IN SITU HYBRIDIZATION PROTOCOL
J. HALL MODIFIED FROM L. HILEMAN AND E. KRAMER PROTOCOLS;
LAST MODIFIED 01APRIL05

MAKING PROBES:

A. Pick clones and prep plasmids:

1. Pick clone from gene/region of interest:
   Make sure that it is not a 'funny clone' with odd sequences, has long 3'UTR, and preferably does not have NOT1 or SPE internal digest sites
2. Eco-RI digest the clone, make sure it's the right size insert, then gel extract insert using min-elute (Qiagen)
3. PCR using insert fragment diluted 1:20 as a template
4. Clone PCR product using TOPO
5. Screen colonies to determining orientation of inserts –
   a. goal two colonies such that transcription from T7 promoter off one clone will generate sense and transcription off another generate antisense (alternatively, can use one clone and do transcription with T7 and T3).
   b. Screen with m13f-m13r to see if have right insert
   c. Screen with m13f and original forward primer to determine orientation of clone
6. Pick one/two clones and grow 3ml cultures for 8 hours
7. Inoculate the 3m cultures into 25-50ml of LB + carb; grow O/N (ca. 16 hours)
   [***SORVAL should be set to 4C***]
8. Use QIAFILTER MIDIPREP kit to isolate plasmids
9. Final step resuspend pres in 250 ul of dH2O; I USED ELUTION BUFFER – CHECK NOTEBOOK
10. ECO-RI digest maxiprep & Spec to get concentration for linearizing below
11. Confirm orientation of inserts with PCR and/or sequencing

B. Linearize Plasmid:

1. Choose an enzyme that:
   a. Cuts at the opposite end of your insert from the T7 promoter
   b. Does not cut into your insert
   c. Leaves 5' overhang or blunt ends
   d. Has 100% (or close) efficiency in optimal digestion buffer
   e. For TOPO-TA for sequencing vectors – SPE I (or NotI if cannot get both orientation of inserts into clones)
2. Set up digestion:
   15ug plasmid
   10ul 10x buffer
   3 ul restriction enzyme
   Bring to 100ul with dH20
   (If need to set up larger digest, keep 3ul of enzyme, but scale up buffer accordingly)

   let digestion go for 4 hours, then add 2ul more of restriction enzyme, leave O/N
   [***make sure Phenol:Chlorofrom:IAA is in fridge; if there is one there already make sure that color is not pink***]

3. Run out:
   1ul undigested plasmid
   1 ul digested plasmid
   10 ul digested plasmid – ***there should be no evidence of undigested plasmid in this lane***

[Heat inactivate enzyme]

C. Phenol/Chloroform extract linearized plasmid (Volumes for 100ul rxn, scale as needed)

1. Add equal volume Phenol:Chloroform:IAA to linearized plasmid (100ul)
2. Vortex & Spin 1 minute @13K rpm
3. Remove aqueous phase (ca. 100 ul) to a new tube (can back extract – see lena's protocol)
4. Extract aqueous with equal volume Chloroform (100ul)
5. Vortex & Spin 1 minute @13K rpm
6. Remove aqueous phase to **RNASE FREE TUBE**

*******FROM THIS POINT ON, EVERYTHING IS RNASE FREE!!!!*******

7. Add 0.1 volume (ca. 10 ul) 3M Sodium Acetate (NaAC) **RNASE FREE**
8. Add 2 volumes (ca. 200 ul) 100% EtOH **RNASE FREE**
9. Precipitate O/N at -20C

[*make sure centrifuge is in walk-in*]

10. Spin 10 min. 13K rpm @4C
11. Wash pellet with 75% EtOH (ca. 100-150ul) **RNASE FREE**
12. Let pellet dry
13. Resuspend in DEPC H2O, vortex, to approximate 1ug/ul (start with 8ul H2O and then adjust, use 0.5ul in 99.5 H2O, 1:200, to spec.)

D. Transcription RXN

1. **2 transcription rxns per probe** (1 to be hydrolyzed, 1 un-hydrolyzed)
2. Reaction (20ul) [see Lena protocol for info on different % incorporation]
   1ul linearized plasmid (1ug)
   2ul NTP mix (35% DIG or other)
   2ul 10X transcription buffer
   2ul T7 RNA polymerase
   1ul RNAse inhibitor
   12ul DEPC H2O
3. Let transcription run 2hours @ 37C in incubator (or thermocycler)

[prepare TBE gel and RNAse free gel-rig towards end of time;
make sure hydrolysis time calculated, see 8 next page]

4. **Set aside 1ul in 9ul DEPC H20; labeled pre-DNase (or 0.5ul in 9.5ul DEPC)**
5. Add **2ul DNAse** (Amplification grade) to transcription rxn
6. Let DNAse treatment go for 15 minutes @ 37C in incubator (or thermocycler)
7. **Set aside 1ul in 9ul DEPC H20; label post-DNase**
8. Put reaction at -20C while run out pre- versus post-DNAse in TBE gel
   Should see in gel:
   Pre-DNase – high band and 1-2 low bands
   Post-DNase- NO high band and same 1-2 low bands (if high band still there, but back in incubator more time and run out again until no high band).
9. After confirm there is no DNA, **stop rxn with 4 ul 200mM EDTA**

E. Probe precipitation and hydrolysis

1. Add 5ul 5M LiCl **RNASE FREE**
2. Add 150ul 100% EtOH **RNASE FREE**
3. Precipitate 2 hours @-20C (can be longer or O/N)
4. Spin 10 min. 13K rpm @4C
5. Wash pellet with 70% EtOH (ca. 100-150ul) **RNASE FREE**
6. Let pellet dry
7. **Resuspend un-hydrolzyed probe in 20ul 50% deionized formamide, continue protocol for hydrolysis**
8. HYDROLYSIS TIME
   Time of alkaline hydrolysis determines final length of probe:

   Time (in minutes) = (Li – Lf)/(0.11 x Li x Lf)
   Li= initial length of probe (transcript) in **kb**
   Lf= desired length of probe in **kb**

   Many protocols call for a final length of 150bp, some like longer, ca. 400bp, for better specificity and reduced background

9. Resuspend precipitated probe pellet in 50ul 0.1M NaHCO3 pH10.2
10. Hydrolyze @60C for time calculated above
11. Stop hydrolysis with 5ul 5% Acetic acid **RNASE FREE**, then precipitate by
12. Add 5ul 3M NaAC **RNASE FREE**
13. Add 125ul 100%EtOH **RNASE FREE**
14. Precipitate 2 hours at -20C (can be longer)
15. Spin 10 min. 13K rpm @4C
16. Wash pellet with 70% EtOH (ca. 100-150ul) **RNASE FREE**
17. Let pellet dry
18. **Resuspend hydrolzyed probe in 20ul 50%deionized formamide**

F. Quantify probe – according to the protocol

PREPARATIONS FOR IN SITUS:

(need 3 days for all of this; amounts for processing ca. 15 slides)

1. Wash plastic ware (1x with soap, 102x without, dry upside down on paper towels):
    a. At least 20 300ml small Tupperware boxes w/lids
    b. 2-3 large flat boxes
2. Bake glassware O/N at 180C (oven in herbaria)
    a. Erlenmayer flask (500ml) or large beaker (ca. 500ml)
    b. 5-10 stir bars
    c. bottles, various sizes (100ml, 500ml)
    d. 2-3 100ml cylinders, 1 50ml cylinder, 1 25ml cylinder
    e. metal slide holder
    f. upright glass slide holder
    g. spatulas
3. Soak plastics (bottle lids) O/N in 0.1M NaOH/1mM EDTA (don't need EDTA?)
4. Make DEPC water
    a. Add 1ml DEPC-active to 1L miliQ H2O in hood – best to make 5L and distribute between 250ml, 500ml, and 1L screw-cap jars
    b. Mix well and let incubate in hood at room temp O/N
    c. Autoclave 20min to kill DEPC
5. Make 4, 2L bottles of 'clean water' – MiliQ water direct from purifier, then autoclave
6. Check that there are enough stock solutions
7. Walk through protocol and make sure that you have everything including working buffers

Day before first day of in situs:

1. Small bottle of clean water, tighten lid right after autoclaved, then put in cold room
2. Blocks to be sectioned in cassettes
3. Make up 2L 1X PBS (200ml per 2L – mark on bottle)
4. Set up EtOH series, 2 citri-solve boxes in hood; 1x PBS, 1X PBS w/Glycine, 150mM NaCl
5. Set one of the incubators to 50C
6. 300ml box of Pronase buffer in walk-in 37C room

EK's list:

5x NTE	600ml	1M Na2HPO4	250ml
20x SSG	500ml	1M NaH2PO4	250ml
Tris pH 7.5	500ml	10XPBS	500ml
Tris pH 8	500ml	0.5M EDTA	500 ml (~14ml
Tris pH9.5	500ml	NaOH)	
G3		1M MgCl2	50.8g/250ml
5M NaCl	500ml	DEPC	3L
10x Pronase	250ml	clean H2O	8L
10XGlycine	250ml		

STOCK SOLUTIONS:

10x PBS pH7.0 (1L/600ml)
1.3M NaCL (76g NaCl/45.6g)
70mM Na2HPO4 (70ml 1M/42ml)
30mM NaH2PO4 (30ml 1M/18ml)
(900ml/540ml DEPC)

5M NaCl (500ml)
146.1g/500ml

10x proteinase K buffer (500ml)
100mM Tris pH8
50mM EDTA

10X Pronase buffer (200ml)
500mM Tris pH7.5 (100ml 1M Tris)
50mM EDTA (20ml 0.5M EDTA)

Tris (500ml) make 3: pH7.5, 8, 9
78.78g Tris [NOTE - check MW of your Tris, this may vary]
pH 7.5, ca. 35ml HCl
pH 8, ca. 20ml HCl
pH 9, ca. 1ml HCl

0.5M EDTA (500ml)
93g EDTA

1M MgCl2 (500ml)
101.6g MgCl2

5X NTE buffer (600ml)
2.5M NaCl (87. 6g NaCl)
50mM Tris pH 8 (30ml 1M)
5mM EDTA (6ml 0.5M)
(564ml DEPC)

20X SSC (1L)
3M NaCl (175.32g NaCl)
300mM Na citrate (88.23g Na Citrate)

G3 buffer (250ml)
100mM Tris pH 9.5 (25ml)
100mM NaCl (5ml)
50mM MgCl2 (12.5ml)

100uM Tris, 100mM Nacl (stock for various buffers) (1L)
100ml of 100mM Tris pH 7.5
30ml of 5M NaC

EtOH series for 300ml containers
(note: 5M NaCl crashes out of solution at greater than 85% EtOH):

	Clean H2O	100% EtOH	5M NaCl
95% EtOH	15ml	285ml	N/A
85% EtOH	36ml	255ml	9ml
70% EtOH	81ml	210ml	9ml
50% EtOH	141ml	150ml	9ml
30% EtOH	201ml	90ml	9ml
150mM NaCL	291ml	N/A	9ml

How much stock solution needed per round 15 slides (down to 10 after probe)?

200ML 10XPBS – 2L WORKING
45ML 5M NACL – DEHYDRATION SERIES
CA. 2L 100% ETOH – DEHYDRATION SERIES
30-60ML PRONASE BUFFER
30ML 10XPBS W/GLYCINE (0R 0.6G)
60ML TRIS 7.5 – BLOCK, BSA BUFF
25ML 5M NACAL – BLOCK, BSA BUFF
25ML TRIS 9.5 – G3
12.5 ML MGCL2 – G3
12ML 20X SSC BUFF – 1.2L WORKING
300ML 5X NTE – 1.5L WORKING

DAY 1, P1 OF 3

- Start early

Sectioning:

- Section as much material as needed/possible. EK typically does 15 slides (paired down to 10 when add probe)
- Sections should be 8um thick
- Add clean water to Fisher PROBE ON PLUS sides, add sectioned material to 5 slides – serial sectioning, then start another 5, when done with 2nd set, pull water off of 1st and start third etc.. after pull water off, after another 15 minutes use kimwipe to get all water off
- 4 hours dry time starts AFTER kimwipe stage

When Slides are fixing:

1. Make 4% Paraformaldehyde
    a. 12g paraformaldyde in 300ml 1X PBS
    b. Wear 2 set gloves and mask when measuring out paraformaldehyde- use three large weigh boats (2 to be used as cover when moving between hood and scale)
    c. Boil PBS in erlenmayer flask (or screw top bottle) in microwave – it needs to be HOT
    d. Then add Paraformaldehyde to flask and still until dissolved (or add PBS to paraformaldehyde already in beaker with stir bar)
    e. Let cool to temp and either put in the 300ml plastic container (or screw cap)
2. Turn on Hybridization oven to HYB temperature (38C-44C); note that temp on oven reads approximately 3C higher than actual temperature
3. Set heat block to 80C – move over to my bench
4. Pronase (or Proteinase) in fridge to thaw;
    a. Concentration of proteinase K MUST be determined experimentally
    b. LH using 1.25ug/ml = 37.5 ul, 10mg/ml protK in 300ml buffer
    c. Be sure to use appropriate buffer (e.g. Pronase versus Proteinase!)
5. 590ML clean water in anhydride containter in hood
6. 1.5L 1X NTE and put in 37C shaker – 300ml 5X stock
7. 1.2L 0.2xSSC and put in incubator at 50C – 12ml 20X stock
8. Determine probe concentrations for 40ul total & dilute
    a. 1X=0.5ng/ul/kb (e.g. 0.5 x 100 (fisher slide) x 0.3 (kb of probe) = 15ng probe/slide)
    b. add appropriate ul of probe (e.g. 1X,5X, etc.) for a total volume of 40ul probe
    c. keep in freezer until need them
9. LH protocol prepare Glycine (0.6g Glycine in 300ml PBS – takes times to go into solution)

DAY 1, P2 OF 3

CITRISOLV	10 MIN	HOOD
CITRISOLV	10 MIN	HOOD
100% ETOH	2 MIN	CHANGE TO FRESH 100% ETOH AFTER THIS TIME
100% ETOH	1 MIN	
95% ETOH	1 MIN	
85% ETOH, 150 MM NACL	1 MIN	
70% ETOH, 150 MM NACL	1 MIN	
50% ETOH, 150 MM NACL	1 MIN	
30% ETOH, 150 MM NACL	1 MIN	
150 MM NACL	2 MIN	
1X PBS	2 MIN	***SLIDES TO 37C SHAKER***
PROTEINASE K TREATMENT	20 MIN **@ 37C**	CHANGE PBS; SET UP 40UL PROBES FOR SLIDE PAIRS AND KEEP @-20C (SEE ABOVE RE: CONCENTRATION)
1X GLYCINE (1XPBS W/GLYCINE)	2 MIN	
1X PBS	2 MIN	
4% PARAFORMALDEHYDE	20 MIN	HOOD; CHANGE PBS; MAKE ACETIC ANHYDRIDE BUFFER: 590ML CLEAN H2O 7.8ML TREITHANOLAMINE 2.4ML HCL CHECK PH IS 8.0, LET STIR UNTIL USE
1X PBS	2 MIN	HOOD
ACETIC ANHYDRIDE	10 MIN	HOOD PLACE SLIDE RACK IN ACETIC BUFFER, START STIRRING @ SPEED 10; SLOWLY DROP 3.6ML ACETIC ANHYDRIDE – CONTINUE STIRRING FOR 10 MIN; CHANGE PBS (OLD PBS – FORM WASTE IN HOOD); SET UP BENCH FOR PROBES; LARGE PLASTIC CONTAINER W/WET TOWELS AND PIPETTES
1X PBS	2 MIN	HOOD
150MM NACL	2 MIN	DISCARD PBS IN ANHYDRIDE HOOD WASTE
30% ETOH, 150MM NACL	1 MIN	
50% ETOH, 150MM NACL	1 MIN	
70% ETOH, 150MM NACL	1 MIN	
85% ETOH, 150MM NACL	1 MIN	
95% ETOH	1 MIN	
100% ETOH	1 MIN	
100% ETOH	2 MIN	

100 MINUTES TOTAL

DAY 1, P.3 OF 3

1. Allow sections to dry (lean vertically for about 10 min)
2. Put HYB in 80C heat block
3. Look at slides (under microscope) and choose which slides to use for sense and anti-sense and for which concentrations (Fill out 'in situ worksheet')
4. Add 40ul probe solution to 80C for two minutes (may want to stagger depending how fast at sandwiching)
5. Add 200ul HYB solution to 40ul probe solution; mix gently by pipetting without generating bubbles
6. Make slide sandwiches (this is tricky, get a "pro" to help you the 1st time)
    a. Take ca. ½ probe+HYB and put in line down middle of 1st slide, smooth out w/tip
    b. Make T with ½ probe+HYB on 2nd slide
    c. Flip (fast!) T slide over and slowly (with gentle bouncing!) lower it onto the 1st slide trying not to have any bubbles
7. Place slides sandwiches elevated in humid chamber (flat container with wet paper towels) and put in hybridization oven overnight (not agitated!)
8. Make sure that everything set up for DAY 2
    a. 0.2x SSC boxes next to Hybridization oven
    b. NTE boxes in 37C shaker

Day 2: Post-hybridization

During the 1st two stringency washes, do the following:

1. Make 1% Block (100 ml for 10 slides)
   1g blocking reagent (from Roche, in fridge)
   10ml, 1M Tris pH 7.5 (final conc. 100mM Tris pH 7.5)
   3ml, 5M NaCl (final conc. 150mM NaCL)
   stir for > 1 hour to go into solution, heat ca. 6

   (can add 1g Block to stock 100mM TrispH7.5, 150mM NaCl)

2. BSA buffer = BTNT (500ml)
   (can also use stock 100µM TrispH7.5, 150mM NaCl)
   5g BSA (Sigma #A3059 – make sure fraction V)
   50ml, 1M Tris pH 7.5 (final conc. 100mM Tris pH 7.5)
   15ml, 5M NaCl (final conc. 150mM NaCl)
   (433.5ml clean water)
   1.5ml, Triton X100
   stir (no heat) approximately 30 minutes to go into solution
3. Genius3 buffer (250 ml)
   25ml, 1M Tris pH 9.5 (final conc.100mM Tris 9.5)
   5ml, 5M NaCl (final conc. 100mM NaCl)
   12.5ml, 1M MgCl2 (final conc. 50mM MgCl2)
   207.5ml clean water
4. Set aside 10 ml of BTNT & G3 in 15ml falcon tubes
5. Set orbital shaker to 37C
6. RNAse to thaw

DAY 2, p.2

- Remove slides from hybridization oven, ***immediately adjust oven to wash temp of 50C***
- Dip slides in pre-warmed 0.2X SSC (in a 300ml plastic container) to separate (be careful not to rub slides together), rinse and place in slide rack sitting in 2nd 300ml plastic container filled with pre-warmed 0.2X SSC buffer

0.2X SSC, 50-55C	1 HOUR	W/GA	SEE PREVIOUS PAGE
0.2X SSC, 50-55C	1 HOUR	W/GA	USE SAME CONTAINER; SEE PREVIOUS PAGE
1X NTE, 37C	5 MIN	W/GA	IN 37C SHAKER
1X NTE, 37C	5 MIN	W/GA	IN 37C SHAKER; USE SAME CONTAINER
RNASE A, 37C	20 MIN	W/GA	IN 37C SHAKER; 600UL RNASE IN 300ML 1X NTE BUFFER
USE POST-RNASE CONTAINERS FROM THIS POINT ON!			
1X NTE, 37C	5 MIN	W/GA	IN 37C SHAKER
1X NTE, 37C	5 MIN	W/GA	IN 37C SHAKER
0.2X SSC, 50-55C	1 HOUR	W/GA	MOVE CART W/SHAKER NEAR HYB OVEN
1X PBS, RT	5 MIN		(CAN LEAVE O/N AT RT)
PLACE SLIDES IN BOTTOM OF LARGE PLASTIC CONTAINER			
1% BLOCK, RT	45 MIN	W/GA	
1% BSA BUFF, RT (=BTNT)	45 MIN	W/GA	MAKE ANTI-DIG IN BSA BUFFER: 8UL IN 10ML BTNT SET ASIDE IN 15ML TUBE
ANTI-DIG ANTIBODY	2 HOURS		PUT ANTI-BODY BUFFER IN LARGE WEIGH BOAT; SANDWICH SLIDES AND ALLOW CAPILLARY ACTION TO PULL ANTI-BODY SOLUTION UP; DRAIN ON STACK OF KIMWIPES AND PULL UP AGAIN; REPEAT AND PUT IN 'HUMID CHAMBER'
DRAIN & SEPARATE SLIDES, PLACE IN BOTTOM OF LARGE PLASTIC CONTAINER			
1% BSA BUFF, RT	15 MIN	W/GA	
1% BSA BUFF, RT	15 MIN	W/GA	USE SAME CONTAINER
1% BSA BUFF, RT	15 MIN	W/GA	USE SAME CONTAINER
1% BSA BUFF, RT	15 MIN	W/GA	USE SAME CONTAINER; THAW LEVAMISOL IF USING
G3 BUFF, RT	10 MIN	W/GA	USE SAME CONTAINER, BUT RINSE W/50ML G3 1ST; MAKE COLOR SUBSTRATE IN 10ML G3: 90UL (UP TO112UL) NBT/BCIP MIX LIGHT SENSITIVE – MAKE LAST MINUTE OR COVER W/TINFOIL
NBT/BCIP, COLOR SUBSTRATE	1-3 DAYS	DARK	MOVE SLIDES TO TALL GLASS SLIDE HOLDER FILLED WITH G3; PUT COLOR SUBSTRATE SOLUTION IN WEIGH BOAT; DRAW UP COLOR SUBSTRATE INTO SLIDE SANDWICHES; PUT IN HUMID CHAMBER PLACE IN DARK

After color reaction is complete:

1. Separate slide sandwiches and stop reaction n TE buffer, 2 minutes
2. Stain tissue in calcofluor for 5-8 minutes in glass slide jar
    a. Stock calcofluor of 1% used 125ul of stock in 62.5ml of 1X PBS to make up 0.002% working solution
3. Wash in 1X PBS for 5min; do not let slides dry out
4. Put a drop of mounting medium of the slide, a coverslip, and observe under the microscope using bright field and fluorescence
    a. Mounting medium is 0.5% of n-propyl gallate (Sigma P3130) in PBS/Glycerol (30% 1x PBS, 70% Glycerol) you can stores the slides in this way for up to 2 weeks at 4C
    b. Can Also Mount with water
5. Take pictures
6. After photographing, you can permanently mount slides in Permount, but you will not be able to see the fluorescence of the calcofluor any longer. Remove the aqueous mounting media by dipping the slides in 1x PBS. Dehydrate though an EtOH series followed by 2 citrisolv washes and mount with Permount

IN SITU WORKSHEET

DATES:
GOALS:
NOTES ON PREPPING (E.G. POTENTIALLY PERTINENT INFO ON STOCK SOLUTIONS, ETC):

BLOCK # AND SECTIONING NOTES:

PROBE NAME:
HYBRIDIZATION TEMP OVEN SETTING:
HYBRIDIZATION TEMP (ACTUAL, STABILIZATION TEMP. CHECK IN A.M.)
HYBRIDIZATION TIME IN: TIME OUT:

PROBE NAME	SENSE/ ANTISENSE	APPROX. PROBE LNGTH	# UL IN PROBE	SLIDES	COMMENTS

DAY(S) FOR COLOR REACTION:

NOTES ON