GUDMAP – SOP "Fluorescent ISH – Freedom Evo" – Gaido Lab - GenePaint System The Hamner Institutes for Health Sciences – CIIT at the Hamner

Last update: March 19, 2007

For questions and comments, please contact:
gaido@thehamner.org *Kevin Gaido*
Updated By: Janan Hensley

DAY 1:
Acetylation of frozen sections cut at 10µm
Assembly of hybridization chambers
Daily Solutions
Starting the run
Steps (Prehyb, hyb, post-hyb, probe detection reactions)

DAY 2:
Disassemble hyb chambers
Clean-up
Shut-down

The Tecan script was supplied by Tecan and modified for FISH by Michael Kelso.

Unless otherwise stated the following protocol requires ultra-pure water (Milli-Q).

For list of solutions & chemicals see appendix.

Essential Practices

1. Always wear gloves and use Rnase-free reagents and materials.
2. Always protect the fluorescent (Cy3) and other sensitive chemicals from direct light. As most fluorescent substrates are light sensitive they lose their sensitivity as they are exposed to light.

DAY 1

Tissue Preparation for FISH in Freedom Evo (Acetylation)

Make 200mL of each solution unless otherwise noted.

1. Isolate tissue.
2. Freeze tissue in OCT by placing tissue in plastic molds with OCT, arranging the tissue as desired, and freezing the mold on dry ice.
3. Store at -80^{0}C
4. Section tissue into 10µm sections. Be sure to use "precleaned Superfrost plus" slides (Fisher 12-550-15). Label slides with a pencil. Store in a plastic bag with desiccant at -20^{o}C, overnight (or up to 3 weeks).
   When ready to acetylate:
   Heat fix slides at 50ºC for 2 minutes (or before using).
5. **Fix:** Fix sections in 4% PFA / PBS for 10 min RT (make up fresh every time).
   (40 mL PFA/160 mL PBS DEPC H20.)
6. Rinse in 1X DEPC PBS for 3 minutes, 3X.
7. **Acetylation:** In a container, on a stirrer, combine: **Tissue Tek holds 200mL**

H_2O (Depc)		**200mL**
Triethanolamine (dissolve in H20 & HCl before hand)	10mM final	**2.66mL**
37% HCl		**350µl**
Acetic Anhydride	0.5% final	**750µl (add last)**

Immerse sections in the above solution for 10 min room temperature (add 750µl acetic anhydride, incubate at room temperature for 5 minutes, after 5 minutes add another 750µl of acetic anhydride and incubate at room temperature for 5 minutes.) *Acetic anhydride is only good for 5 minutes, adding 2x @ 750µl each gives a final concentration of 0.5%.*

KEEP ACETIC STEP ON STIRRER AT ALL TIMES DURING INCUBATION!

8. Rinse in DEPC PBS for 3 minutes, 3X.
9. Series dehydration (1-2 minutes for each, **see below steps 9a – 9f**)

DEHYDRATION STEPS

9a. 70% EtOH DEPC H20
9b. 80% EtOH DEPC H20
9c. 95% EtOH DEPC H20
9d. 95% EtOH DEPC H20
9e. 100% EtOH DEPC H20
9f. 100% EtOH DEPC H20

10. Air dry and store in sealed boxes at -80^{0}C or use immediately for FISH. (<1 month storage, 3-5% degradation per month).

NOTE: EtOH solutions are only good for up to 2 weeks after diluting, make fresh every two weeks.

Assembly of Hybridization chambers

1. Thaw the desired number of acetylated slides from the -80ºC freezer, if acetylated before hand, in a 37ºC incubator for about five minutes. If slides were acetylated the same day as the run then slides do not need to thaw at 37ºC.
2. Make sure the area is Rnase free and collect all needed equipment and tools. Including: assembly platforms, aluminum assembly frames, assembly clips, Tecan spacers, glass back plates, acetylated slides and scissors.
3. Begin placing aluminum assembly frames onto the assembly platforms in their respective grooves. Begin laying the slides down into the aluminum assembly frame with the label toward the centre of the workbench facing upward. Make sure slides are against the top of the assembly frame.
4. Place the Tecan spacers on top of the slides. It is important for the spacers to be placed within the frame with equal overhang to prevent damage to slides, and to ensure proper solution flow through the hybridization chamber.
5. Carefully lay the back plate on top of the slide and spacer, against the top of the assembly frame, with the etched reservoir facing downward onto the label.
6. Fasten the back plate to the assembly frame with the assembly clip. To clip the back plate to the frame, hold the back plate onto frame with slight pressure with one hand and hook one end of clip and pull over and down with the other hand. An audible click should be heard when done correctly. Two assembly clips per aluminum assembly frame are used. One is placed just below the etched reservoir and the other about a half-inch above the bottom of the glass back plate.
7. With a pair of scissors, cut the overhang of the spacers off by carefully cutting along the top of the assembly frame and along the bottom of the slide. Be careful not to cut the slide with scissors, may cause slide to crack.
8. Place the hyb chambers in their respective position on the platform.

GUDMAP – SOP "Fluorescent In Situ-Hybridization" (CIIT at the Hamner)

Last update: March 19, 2007
For questions and comments, please contact:
gaido@thehamner.org Kevin Gaido
Updated By: Janan Hensley

Preparation for the FISH run

Waterbaths, incubators, & heatblocks must be turned on to allow time to warm to desired temperature. Remove solutions from 4ºC storage (Formamide, Peroxide, 1X MWB) and equilibrate to RT before using.

DAY 1 continued: Daily solutions

FISH daily solutions			Total volume			
			To be heated – 65ºC for 30 minutes before filter and degas steps			
1. Make sure waterbath is at 65ºC.			To be filtered – use whatman paper			
2. Dry slides for 5 minutes at 37ºC.			To be degassed			
			To be added last			
1st DAY SOLUTIONS						
Solution	**Amount**	**units**	**Reagent**	**catalog #**		**NOTES**
1X PBS	150	mL	1X PBS DEPC treated			
	750	µL	10% Tween			
	150	mL	Total volume			
0.2M HCl	5	mL	2M HCl	Fisher: A144-212		
	250	µl	10% Tween			
	44.75	mL	ultra pure H20 (Depc)			
	50	mL	Total volume			
5X SSC	74.5	mL	ultra pure H20 (depc)			
preheat to 65ºC	25	mL	20X SSC	Ambion: 9763		
	500	µL	10% Tween			
	100	mL	total volume			
0.1X SSC	99	mL	ultra pure H20 (depc)			
preheat to 65ºC	500	µL	20X SSC			
	500	µL	10% Tween			
	100	mL	total volume			

Formamide I	28	mL	ultra pure H20 (depc)	Roche 11814320001		Bring to RT before using
preheat to 65ºC	7	mL	20X SSC			
	350	µL	10% Tween			
	35	mL	formamide			
	70	mL	total volume			

Formamide II	31.5	mL	ultra pure H20 (depc)			
preheat to 65ºC	3.5	mL	20X SSC			
	350	µL	10% Tween			
	35	mL	formamide			
	70	mL	total volume			

1X NTE	20	mL	5X NTE			
preheat to 65ºC	500	µL	10% Tween			
	79.5	mL	ultra pure H20 (depc)			
	100	ml	total volume			

20mM iodoacetamide (add at end of 1st day)	185	mg	iodoacetamide (added last)	Sigma I-1149		IDO not stable, add last
	50	mL	1X NTE			Take 50mL 1X NTE from solution made in previous step
	—		10% Tween (already in 1X NTE)			
Degas without IDO, add IDO after degas step	50	mL	total volume			

1X TNT	20	mL	10X TNT			
	1	mL	10% Tween			
	179	mL	ultra pure H20 (depc)			
	200	mL	total volume			

Blocking solution

4% sheep serum	2.4	mL	sheep serum	Gibco 16070-096 or Sigma S-2263 (100mL)		Sheep serum is aliquoted into 5mL aliquots and stored in -20ºC
	6	mL	10X TNT			
filter, degas	300	µL	10% Tween			
add at end of 1st day	51.6	mL	ultra pure H20			
	60	mL	total volume			

TNB	7	mL	10X TNT			
preheat to 65ºC	350	µl	10% Tween			
	63	mL	ultra pure H20 (depc)			
	0.35	g	blocking reagent	Perkin Elmer		Add blocking reagent before heating so it dissolves into solution
Degas	70	mL	total volume			Degas after heating

1X Maleate wash buffer	99.5	mL	1X MWB			Stored at 4ºC, bring to RT before using.
(MWB)	500	µl	10% Tween			
prepare fresh weekly	100	mL	total volume			

1% BR	0.5	g	blocking reagent	Roche: 11096176001		Stored at -20ºC
preheat to 65ºC	50	mL	1x maleate wash buffer			
filter, degas	50	mL	total volume			**Add at end of 1st day**

TMN	94.5	mL	TMN			
filter	500	µl	10% Tween			
prepare levamisole 10mg/mL	5	mL	levamisole 10mg/mL			
	100	mL	total volume			

pre-HYB	15	mL	Formamide			
	7.5	mL	20X SSC			
	7.5	mL	ultra pure H20 (depc)			
	15	µl	tRNA 10mg/ml	Ambion # 7119		Stored at -20ºC
	15	µl	Heparin 100mg/ml	Sigma: H-3149		Stored at -20ºC
	30	mL	total volume			

In situ **Hyb mix**	15	mL	*in situ* hyb mix	Ambion B8807G		
heat at 65C (10 minutes)	enough for 12 slides					
cool to RT						
add 15µl 100mg/ml DTT,						Stored at -20 ºC
add 15µl 10mg/ml tRNA						Stored at -20ºC

Probe	300 ng/mL					
vortex solution, **heat at 80ºC for 30 seconds (heat block), then heat at 65ºC** for about 10 minutes (waterbath), cool, add to rack	1	mL	*in situ* hyb mix			
	1	mL	total volume			quick spin after heating

Anti-digoxigenin-POD	**8.5mL TNB**	**see note at right**	Take from TNB		700µl x 12 slides = 8.4mLs TNB (plus 100µl extra) = 8.5mL
(1:500)	----------	10% Tween	In TNB already		
Add 1mL autoclaved water for a new kit	**17µl**	anti-digoxigenin-POD	Roche: 11207733910		Anti-DIG(1:500) = 8.5mL/500 = 17µl
Add antibody at end of first day	8.517mL	total volume			

0.7% H_2O_2	1.16	mL	H_2O_2	Sigma: H-0904		Stored at 4ºC, bring to RT before using.
Add at last moment	50	mL	MeOH	Fisher: A412-4		

DAY 2 SOLUTIONS

TSA-Cy3 (1:50)	62	µl	TSA-Cy3	TSA-Plus Cyanine 3/Fluorsecein system Perkin Elmer: NEL744		tyramide- biotin (1:50) = 3.1mL/50 = **62µl**
(TSA)	3.1	mL	TSA-Plus Cy3 buffer	***12 slides x 250µl = 3mL***		250µl/slide (12 slides) = 3mL + 100ul extra = **3.1mL**
Add 1.2mL DMSO to TB for a new kit	3.1	mL	total volume			

4% PFA	10	mL	5X PFA	Sigma P6148 (500g)		Aliquot 5X PFA into 5mL portions and freeze at -20C. To make just add PBS. Heat soln at 65ºC for 10 min for PFA to go into solution (if necessary).
	0.25	µl	10% Tween			
	40	mL	PBS			
	50	mL	total volume			

Starting the FISH run

1. Flush the instrument. This can be done by pressing the "flush instrument" button in the menu bar of the Gemini program, and clicking "OK" when the description box comes up. The robot should flush about 50 ml of system liquid.

2. Wipe the tips with 100% ethanol using a Kimwipe.

3. Make sure the two water baths are on.

4. Fill up the large system liquid water with ultra pure (Milli-Q) the ***night before a run, allow the liquid to degas naturally***. Do not add system liquid the same day unless the water is degassed, you do not want bubbles in the system liquid which may cause a run to fail.

5. Empty out the waste containers.

6. Open Evoware, select the script you want to run.

7. Press the start button and the script will begin and ask for the number of slides to be processed. Before entering the slides number and pressing enter, prepare the methanol peroxide solution and place into receptacle.

Fluorescent *In Situ*-Hybridization Steps

Prehybridization steps

"Washing" means adding the amount of solution specified in the protocol to all flow-through chambers. "Repeated washing" means multiple cycles of washing. By contrast, "incubation" means that added solutions were left in the chambers for the amount of time specified in the protocol below. All solutions were provided in suitable containers located on the platform.

1. Wash 5 times for 5 minutes with MeOH containing 0.7% hydrogen peroxide.
2. Wash 7 times with 300µL of PBS.
3. Incubate 2 times for 5 minutes of 0.2N HCl.
4. Wash 7 times with PBS.
5. Incubate 2 times for 15 minutes with pre-hybridization solution.
6. Incubate 1 time for 15 minutes while the temperature is raised to 64°C (no solution is delivered, only a timer is set within the script, this gives the incubator time to warm before probe hybridization).

Hybridization step

1. The robot automatically delivers the probe to the specified hybridization chambers. Incubate probe at 64°C for 5.5 hours. After 2.5 hours a second aliquot of probe is pippetted. Adding fresh probe halfway through the incubation increases the signal strength of weakly expressed genes.

Post hybridization stringency washes

Post hybridization washes are carried out at 62°C. Temperature reduction to room temperature is carried out while in cycle 1 of step 1 below:

Stringency wash solution is prewarmed for 1.5 hours (no longer) on the robot platform in a heated container so that they reach approximately 60°C. This is necessary to avoid degassing that would occur if room temperature solutions were added to a 62°C hybridization chamber.

1. Incubate 5 times for 5 minutes with 5x SSC.
2. Incubate 5 times for 10 minutes with 2x SSC with 50% formamide (Formamide I).
3. Incubate 5 times for 12 minutes with 1x SSC with 50% formamide (Formamide II).
4. Incubate 4 times for 8 minutes with 0.1x SSC.

Probe detection reactions

Temperature reduction to room temperature is carried out while in cycle 4 of step 4 in the above stringency washes. All post-hybridization steps are carried out at room temperature.

1. Wash 4 times for 5 minutes with NTE
2. Incubate 6 times for 5 minutes with 20mM iodoacetamide in NTE
3. Wash 4 times for 5 minutes with NTE
4. Wash 2 times for 5 minutes with TNT solution
5. Incubate 6 times for 5 minutes with 4% sheep-serum
6. Wash 4 times for 5 minutes with TNT solution
7. Incubate 2 times for 10 minutes with TNB blocking buffer (PerkinElmer Life sciences, FP1020)
8. Wash 2 times for 5 minutes with TNT solution
9. Wash 2 times for 5 minutes with maleate wash buffer (MWB)
10. Incubate 2 times for 10 minutes with 1% blocking reagent (Roche)
11. Wash 2 times for 5 minutes with MWB
12. Wash 2 times for 5 minutes with TNT solution
13. Incubate 3 times for 5 minutes with TMN solution
14. Wash 4 times for 5 minutes with TNT solution
15. Incubate 4 times for 10 minutes with TNB blocking buffer.
16. Incubate 2 times for 30 minutes with Anti-DIG-POD in TNB blocking buffer
17. Incubate 6 times for 5 minutes with TNT solution
18. Incubate 1 time for 30 minutes with tyramide-biotin diluted with amplification diluent's buffer (all in TSA Plus Cy3 kit, PerkinElmer Life sciences NEL744).
19. Wash 4 times for 5 minutes with TNT solution
20. Wash 2 times for 5 minutes with TMN solution.
21. Wash 3 times with system liquid water.
22. Wash once with NTE.
23. Incubate once for 10 minutes with 4% PFA
24. Wash 3 times with system liquid water

Disassembly of Hybridization chambers

1. Remove hybridization chambers from the robotic platform.

2. Use the clamp remover to remove clamps from assembled hybridization chambers. Place clamps into a small container to be rinsed.

3. Place unclamped hybridization chambers face-down into MilliQ water one at a time. Do not allow chambers with slides to set in water for too long. Place slide frames into a container to be rinsed later.

4. Carefully remove slides from water and back plates, let spacers float off and place them, in the metal slide racks. Take care not to scratch the tissue sections.

5. Rinse the slide racks briefly in ultra-pure (Milli-Q) water.

6. Allow the slides to dry in a fume hood at RT for ~10 minutes.

7. Coverslip with VectaShield Mounting Medium with Dapi (Vector Laboratories, Inc. cat# H-1200). (use only 1-3 drops per slide, depending on the number of sections per slide)

Cleaning of Hybridization chambers

1. Take back plates from the water and place them into containers with ethanol. Leave them overnight.
2. Take back plates out of ethanol and place them in stainless steel containers, and put them in a dishwasher to be cleaned.
3. Rinse slide holders and clamps twice in deionized water and leave them out to dry.
4. Remove stainless steel containers from dishwasher; place the containers in external stainless steel box and autoclave.

Shut-down

1. Empty out the waste containers.
2. Fill up the system liquid container.
3. Turn off the robot; water-baths can stay on stand-by.

GUDMAP – SOP "Fluorescent ISH – Freedom Evo" –
Gaido Lab - GenePaint System
The Hamner Institutes for Health Sciences – CIIT at the Hamner

Last update: March 19, 2007

For questions and comments, please contact:
gaido@thehamner.org *Kevin Gaido*
Updated By: Janan Hensley

Stock solutions

Depc H20: In fume hood, add 1mL DEPC to 1L of H20. Allow DEPC to dissolve for 4 hours or O/N with shaking at 4ºC. Autoclave on liquid cycle, allow DEPC H20 to equilibrate to RT before using.

10x PBS: For 1L add the following solids components:

Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g
KCl	2 g
NaCl	80 g

Add ultra-pure water to a volume of 800mL and stir until solid components dissolve. Adjust pH to 7.5, add ultra-pure water to a final volume of 1000mL. Autoclave and store in 1L bottle at RT .

1X PBS DEPC: Add 1 package of Sigma's powdered PBS (P3813-10Pak) to 1L of H20, dissolve by stirring. Proceed with directions for Depc H20 as stated above.

20% (5X) PFA: 1L (Note: PFA is extremely hazardous).
Add 1mL 2N NaOH to approximately 500 mL autoclaved water and heat the water to 65º C. Under the hood, dissolve 200 g paraformaldehyde in the water while heating and stirring. Add 100 mL 10x PBS and let the solution cool to room temperature. Adjust pH to 7.4 using approximately 1 mL 2M HCl. Adjust the volume to 1 L with H20. Aliquot out to 50mL or 10mL conicals. Store at -20º C for up to 6 months, or at 4º C for up to one week. Dilute to 4% (1x) for use. pH: 7.4.

4% PFA in PBS: 40mL of 5X PFA added to 160mL PBS DEPC H20. (acetylation)

Acetylation Buffer: 200mL DEPC H20, 2.66mL Triethanolamine, 350µl 37% HCl.

750µl (x2) is added when slides are incubating. (acetylation)

70% EtOH: Make 200mL each container for acetylation. Mix 140mL EtOH plus 60mL DEPC H20. Discard after 2 weeks.

80% EtOH: Make 200mL each container for aceylation. Mix 160mL EtOH plus 40mL DEPC H20. Discard after 2 weeks.

95% EtOH: Make 200mL each container for acetylation. Mix 190mL EtOH plus 10mL DEPC H20. Discard after 2 weeks.

100% EtOH: Make 200mL. Discard after 2 weeks.

20x SSC: Pre-made: Ambion cat# 9763 (1L)

OR for 4L add the following solid components:

Citric acid	325.8g
NaCl	701.2g

Add ultra pure water to a volume of 3600 mL and stir until solid components dissolve.
Adjust pH to 7.0, add H20 to a final volume of 4000mL. Autoclave and store in 1L bottles at room temperature.

5x NTE: For 1L add the following solids components:

NaCl	146.25g
Tris	6.05 g
EDTA	9.3 g

Add ultra-pure water to a volume of 800mL and stir until solid components dissolve. Adjust pH to 8 (fine tune the pH), and add ultra-pure water to a final volume of 1000mL. Autoclave and store in 1L bottle at RT .

10x TNT: For 1L add the following solids components:

Tris (Trizma base)	121.5 g
NaCl	87.95 g

Add ultra-pure water to a volume of 800mL and stir until solid components dissolve. Adjust pH to 7.5, add ultra-pure water to a final volume of 1000mL. Autoclave and store in 1L bottle at RT .

Sheep serum:
Heat the inactive sheep serum for 30 minutes at 55º C on a shaking water bath.

Allow the solution to cool and aliquot. Store at -20º C.

TNB: Add 0.25g of Perkin Elmer blocking powder to 50mL 1xTNT.

Heat it in 65ºC waterbath for about 1 hour. Needs to be degassed. Make sure it has cooled down to RT before adding primary antibody (do not cool rapidly or with ice).

Maleate Wash Buffer (1X MWB): For 1L add the following solids components:

NaCl	8.765 g
Maleic Acid	11.61 g
NaOH	8 g

Dissolve in 800m L ultra-pure water and adjust pH to 7.5. Add ultra-pure water to 1000mL. Store at 4°C. Discard after 1 week.

Blocking Reagent 1%: For 50mL add the following solids components:

0.5g of Roche blocking powder and fill up to 50mL with MWB.

Heat it in 65ºC waterbath for about 1 hour. Need to be filtered and degassed. Before adding secondary antibody make sure it is at RT.

TMN: Dissolve:
12.125 g Tris (Trizma Base)(0.1M)
5.85 g NaCl (0.1M) in 800mL of ultra-pure water,

Adjust the pH to 9.5.
Add 10.11g $MgCl_2(6H_2O)$ and readjust the pH to 9.5. Add ultra-pure water to 1 L.

Appendix: List of chemicals

Chemical	Catalog #	Amount
DEPC	Sigma D5758	50mL
PBS	Sigma P3813	10 pack (makes 10 – 1 Liters)
2M HCl	Fisher A144-212	2.5L
Tween 20	Fisher BP337	500mL
20X SSC	Ambion 9763 (ABI)	1L
Formamide *	Roche 11814320001	500 mL
Iodoacetamide **	Sigma I-1149	100 g
Sheep (lamb) serum *	Gibco 16070-096 or (Sigma S-2263 (100mL))	500 mL
Roche Blocking reagent	Roche 11096176001	50g
Levamisole *	Sigma L9756	10 g
tRNA (yeast)	Ambion 7119	0.5mL
heparin	Sigma H-3149	100K units (grade 1A from Porcine intestinal mucosa)
In situ Hybridization buffer *	Ambion B8807G	100 mL
Hydrogen peroxide (H_2O_2) *	Sigma 216763	500 mL
Methanol (MeOH) *	Fisher A412-4	4 L
TSA-Plus Cy3 (Cyanine 3 Tyramide) *	Perkin Elmer NEL744	50-150 slides
1X Plus amplification diluent *	Perkin Elmer NEL744	15mL (included in TSA-Plus Cy3 kit)
Paraformaldehyde * *	EMS 19208	0.5 kg
Triethanolamine	Fluka Biochemika 90279	500mL
HCl (concentrated)	Sigma 258148-500mL	500mL
Acetic anhydride	Sigma A6404-200mL	200mL
Anti-digoxigenin-POD *	Roche 11207733910	1 mL
Potassium chloride (KCl) *	Sigma P4504	500 g
Sodium phosphate (Na_2HPO_4) *	Sigma S0876	500 g
Potassium phosphate (KH_2PO_4) *	Sigma P5379	100 g
Sodium citrate (citric acid) *	Sigma C7254	1 kg

Tris (Trizma) *	Sigma T1503	1 kg
Blocking reagent [TNB] *	NEN FP1012	10 g
Maleic acid *	Sigma M0375	500 g
Magnesium chloride ($MgCl_2$*$6H_2O$) *	Sigma M0250	500 g
Sodium hydroxide (NaOH) *	Sigma S5881	1 kg
EDTA *	Sigma E4884	500 g
Diethylpyrocarbonate (DMSO)*	Sigma D8418	50mL
Sodium Chloride (NaCl)	Sigma S3014	500g
DTT – DL-Dithiothreitol	Sigma 43815	1g
OTHER SUPPLIES		
Superfrost Plus slides	Fisher 12-550-15	
Corning cover glass No. 1	Corning 2935-224	10pks/case
VectaShield Mounting medium for Fluorescence with Dapi	H-1200	10mL

*Handle all chemicals with care.

Solution descriptions

***In situ* Hybridization buffer** (Ambion B8807G, ABI)

SSC (0.3M NaCl 0.03M sodium citrate trisodium citrate dehydrate pH 7.0).

NTE (5mM EDTA, 10mM Tris, 500mM NaCl, pH 7.6)

TNT solution (100mM Tris 150mM NaCl, pH 7.6)

TNB blocking buffer (0.5% blocking reagent in TN (PerkinElmer Lifesciences, FP1020) pH 7.6).

MWB (100mM maleate, 150mM NaCl, pH 7.5)

Blocking reagent 1% (1% BR) (Roche 110961760001) containing MWB

TMN solution (0.1M Tris, 0.05M $MgCl_2$, 0.1M NaCl, pH 9.5)

Summary of Tecan script

cycles	T (min)	volume	Reagent	l. class	temperature
5	5 min.	300	H2O2 in MeOH	ethanol	24º C
7	5 min.	300	PBS (1)	water	24º C
2	5 min.	300	0.2M HCl	water	24º C
7	5 min.	300	PBS (3)	water	24º C
2	15min	300	hyb-mix (1)	hyb-mix	24º C
1	15 min.		Heat up to 64º C	hyb-mix	64º C
1	330 min	300	probe hybridization	probe	64º C
5	5 min.	300	5 x SSC	water o/n	62º C
5	10 min.	350	formamide I	water o/n	62º C
5	12 min.	350	formamide II	water o/n	62º C
3	8 min.	300	0.1 x SSC (1)	water o/n	62º C
1	8 min.	300	0.1 x SSC (2)	water o/n	24º C
4	5 min.	300	NTE (1)	water o/n	24º C
6	5 min.	300	20 mM iodoacetamide (1)	water o/n	24º C
4	5 min.	300	NTE (2)	water o/n	24º C
2	5 min.	300	TNT (1)	water o/n	24º C
6	5 min	300	4% sheep serum (1)	water o/n	24º C
4	5 min.	200	TNT (2)	water o/n	24º C
2	10 min.	300	TNB blocking buffer	water o/n	24º C
2	5 min.	200	TNT (3)	water o/n	24º C
2	5 min.	300	maleate wash buffer (1)	water o/n	24º C
2	10 min.	350	blocking reagent	water o/n	24º C
2	5 min.	300	maleate wash buffer (2)	water o/n	24º C
2	5 min.	250	TNT (4)	water o/n	24º C
3	5 min.	350	TMN	water o/n	24º C
4	5 min.	200	TNT (2)	water o/n	24º C
4	10 min.	300	TNB blocking buffer	water o/n	24º C
2	30 min	350	antiDIG	water o/n	24º C
6	5 min.	200	TNT (3)	water o/n	24º C
1	25 min	250	TSA-Cy3	-	24º C
4	5 min.	250	TNT (4)	water	24º C
2	5 min.	400	TMN	water	24º C
3	x	400	System liquid (1)	System liquid	24º C
1	x	300	NTE (3)	water	24º C
1	10 min.	200	4% PFA (2)	water	24º C
3	x	400	System liquid (2)	System liquid	24º C

Platform layout

Left front Left rear

PreHyb	HCl	MeOH/PFA
TMN		Serum
NTE	TNB	TNT (extra)
MWB	BR	IDO

PBS
TNT

	Anti-DIG	TSA													

5 x SSC	Formamide I
0.1 x SSC	Formamide II

Right Front Right rear