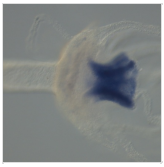

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Cassiopea In Situ Hybridization

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1 Works for me dx.doi.org/10.17504/protocols.io.biz4kf8w

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ABSTRACT

This is a protocol for *in situ* hybridization using Digoxigenin-labeled RNA probes to genes of interest. A separate protocol describes the design and synthesis of those probes. The purpose of this protocol is to visualize the spatial expression of genes.

This protocol was written up by Bailey M. Steinworth and is based on the following publications:

Wolenski FS, Layden MJ, Martindale MQ, Gilmore TD, Finnerty JR. 2013. Characterizing the spatiotemporal expression of RNAs and proteins in the starlet sea anemone, Nematostella vectensis. Nat. Protoc. 8:900–915.

Sinigaglia C, Thiel D, Hejnol A, Houliston E, Leclère L. 2018. A safer, urea-based in situ hybridization method improves detection of gene expression in diverse animal species. Dev. Biol. 434:15–23.

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Preparation – part I
Make stock solutions (or ensure you have an adequate volume already):
500 ml Maleic Acid Buffer
1 L DEPC-treated 1X PTw + 1L DEPC-treated 1X PTx
500 ml 20X SSC (pH 7.0; from manufacturer)
Prepare an aliquot of PTw for fixation by adding drops of NaOH until pH=8.8

*Use **RNAse-free equipment and fresh solutions for all Day 1 procedures** (through hybridization).

"Quick washes" take just long enough for embryos to settle. "Long washes" are 5 min at RT on rocker table.

-DAY 1-

Preparation
Make fresh & keep all solutions on ice:
4% paraformaldehyde (dilute 16% PFA 1:4) + 0.3% glutaraldehyde in PTw at pH=8.8 – 1 ml per sample
4% paraformaldehyde in PTw at pH=8.8
1% Triethanolamine (100ul in 10ml PTw) – 2ml per sample
1% Triethanolamine + 3ul/ml acetic anhydride – 1ml per sample
1% Triethanolamine + 6ul/ml acetic anhydride – 1ml per sample

Fixation
1.Immobilize animals in 7% MgCl2 in filtered sea water (FSW).
2.Transfer animals to 2 mL screw-cap tube and remove excess liquid.
3.Quickly add 1 mL of the 4% PFA with glutaraldehyde (made with PTw at pH=8.8) & incubate 1 minute 30 seconds.
4.Remove fixative and add 1 mL of 4% PFA **without** glutaraldehyde (made with PTw at pH=8.8). Incubate at 4°C for 1 hour with rocking.
5.Remove fixative do 2 quick washes and 1 long wash in PTw (pH=7.4; all PTw from this point on will be pH=7.4).
6.Do 1 quick wash and 1 long wash in PTw.
7.Transfer to 100% methanol and store at -20°C at least overnight (can be stored for several months).

Pretreatment
8.Rehydrate tissue:
a.Remove 250 ul methanol, replace with PTw.
b.Remove 500 ul, replace with 100% PTw.
c.Remove all liquid, do 2 quick washes and 1 short wash in PTw.
d.Do 1 quick wash and 1 short was in PTw.
9.Remove PTw, do:
a.2 long washes in 1% Triethanolamine
b.1 long wash in 1% Triethanolamine + 3ul/ml acetic anhydride
c.1 long wash in 1% Triethanolamine + 6ul/ml acetic anhydride
10.Remove final triethanolamine wash, do 2 quick washes in PTw and 1 long wash in PTw.

Prehybridization
11.Remove as much liquid as possible without allowing embryos to dry out (remember to collect all hybe buffer in the **ureawaste container**). Add 1 ml hybe buffer **with salmon sperm** (per well) - incubate for 10 minutes at RT on the rocker.

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12.Remove hybe buffer (collect in urea waste), add a new 1 ml of hybe buffer and place the plate in incubator at hybe temp at least 1 h.

Hybridization

13.Turn on heat block to 90˚C. Dilute probe stock (see Riboprobe Synthesis protocol) to a final concentration of 0.05 - 10 ng/ml (usually **1.0 ng/ml**) in hybe buffer (with salmon sperm DNA/tRNA) in a 2ml screw cap tube. (DIG- or FL-labeled probes should be stored as **100 ng/ul** stock in hybe buffer at -20˚.) Denature probe at 80-90°C for a maximum of 10 minutes; you will need to do this each time you use the probe. Remove old hybe buffer from each well (discard in urea waste) and add probe (~300-500ul per well). Return plate to incubator and hybridize overnight. Seal the plate with tape and place into a Tupperware with a damp paper towel to prevent evaporation. (Save empty probe tubes in the freezer.)

-DAY 2-

14.Warm hybe buffer (no salmon sperm DNA) and 2X SSC (pH 7.0) stock to hybe temp; make 40ml stocks of the hybe/2X SSC dilutions as indicated below and keep them all at hybe temperature. Warm 0.02X SSC stock to hybe temp.
15.Remove probe from wells and replace in freezer tube (each probe can be reused at least 4-5 times). Add 500ul of warm hybe buffer to each well and replace the plate in the hybe oven for 10 mins. Repeat the previous step but incubate for 40 mins. While your plate is incubating, make 40ml stocks of 0.02X SSC/PTw solutions (below) and store at RT.

Washes

16.Remove hybe buffer and replace with the following (use ~500ul per well per wash):
30 min in 75% hybe/25% 2X SSC at hybe temp
30 min in 50% hybe/50% 2X SSC at hybe temp
30 min in 25% hybe/75% 2X SSC at hybe temp (return all hybe/2X SSC solutions to the freezer after use)
30 min in 100% 2X SSC (pH 7.0) at hybe temp (store 2X SSC at RT after use)

- no need to collect solutions in urea waste after this step–

2 x 20 min in 0.02X SSC at hybe temp (leave the 0.02X SSC out of the hybe oven after second wash)
1 x 20 min in 0.02X SSC at RT on rocker (store 0.02X SSC at RT after use)
10 min in 75% 0.02X SSC/25% PTw at RT on rocker
10 min in 50% 0.02X SSC/50% PTw at RT on rocker
10 min in 25% 0.02X SSC/75% PTw at RT on rocker
10 min in 100% PTw at RT on rocker

Visualization of Probe

17.Wash 5 x 10 min with PBT at RT on rocker. While in the last wash, make enough blocking buffer for the next two steps. **If doing fluorescent ISH, switch to fluorescent detection protocol after this step.**
18.Block in Roche (Boehringer-Mannheim) Blocking buffer (diluted to 1X with maleic acid buffer) for (at least) 1 hr at RT on rocker (or overnight at 4°C). While blocking, dilute antibody **(anti-DIG AP, Fab fragments**, if doing basic NBT/BCIP reaction) 1:5000 in blocking buffer (e.g., 1.5ul antibody in 7000ul blocking buffer) and place on rocker at 4˚C for at least 1 h prior to use.
19.Remove old blocking buffer from wells and replace with buffer + antibody and incubate at 4°C overnight on rocker. (Can also incubate 1-4 hrs at room temp.)

-DAY3-

20.Remove antibody (no need to collect/reuse), do 2 quick and 1 slow (**20 mins**) washes in 500ul PTx
21.Repeat previous step a total of five times; make AP buffer – with and without $MgCl_2$.
22.Wash 1x for 10 minutes in 500ul AP Buffer **without $MgCl_2$**
23.Wash 2x for 10 minutes in 500ul AP buffer with $MgCl_2$. While in final wash, make AP substrate solution.
24.Develop in 300ul AP substrate solution at RT or 4˚C. Wrap your plate in foil or place it in a box with a cover to keep it dark. Monitor color development by checking embryos under dissecting scope every 30 mins. This process may take several hours or several days; you may want to develop at RT during the day and at 4˚C overnight. **NOTE**: you will need to replace the developing solution several times (approx. every 30 mins) during the first day of development and once/day for the remaining days of development. Always replace with fresh developing solution before the old developing solution starts to turn orange/purple.

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Stopping the Reaction

25.Because each probe will react with embryos at a different rate, you may need to stop some wells before others. To stop some wells, remove AP solution and wash 5X with PTw. These wells will be fine while the rest keep developing. If the PTw becomes purple while waiting for the rest of the wells to finish, just replace with new PTw.
26.When the last wells are finished, you will need to fully stop and clear the plate. Warm the hybe buffer (no sperm/tRNA) to 63˚C. Stop the AP reaction by removing the AP substrate solution from the remaining wells and washing embryos 5X with 500ul PTw at RT.
27.Remove PTw and wash for an additional 30 min (max 4h) in 500ul hybe buffer at hybe temp.
28.Remove 250ul of hybe buffer, replace with PTw at RT (remember to collect hybe waste).
29.Remove all hybe/PTw and replace with PTw. Repeat until wells look clear of hybe buffer.
30.Remove PTw from wells and replace with 500ul of 80% glycerol (1ml 10X PBS + 9ml glycerol). Place plate on rocker at RT to swirl all embryos into the center. Move plate to 4˚C rocker overnight if they are still dispersed. (DO NOT use less than 500ul or embryos will not swirl toward center.)
31.Mount in 80% glycerol on glass slides and image using the Zeiss M2 microscope.

Notes/FAQs

·Hybridization temperature can range from 55 to 65˚C, depending on probe and stringency needed.Start with 63°C and adjust if necessary.
·To reduce the stringency of washes, use a higher salt concentration than 0.05x SSC (such as 0.2x SSC), which can speed up development time but may cause an increase in background.
·Developing is a key step, be patient and refresh substrate solution before it turns purple.Some probes come up in minutes (in which case you should try to develop it slower at 4°C to achieve the best signal to noise) while others may take up to several months.
·For best results, do prehybridization overnight and hybridization for 48h or more.
·Do all washes/etc in well plate on a tilted surface to prevent loss of embryos. Do not let embryos dry out while you're removing your wash solution.
·Ideal glycine concentration can vary from 0.2-0.4% (0.04 – 0.08g in 20 ml ok)
·Make 2X SSC by diluting manufacturers 20% SSC stock (pH 7.0) in DI water. Make 0.02X SSC by diluting 2X SSC in DI water.
·Before using salmon sperm, boil it for 5-10mins at 90˚ in heat block; do not boil tRNA before use. Allow it to come to RT (~5mins) before use.
·The SDS in the hybe buffer will precipitate out of solution at -20˚C, this is ok. Hybe buffer can be frozen and thawed multiple times without loss of function. Make sure the hybe buffer has come up to 63°C and is fully mixed/homogeneous before use!
·Crystals in AP substrate solution are bad. To prevent formation of crystals, always use a new tube and a clean tip to make your solution and be sure to wash embryos adequately (contaminants in wells will seed crystal formation). Change your AP substrate solution every 30 mins for the first day of development to further prevent the formation of crystals.
·It is critical to maintain stringent RNAse-free techniques for Day I of this protocol (to preserve the RNA quality). Use filter tips and DEPC-treated solutions for Day I. Once tissues go into the hybridization step they are no longer susceptible to RNA degradation so no need to use filter tips or DEPC-treated solutions after Day I.
·Embryos may start to float as you go into the SSC solutions (Day II). Use extra care to avoid removing them during washes.
·If tissues appear to be developing too quickly and you are concerned that they may over-develop overnightyou can replace the AP substrate solution with AP buffer with MgCl2 (no NBT/BCIP) and leave them in the fridge overnight. They will still develop but only at an extremely slow rate.
·Stopping your wells with PTw is technically reversible, but not recommended. If you stop your wells in PTw and need to re-start the development process, simply start over with AP buffer (no MgCl2) and go through the protocol as before (AP buffer with MgCl2, then AP substrate solution).
·TEA is extremely viscous. Use a filter tip when pipetting out of the TEA aliquot. Never pipette directly out of the TEA bottle.
·Proteinase K is an endopeptidase that non-specifically breaks down peptide bonds and is thus useful for removing any proteins that may inhibit binding of your probe to your endogenous RNA sequence. It is also useful for inactivating DNAses and RNAses in your sample. Every new aliquot of proteinase K should be tested for efficiency; the digestion times in the table on page 1 might need to be adjusted for each new aliquot.
·1% TEA has a pH of 10.0 and is very effective in neutralizing acidic residues and solubilizing/emulsifying fatty acids and other non-water soluble molecules.
·Acetylation of any positively charged overhangs with acetic anhydride (in TEA) is an important step for reducing background.
·SSC (saline sodium citrate) buffer is used in decreasing concentrations (20X in hybe buffer, 2X in the first set of washes, 0.02X in the second set of washes) to increase the stringency of your post-hybridization washes. High temperature and low salt concentration are more stringent conditions than low temperature and high salt and will be most effective at reducing non-specific hybridizations. If your probe and target are 100% identical, use the highest stringency protocol possible.

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Solutions

Hybe buffer			final concentration
Urea	9.6 g	2.4 g	4 M
20X SSC (pH 4.5)	10 ml	2.5 ml	5X
20 mg/ml heparin	0.1 ml = 100 ul	25 ul	50ug/ml
100% Tween-20	0.1 ml	25 ul	0.10%
20% SDS	2.0 ml	0.5 ml = 500 ul	1.00%
[10 mg/ml salmon sperm DNA]	**[0.2 ml]**	**0.05 ml = 50 ul**	**[100ug/ml]**
[10 mg/ml tRNA] - optional	**[0.2 ml]**	**0.05 ml = 50 ul**	**[100ug/ml]**
DEPC H2O	Fill to 40 ml	Fill to 10 ml	

DEPC H_2O To 1L of dH_2O in an autoclavable bottle, add 1ml DEPC. Close lid tightly, wrap in parafilm, and shake vigorously for 30s.

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Incubate at RT for 1h. Remove parafilm and loosen lid before autoclaving. Autoclave on the L45 cycle. From Ambion website: "Autoclaving does inactivate DEPC by causing hydrolysis of diethylpyrocarbonate. CO2 and EtOH are released as reaction by-products. DEPC has a half-life of approximately 30 minutes in water, and at a DEPC concentration of 0.1%, solutions autoclaved for 15 minutes/liter can be assumed to be DEPC-free."

10x PBS18.6 mM NaH_2PO_4(2.56 g NaH_2PO_4-H_2O)
84.1 mM Na_2HPO_4(11.94 g Na_2HPO_4-H_2O)
1,750 mM NaCl(102.2 g NaCl)
Mix phosphates in about 800 mL of dH_2O in a 1L autoclavable bottle.Check pH. It should be 7.4 ± 0.4. If more than 0.4 off, start over. Otherwise adjust pH to 7.4 with NaOH or HCl. Add the NaCl, DEPC treat, and autoclave.

PTw1x PBS + 0.1% Tween-20 detergent
(100 ml 10x PBS, 900 ml dH_2O; DEPC treat and autoclave. When cool, add 1 ml RNAse-free 100% Tween.)

PTx1x PBS + 0.1% Triton-X detergent
(100 ml 10x PBS, 900 ml dH_2O; DEPC treat and autoclave. When cool, add 1 ml RNAse-free 100% Triton-X.)

PBT1x PBS + 0.1% Triton X + 0.1% BSA (store at 4˚C)
(100 ml 10x PBS, 900 ml dH_2O; no need to DEPC treat. Add 1 ml 100% Triton X-100 and 1g BSA. Filter sterilize and store at 4˚C.)
HeparinMake 20 ml stock (400mg heparin in 20ml DEPC-treated water), filter sterilize using a syringe filter, aliquot and store at 4°C. Do not autoclave.

20X SSC 0.3 M Na citrate + 3 M NaCl
(pH 4.5)(To 800ml dH_2O add 175.3 g NaCl and 88.2 g Na citrate; **pH to 4.5** and autoclave.)

Maleic Acid To 400ml dH_2O add 6.905 g maleic acid and 4.38 g NaCl (or 75mls of 1M NaCl); pH to 6.5 with NaOH pellets, then **Buffer** to 7.4 with 5M NaOH, then to 7.5 with 1M NaOH. Autoclave.

Roche Blocking Make 10X stock (from powder) in maleic acid buffer. Autoclave and store at -20 (in 15ml Falcon tubes). Working **Buffer**solution: thaw one 10X aliquot and retain at 4˚C; for day of use, dilute to 1X in maleic acid buffer.

Alkaline Phosphatase buffer Make two aliquots: one with $MgCl_2$, one without. Prepare just prior to use.

		2ml	5ml	10ml	12ml	50ml	[FINAL]
2M	**NaCl**	0.1	0.25	0.5	0.6	**2.5 ml**	100 mM
1M	**MgCl2**	0.1	0.25	0.5	0.6	**2.5 ml**	50 mM
1 M	**Tris, pH 9.5**	0.2	0.5	1.0	1.2	**5.0 ml**	100 mM
100%	**Tween**	0.01	0.025	0.05	0.06	**0.25 ml**	0.5%
	DI water	Fill to 2 ml	Fill to 5 ml	Fill to 10 ml	Fill to 12 ml	**Fill to 50ml**	

AP Substrate Solution
To AP buffer (with $MgCl_2$), add 3.3 ml/ml NBT (stock: 50 mg/ml in 70% dimethyl formamide:30% water) and then 3.3 ml/ml BCIP (stock: 50 mg/ml in dimethyl formamide; stocks are stored at -20˚ in non frost-free freezer). Keep this solution dark and make fresh every time. Use in 300ul aliquots to conserve NBT and BCIP.

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