$^{o}$C until ready to cut.

⇒Fresh tissues snap frozen immediately after removal, also work well. In this case quickly place the frozen tissue (before it thaws) in an embedding mold with OCT and immediately freeze in a methanol/ dry ice bath.

⇒ In my experience, the widely used method of prefixing sections with paraformaldehyde/PBS and cryoprotecting in sucrose/PBS makes the sections more difficult to cut. Also, I find that the preservation of morphology on snap frozen sections is similar (e.g. colon tumor) or better (e.g. normal colonic mucosa) than with the prefixing method.

***Part C: In Situ Detection***

**Day 1**

1. Remove sections from -80$^{o}$C freezer and place in cryostat set at -20 $^{o}$C. Allow temp to equilibrate (~10-15min). Meanwhile, in 50ml conical tubes prepare fresh 4% Paraformaldehyde/PBS on ice (see stocks).

⇒All of the solutions for Day 1 are prepared in RNAse-free 50ml conical tubes, which serve as disposable slide holders. Each 50ml tube can fit 2 outward-facing slides. 30ml of solution is sufficient to immerse sections. Note: Slides fit better in 50ml conical tubes from certain companies (e.g. Fisherbrand).

2. Cut sections onto high quality RNAse-free microscope slides (e.g. CSS-100 silylated slides from CEL Associates) and store in cryostat (-20 $^{o}$C) until all are cut. I would not recommend charged slides (e.g. Fisher's ProbeOn Plus slides) since they tend to repel tissues that are rich in extracellular matrix such as normal colon submucosa.

3. Fix in 4% paraformaldehyde/PBS 1hour at RT.

⇒Non-specific background staining observed in certain tissues can sometimes be reduced by longer incubations with Paraformaldehyde.

4. Rinse with PBS 2X 5 min on ice.

5. Inactivate endogenous Alkaline Phosphatase activity with 0.2N HCl for exactly 5 min at RT.

6. Immediately immerse sections in Dako's ready-to-use Pepsin (see stocks) diluted 0.1-10% from stock in fresh pre-warmed 0.2N HCL. Incubate exactly 5 min at 37$^{o}$C.

⇒Each new batch of pepsin needs to be titrated. In general, the best results are obtained by using the highest concentration of pepsin that can be achieved without having any tissue come off during the hybridization.

7. Rinse with PBS 1X 5 min on ice.

*8. RNAse control (Optional). To this control only, add RNAse A/T1 cocktail (Ambion cat # 2288) diluted 1/35 in TNE buffer. Incubate at 37$^{o}$C for 30 min. Afterwards, rinse with PBS 2X 5 min on ice. The other sections are stored in PBS on ice during this incubation.*

9. Acetylate. Pre-rinse sections by placing slides in 0.1M Triethylnolamine (pH 8.0) for 4-5 minutes (see stocks). To a separate set of 50ml tubes, add 75ul Acetic Anhydride (sigma Cat # A-6404) to each 50ml tube containing 30ml of 0.1M Triethylnolamine,

mix, and immerse slides in 0.25% Acetic Anhydride/ Triethylnolamine solution for 10 min RT.

10. Rinse with PBS 1X 5min.

11. Equilibrate for 5-10 min in 5X SSC (Gibco cat # 15557-028).

12. Prehybridization. Depending on the size of the section, place a small (Molecular Probes cat # C-18156) or large (cat # C-18161) CoverWell incubation chamber gasket upside down on a Kim wipe. Add ~150 (or 380ul) of mRNA hybridization buffer (DAKO cat # S3304) to the chamber. Remove slide from SSC, shake off excess liquid, invert slide so sections are facing downwards, and carefully squeeze onto CoverWell. To ensure a good seal, apply excessive downward pressure (the glass will not break if pressure is applied evenly on a flat surface). Prehybridize 1-2 hours at $55^{o}$C.

⇒Many protocols calculate the optimal Hybridization Temp [the formula that comes with Dako's mRNA hybridization solution after simplification is: HT = 41+ 0.41(%GC)-500/n where %GC is the percentage of GC in the probe (e.g. 60) and n represents the length of the probe in basepairs. Empirically, the hybridization temperature appears to have very limited effects on signal to noise. Therefore, I essentially always use $55^{o}$C and would only recommend raising it to 60-$65^{o}$C if probe-dependent background persists.

13. In a 1.5ml RNAse-free microfuge tube add digoxigenin-labeled riboprobe to fresh hyb. solution (final concentration 100-200 ng/ml) ensuring a dilution of at least 1/50. Vortex, then denature probe at $95^{o}$C for 3 min. and place immediately on ice. Add 150ul (or 380ul) to new Coverwell incubation chamber gasket. Discard gaskets from prehybridized sections and seal on new gaskets containing riboprobe. Incubate overnight at the hybridization temperature.

**Day 2**

1. Rinse excess probe from slides by incubating in 50ml tubes containing 30ml 2X SSC for 5 min in a $45^{o}$C water bath.

⇒For certain transcripts, particularly abundant ones, non-specific background due to the binding of riboprobe can be eliminated completely (without reducing the signal) by titrating the concentration of probe. However, for less abundant transcripts, probe-related background often persists even after probe titration. This may be due to non-specific binding of the probe to nucleotides but may also be due to non-specific binding to proteins and carbohydrates. In either case, digestion of unbound probe often helps (for some probes the only way to eliminate the non-specific binding is to design a new probe against a non-overlapping region of the gene).

⇒All rinses on day 2 are performed in 50ml conical tubes up until step 9. Once the biotin tyramide has been deposited, coplin jars are fine.

2. Remove formamide traces by rinsing 1 time with 2X SSC, and then twice in TNE buffer each for 5 min at 45$^{o}$C (see stocks).

3. Incubate with 250ul RNase A/T1 cocktail (Ambion cat # 2288) diluted 1/35 in TNE buffer at 37$^{o}$C for 1-2hr. Before adding the RNase I quickly wipe the outside edges of the slide with a Kim wipe. To prevent drying I immediately add a drop of RNase to each section. Next, I circumscribe the outside edges of the slide with a heat-resistant pap pen (e.g. RPI's Super HT cat #195505), then add RNase cocktail to each section.

4. Stringently wash slides with 30ml 2X SSC, 50% formamide DI (cat# AB600 American Bioanalytical) twice for 20 min at the hyb. temp.

5. Stringently wash slides with 30ml 0.08X SSC once for 20 min at the hybridization temperature. I make this final wash solution using Dako's 5X SSC Stringent Wash Concentrate, which also contains detergent and blocking agent (found in GenPoint kit, cat # K0620; also sold separately, cat # S3500).

6. Rinse twice with 1X TBST (see stocks) for 3 min at RT.

7. Quickly dry around the sections with a Kim wipe, then re-circle the sections with a pap pen. Before the sections dry, add 150ul of blocking buffer. Incubate for 30 min at RT.

⇒Before using blocking buffer, be sure to add 1/20 dilution of rabbit immunoglobulin fraction- see stocks.

⇒ Before dilution all antibodies should be spun in a microfuge (e.g. 5 min. max. speed) to prevent precipitate-related background!

8. Incubate sections with rabbit HRP-anti-DIG (Dako cat # P5104) diluted 1:150 in Blocking buffer for 30-45 min at RT.

⇒A higher concentration of antibody (e.g. 1:100 dilution) should be used for a single amplification cycle- see below.

9. Wash 3 X 4 min with 1X TBST with agitation.

10. Add one drop of ready-to-use biotinyl-tyramide (from DAKO GenPoint kit; cat # K0620) directly to slides. Incubate in dark 8 min at RT.

⇒DAKO's biotinyl-tyramide gives a much more intense signal than NEN's biotinyl-tyramide (from NEN renaissance kit).

11. Wash 3 X 4 min with 1X TBST with agitation.

⇒For relatively abundant transcripts, one biotin-tyramide amplification cycle is sometimes sufficient (can skip steps 12-15). However, for less abundant transcripts (i.e. most transcripts) the second cycle can provide the level of sensitivity required for a robust signal with little or no increase in background.

12. Add to sections rabbit HRP-anti-biotin (Dako cat # P5106) diluted 1:150 in Blocking buffer for 20 min at RT.

13. Wash 3 X 4 min with 1X TBST with agitation.

14. Add one drop of ready-to-use biotinyl-tyramide (from DAKO GenPoint kit) directly to slides. Incubate in dark 5 min at RT.

15. Wash 3 X 4 min with 1X TBST with agitation.

16. For detection by Fast Red, incubate with rabbit AP-anti-Biotin (Dako cat # D5107) diluted 1:75 with blocking buffer for 20min at RT (in dark).

17. Wash 3 X 4 min with 1X TBST with agitation.

18. Dissolve Fast Red tris tablets (Sigma, cat # F-4648) in dH2O (1 pellet/ml). Next, add Fast Red chromagen tablets to buffer (1 pellet/ml) and vortex to dissolve. Filter through a 0.2um filter. Add 150ul (or 380ul) to a small (or large) inverted CoverWell incubation chamber and press sections onto the solution. Store at RT in dark. Optimal staining usually takes ~20 min. to 1 hour. Stop reaction by washing twice in dH20 for 2-3 min. at RT.

19. In a coplin jar, counterstain for 1 min with Mayers Hematoxylin (Dako, cat # S3309). Rinse with dH2O. Blue the nuclei by incubating in 0.08% ammonium hydroxide for 1 min. Rinse twice in dH20 for 2-3 min.

20. Shake of excess water and add one drop of Supermount permanent aqueous mounting medium (e.g. Biogenex cat # HK079-7K) to section. This step dramatically enhances nuclei staining by hematoxylin. To dry, store in dark at $4^{o}$C overnight.

21. Place glass coverslip on a Kim wipe, add one drop of Permount (Fisher), invert slide and press section onto coverslip.

## Stocks

1. **4% paraformaldehyde in 1X PBS**: For 50ml mix one vial containing 10ml of 20% paraformaldehyde (EMS, cat #15713) with 5ml of 10X PBS (Ambion, cat # 9625) and 35ml of DEPC water (Quality Biological, cat # 351-068-131).

2. **0.1M Triethylnolamine (pH 8.0)** For 8 slides dissolve 4.17g Triethlynolamine-HCl (sigma cat # T-9534) in 225ml of DEPC-treated water (RNAse-free T75 tissue culture flasks are convenient). Adjust the pH to 8.0 by adding ~600-700 ul of 50% sodium hydroxide

3. **Blocking buffer**: First make 100mM Tris HCl (pH 7.5), 150mM NaCl, 1% blocking reagent (highly purified casein) from the Digoxigenin Nuleic Acid Detection Kit (Boehringer Mannheim cat # 1175 041). With pipetting blocking reagent will dissolve at 55$^{o}$C in <1 hour. Store at 4$^{o}$C and use within a month (longer storage may lead to higher background). Immediately before adding to sections spin rabbit immunoglobulin fraction (Dako cat # X0903) in microfuge and add 1/20 dilution to the blocking buffer. To remove any precipitated blocking reagent heat to 37$^{o}$C 5-10 min, and spin in tabletop centrifuge 5 min. Store at RT during assay. Note: Do not add sodium azide to blocking solution as this can inactivate HRP over long periods of time!

4. **TNE buffer**: 10mM Tris HCl (pH 7.5), 500mM NaCl, 1mM EDTA.

5. **Pepsin**: The stock solution is made by dissolving one 2g ready-to-use Pepsin powder packet (DAKO cat # S3002) in 250ml of 0.2N [i.e. 0.8% (w/v)]. Freeze small aliquots (e.g. 10ml) at –20$^{o}$C. When ready to use, thaw aliquot and dilute to optimal concentration in 50ml tubes with 0.2N HCl. Heat to 37$^{o}$C before adding slides.

6. **TBST:** Add 111ml of Dako's 10X Tris Buffered Saline with Tween 20 (cat # S3306) directly to 1L of DEPC water (Quality Biological, cat # 351-068-131).

### Tips for preventing background

1. Strong patches of non-specific background can occur when biotin, rather than digoxigenin, is used to label the riboprobe. Biotin-labeled probes seem to bind non-specifically to certain tissues e.g. some colon epithelial cells, and necrotic tissues. This background may be due to endogenous biotin, but is more likely due to endogenous streptavidin-like activity that non-specifically binds Biotin-labeled probes.

2. Some protocols suggest the need to inactivate endogenous peroxidase activity. However, with this protocol endogenous peroxidase activity appears to be completely inactivated by the pepsin treatment. Furthermore, treatment with hydrogen

peroxidase/PBS (often used to inactivate endogenous peroxidase) results in noticeably higher background. I would strongly recommend not including such a step.

3. I usually make 500-600bp probes across the entire known gene sequence, and then compare each probe individually. Sometimes all probes perform equally well, but often variability in signal to noise is observed. Combining individual probes with high signal to noise results in a noticeably improved signal without any increase in background.

4. Never let the sections dry out.

**If you find conditions which either simplify the current protocol or make it more robust, please contact me so that I can pass the good news onto others. My e-mail address is: stcroix@jhmi.edu**

Thanks, and best of luck!
Brad