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RNA in situ hybridization

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ABSTRACT

This protocol provides details on tissue harvest and fixation, RNA probe generation, and RNA *in situ* hybridization for use in sectioned axolotl tissue. This protocol was modified from Brent et al, A somitic compartment of tendon progenitors, Cell 2003

GUIDELINES

All tissue harvest, probe generation, and day 1 of RNA *in situ* hybridization should be performed using RNase-free reagents and tools.

MATERIALS

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- OneTaq DNA Polymerase - 1,000 units **New England Biolabs Catalog #M0480L**
- RNeasy Mini Kit **Qiagen Catalog #74104**
- RNase Zap **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R2020-250ML**
- Deoxynucleotide Solution Set - 25 umol of each **New England Biolabs Catalog #N0446S**
- Formamide, deionized **American Bio Catalog #AB00600-00500**
- BCIP **Sigma – Aldrich Catalog #B6149**
- Lamb Serum, New Zealand origin **Thermo Fisher Scientific Catalog #16070096**
- T7 RNA Polymerase **Merck MilliporeSigma (Sigma-Aldrich) Catalog #10881767001**
- SP6 RNA Polymerase **Merck MilliporeSigma (Sigma-Aldrich) Catalog #10810274001**
- DIG RNA Labeling Mix **Merck MilliporeSigma (Sigma-Aldrich) Catalog #11277073910**
- Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #3335402001**
- pGEM®-T Easy Vector Systems **Promega Catalog #A1360**
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**

STEP MATERIALS

- QIAquick Gel Extraction Kit **Qiagen Catalog #28704**
- RNase Zap **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R2020-250ML**
- RNeasy Mini Kit **Qiagen Catalog #74104**
- RNase-Free DNase Set **Qiagen Catalog #79254**
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**
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- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**
- Tissue-Tek® O.C.T. Compound, Sakura® Finetek **VWR International Catalog #25608-930**
- RNase Zap **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R2020-250ML**
- Tissue-Tek® O.C.T. Compound, Sakura® Finetek **VWR International Catalog #25608-930**
- Superfrost Plus Microscope Slides **Thermo Fisher Scientific Catalog #4951PLUS4**
- Proteinase K, recombinant, PCR grade **Merck MilliporeSigma (Sigma-Aldrich) Catalog #3115836001**
- Acetic anhydride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #320102-1L**
- Triethanolamine hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T1502**
- Formamide, deionized **American Bio Catalog #AB00600-00500**
- Bel-Art™ Polyethylene Utility Bags **Fisher Scientific Catalog #22-260173**
- Texwipe™ KOGO™ TPR Cleanroom Adhesive Tapes **Fisher Scientific Catalog #18-366-432**
- Plastic staining dish **VWR International Catalog #25608-904**
- RNase A **Merck MilliporeSigma (Sigma-Aldrich) Catalog #10109142001**

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☒	Lamb Serum, New Zealand origin	Thermo Fisher Scientific Catalog #16070096
☒	Blocking Reagent	Merck MilliporeSigma (Sigma-Aldrich) Catalog #11096176001
☒	Anti-Digoxigenin-AP, Fab fragments	Merck MilliporeSigma (Sigma-Aldrich) Catalog #11093274910
☒	5-Bromo-4-chloro-3-indolyl phosphate disodium salt	Merck MilliporeSigma (Sigma-Aldrich) Catalog #B6149
☒	Nitrotetrazolium Blue chloride	Merck MilliporeSigma (Sigma-Aldrich) Catalog #N6639
☒	Polyvinyl alcohol mounting medium with DABCO®, antifading	Merck MilliporeSigma (Sigma-Aldrich) Catalog #10981
☒	Thermo Scientific™ Richard-Allan Scientific™ Cover Glass	Thermo Fisher Scientific Catalog #102460
☒	Depc (Diethyl Pyrocarbonate)	VWR International Catalog #80730-888
☒	Lamb Serum, New Zealand origin	Thermo Fisher Scientific Catalog #16070096
☒	Dextran Sulfate, 50% Solution	American Bio Catalog #AB00426-00200
☒	Yeast tRNA	Thermo Fisher Scientific Catalog #15401029
☒	Formamide, deionized	American Bio Catalog #AB00600-00500
☒	Ficoll® 400	Merck MilliporeSigma (Sigma-Aldrich) Catalog #F2637
☒	Polyvinylpyrrolidone	Merck MilliporeSigma (Sigma-Aldrich) Catalog #PVP40
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- ☒ SP6 RNA Polymerase **Merck MilliporeSigma (Sigma-Aldrich) Catalog #10810274001**
- ☒ DIG RNA Labeling Mix **Merck MilliporeSigma (Sigma-Aldrich) Catalog #11277073910**
- ☒ Tricaine methanesulfonate MS222 **Merck MilliporeSigma (Sigma-Aldrich)**

I. Tissue Preparation and Sectioning

- 1 Narcotize animals in 0.1% Tricaine prepared in 40% Holtfreter's. Remove tissue of interest. Deposit directly into 4% PFA prepared in 1X DEPC PBS.

Note

All solutions and tools/bottles should be RNase-free, being prepared with DEPC-treated water or being cleaned with RNase Zap, respectively.

After survival surgeries always keep animals overnight in 40% Holtfreter's supplemented with 0.5% sulfamerazine.

- ☒ Tricaine methanesulfonate MS222 **Merck MilliporeSigma (Sigma-Aldrich)**

- 2 Fix tissues in 4% PFA prepared in 1X DEPC PBS for 1-2 hr at room temperature with gentle rocking or overnight at 4°C with gentle rocking.

- 3 Remove 4% PFA/1X DEPC PBS and wash in 1X DEPC PBS by gently rocking tissue in 1X DEPC PBS for 30 min.

- 4 Transfer tissue through a sucrose series, starting with 5% sucrose in 1X DEPC PBS, then 10%, 20%, and eventually 30%. Change to higher percent sucrose when tissue sinks.

Note

Once tissue sinks in 30% sucrose in 1X DEPC PBS proceed to the next step. Alternatively, tissue can be rocked overnight in 30% at 4°C.

- 5 Add approximately equal volume of OCT compound to the 30% sucrose/1X DEPC PBS. Use a gentle inversion rocker to let it equilibrate for at least 30 minutes.

- ☒ Tissue-Tek® O.C.T. Compound, Sakura® Finetek **VWR International Catalog #25608-930**

- 6 Prepare a dry-ice/ethanol "bath" and RNase Zap forceps.

- ☒ RNase Zap **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R2020-250ML**

- 7 Remove tissue from 30% sucrose/OCT solution and move into tissue mold. Fill mold with OCT and orient tissue as desired.

- ☒ Tissue-Tek® O.C.T. Compound, Sakura® Finetek **VWR International Catalog #25608-930**

- 8 Place mold with tissue into dry ice/ethanol bath to freeze OCT.

- 9 After freezing, move tissue into plastic bags and then store at -80°C until sectioning

- 10 Section tissue on a cryostat. Sections can be 10-20 microns (usually 16 microns). Collect sections on Superfrost Plus slides.

Superfrost Plus Microscope Slies **Thermo Fisher Scientific Catalog #4951PLUS4**

- 11 Air-dry the sections 30 min-2 hrs. Store at -80°C or continue on to hybridization.

II. Probe Preparation

- 12 Perform PCR using OneTaq and with T7 and Sp6 primers using pGEM-T easy with probe inserted as template. Do 5+ reactions to ensure ample PCR product. Annealing temperature 40.5°C, extension time time appropriate for length of probe (typically around 30 seconds).

Reagent	Volume (μL)
5x OneTaq Stand Buffer	10
10mM dNTP	1
10μM T7 primer (TAA TACG ACTC ACTA TAGG G)	1
10μM Sp6 primer (ATT TAGG TGAC ACTA TAG)	1
OneTaq	0.25
2ng/μL pGEM-T Easy w/ probe inserted	1
Ultrapure H ₂ O	35.75
Reaction Volume	50

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**

Deoxynucleotide Solution Set - 25 μmol of each **New England Biolabs Catalog #N0446S**

☒ OneTaq DNA Polymerase - 1,000 units **New England Biolabs Catalog #M0480L**

- 13 Gel extract PCR product and calculate concentration of gel extracted product.

☒ QIAquick Gel Extraction Kit **Qiagen Catalog #28704**

- 14 Perform in vitro transcription (3 reactions) with either T7 or Sp6 polymerase depending on anti-sense orientation of probe in pGEM-T Easy. Prepare below reaction and incubate at 37°C for 2 hours.

Reagent	Volume (μL)
10x Transcription Buffer	2
10x DIG	2
Protector RNase Inhibitor	0.5
Sp6 or T7 RNA polymerase	1
template (500ng)	variable
Ultrapure H ₂ O	to 20μL

☒ Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #3335402001**

☒ T7 RNA Polymerase **Merck MilliporeSigma (Sigma-Aldrich) Catalog #10881767001**

☒ SP6 RNA Polymerase **Merck MilliporeSigma (Sigma-Aldrich) Catalog #10810274001**

☒ DIG RNA Labeling Mix **Merck MilliporeSigma (Sigma-Aldrich) Catalog #11277073910**

🕒 02:00:00 incubation

🔥 37 °C incubation

Note

Use RNase-free reagents for all steps and thoroughly RNase Zap all surfaces and pipettes for this step and all subsequent steps of probe preparation.

- 15 Prepare a gel mold and electrophoresis box by spraying thoroughly with RNase Zap followed by washing out with MilliQ water. In addition, RNase Zap and rinse with MilliQ the interior of a bottle and prepare a 2% agarose gel in this RNase-free bottle.

☒ RNase Zap **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R2020-250ML**

- 16 After 2 hour incubation, proceed to RNeasy RNA cleanup with on column DNase I digestion.

☒ RNeasy Mini Kit **Qiagen Catalog #74104**

☒ RNase-Free DNase Set **Qiagen Catalog #79254**

- 17 Combine all three reactions and volume up to 100μL with Ultrapure H₂O.

☒ UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**

- 18 Add 350μL RLT buffer and mix well.

- 19 Add 250μL 100% ethanol and mix well by pipetting. Proceeded immediately to the next step.

- 20 Transfer 700µL to a RNeasy Mini spin column that is in a 2mL collection tube, close lid and centrifuge for 15s at **10,000 rpm** (8,000g). Discard flow through.
- 21 Add 350µL Buffer RW1, close lid, and centrifuge for 15s at 10,000 rpm.
- 22 Combine 10µL DNase I stock solution and 70µL Buffer RDD and mix gently by inverting the tube.
- 23 Add 80µL of DNase I/Buffer RDD solution directly to the RNeasy column membrane, close lid, and let it sit on the bench for 15min at room temperature.
00:15:00 incubation
- 24 Add 350µL Buffer RW1 to column, close lid, centrifuge for 15s at 10,000 rpm. Discard flow-through.
- 25 Add 500µL Buffer RPE to column, close lid, and centrifuge for 15s at 10,000 rpm. Discard flow-through.
- 26 Add 500µL Buffer RPE to column, close lid, and centrifuge for **2 min** at 10,000 rpm. Discard flow-through.
00:02:00 centrifugation
- 27 Place RNeasy column into a new 2mL collection tube, close lid, and centrifuge at max speed for 1 min.
- 28 Place RNeasy column into a 1.5mL collection tube (supplied with kit) and add 50µL of Ultrapure H₂O directly to the column membrane. Close the lid and centrifuge for 1 min at 10,000 rpm.
UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**
- 29 Remove eluate from collection and re-apply to the column for a second wash.
- 30 Remove 2ul of eluate and put into a new 1.5mL tube. Store the remainder of the eluate at -80°C until use.
- 31 Add 8µL of Ultrapure H₂O and incubate at 60°C for 10 minutes.
00:10:00 incubation

60 °C incubation

UltraPure™ DNase/RNase-Free Distilled Water Thermo Fisher Scientific Catalog #10977023

- 32 Add a clear loading dye (e.g. 40% sucrose) and run on the 2% RNase-free agarose gel to confirm size and quality of product.

III. Hybridization

- 33 Prepare required buffers. See note for recipes.

Depc (Diethyl Pyrocarbonate) VWR International Catalog #80730-888

Lamb Serum, New Zealand origin Thermo Fisher Scientific Catalog #16070096

Note

SOLUTIONS

DEPC water

In a 4L Erlenmeyer flask, put 3L deionized water in, add 3 mL DEPC, stir overnight (in the hood, with a little foil cap loosely on top). Autoclave the next day. Two 3L flasks should be enough for an in situ.

4% PFA in DEPC PBS

Make a 5X stock = 20% PFA in 5X DEPC PBS. Freeze in aliquots. When defrosting, some PFA/salts might come out of solution. Defrost in a warm bath and shake once in a while. Dilute 1:5 in a Falcon tube to give the correct concentration. Can be repeatedly freeze-thawed.

10X PBS (1 L)

80g NaCl
2g KCl
14.4 g Na₂PO₄
2.4 g KH₂PO₄

adjust to pH 7.4 with HCl and bring volume to 1 L with water

PBT

1X PBS +0.1% Tween-20

Hybridization Solution

10 mM Tris pH 7.5
600 mM NaCl
1 mM EDTA
0.25% SDS
10% Dextran Sulfate
1x Denhardt's (recipe follows)
200 mg/mL yeast tRNA
50% formamide

**Dissolve Tris and EDTA into DEPC-treated water after DEPC has been autoclaved. Adding Tris and EDTA prior to autoclaving will interfere with the ability of DEPC to eliminate RNases.

store at -20°C (in aliquots)

100X Denhardt's solution (100 mL)

2g BSA
2g Ficoll (MW 400,000)
2g Polyvinylpyrrolidone (MW 40,000)
15mL 20X SSC (3x final conc.)

volume to 100 mL

20X SSC (1L)

175.4g NaCl

88.2g Sodium citrate dihydrate

pH to 7.0, volume, autoclave

10X TEA (triethanolamine HCL)

1M stock pH 8.0

TNE

10mM Tris pH 7.5

500mM NaCl

1mM EDTA

5X MABT (1L)

58g Maleic acid

43.8 NaCl

volume up to 1L

pH to 7.5 with NaOH

filter sterilize

when ready to use, dilute 1:5 to make 1X MAB, then add Tween to equal 0.1% Tween

NTM pH 9.5

100mM NaCl

100mM Tris pH 9.5

50mM MgCl₂

NBT stock

50mg/mL in 70% dimethylformamide (store at -20°C, dark)

BCIP stock

25mg/mL in water (store at -20°C, dark)

HISS

Sheep serum, heat inactivated at 56°C for 30 min, store at -20°

☒ Dextran Sulfate, 50% Solution **American Bio Catalog #AB00426-00200**

☒ Yeast tRNA **Thermo Fisher Scientific Catalog #15401029**

☒ Formamide, deionized **American Bio Catalog #AB00600-00500**

☒ Ficoll® 400 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F2637**

☒ Polyvinylpyrrolidone **Merck MilliporeSigma (Sigma-Aldrich) Catalog #PVP40**

☒ Triethanolamine hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T1502**

- 34 Prepare an oven that can accommodate your slide boxes at 65°C.

🔥 65 °C oven

- 35 Thaw slides at room temperature ("air-dry") for 15-20 min.

🕒 00:15:00

- 36 Rinse slides in 1X DEPC PBS to re-hydrate tissue. This typically requires ~3mL of 1X DEPC PBS

- 37 Fix slides in 4% PFA DEPC 1X PBS for 10 min.

⌚ 00:10:00

38 Rinse in DEPC PBT

39 Wash twice in DEPC PBT, 5 min. each.

40 Treat with Proteinase K in DEPC PBS for 10 min. Do this in a bucket with 200 mL DEPC 1X PBS with 1 µg/mL Proteinase K.

⌚ 00:10:00

⊗ Proteinase K, recombinant, PCR grade **Merck MilliporeSigma (Sigma-Aldrich) Catalog #3115836001**

41 Rinse once in DEPC PBT.

42 Post-fix with 4% PFA 1X DEPC PBS for 5 min.

⌚ 00:05:00

43 Three 5 min washes in DEPC PBT.

44 Acetylation, 10 min, room temperature. Add 625 µl acetic anhydride to 250 mL 0.1M TEA (triethanolamine, prepped with DEPC water) and shake well—use immediately.

⊗ Acetic anhydride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #320102-1L**

⊗ Triethanolamine hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T1502**

45 Rinse in DEPC PBT.

46 Wash twice in DEPC PBT, 5 min each.

47 Rinse in DEPC H₂O.

48 Prepare a humidified slide box. To do this, stand a small slide box on its end and roll a cylinder of paper towels that can fit into the bottom of the box (this will take up about a fifth of the slots). Soak the paper towels in ~25mL of 5X SSC/50% formamide. Set this on your bench with the remaining slots ready to accept the slides.

☒ Formamide, deionized **American Bio Catalog #AB00600-00500**

- 49** Take each slide individually and gently let the water wick off onto a Kimwipe. Wipe the edges of the slide with the Kimwipe so that a border of dry slide surrounds the tissue. Continue on to the next step before the tissue sections dry. This step should only take ~1 min/ slide.

- 50** Apply the probe. Use 150 μ l of probe/hybridization buffer for each slide. Gently apply a coverslip-sized piece of plastic bag material (polyethylene) on each slide. Use 2 μ l of probe for every 100 μ l of hybridization buffer, so here use 3 μ l, which should make about 150 μ l probe. Insert slide into humidified slide box.

☒ Bel-Art™ Polyethylene Utility Bags **Fisher Scientific Catalog #22-260173**

Note

Steps 49 and 50 should be repeated for each slide individually. Placing the slides into the slide holder after applying the plastic coverslip.

- 51** Seal the box with plastic tape along all the edges, being careful not to let the slides tip forward at all.

☒ Texwipe™ KOGO™ TPR Cleanroom Adhesive Tapes **Fisher Scientific Catalog #18-366-432**

- 52 Gently transfer the slide box to the prepared 65°C oven. Stand it upright inside. Incubate overnight.

IV. Post-hybridization washes

- 53 The next day, prewarm all the solutions to the appropriate temperature.
65°C: 5X SSC, 2X SSC, 0.2X SSC, 1X SSC/50% formamide.
37°C: TNE
- 54 Prepare block: 20% sheep serum, volumed with MABT, with 2% (w/v) blocking reagent dissolved. Start dissolving block in MABT first, then add the sheep serum. Incubate in a water bath at 55°C, shake intermittently to get it into solution. To determine how much block to make multiply your total number of slides by 2 to determine mL of block. Block solution will be used for the block step as well as the incubation.
☒ Lamb Serum, New Zealand origin **Thermo Fisher Scientific Catalog #16070096**
☒ Blocking Reagent **Merck MilliporeSigma (Sigma-Aldrich) Catalog #11096176001**
- 55 Once all buffers have come to appropriate temperature remove the coverslips in a bath of 5X SSC (warmed to 65°C) in a glass staining dish that holds slides horizontally. Use a needle to gently break the seal along the edge of the coverslip/slide, which will allow the slips to float to the surface.

Discard plastic coverslips. Do this step quickly.

- 56 Transfer slides to 1X SSC/50% formamide for 30 min at 65°C (in the oven). All steps at 65°C and 37°C should be done in plastic staining dishes.

☒ Plastic staining dish **VWR International Catalog #25608-904**

- 57 TNE 10 min at 37°C.

🔥 37 °C incubation

🕒 00:10:00 incubation

- 58 TNE + RNase A (20µg/mL) for 30 min at 37°C. This is 400 µL of 10 µg/µL stock in 200 mL TNE.

☒ RNase A **Merck MilliporeSigma (Sigma-Aldrich) Catalog #10109142001**

🔥 37 °C incubation

🕒 00:30:00 incubation

- 59 TNE 10 min at 37°C (re-use TNE from the first incubation).

🕒 00:10:00 incubation

🔥 37 °C incubation

- 60 2X SSC 20 min 65°C

🔥 65 °C incubation

🕒 00:20:00 incubation

- 61 0.2X SSC 20 min 65°C.

🕒 00:20:00 incubation

🔥 65 °C incubation

- 62 0.2X SSC 20 min 65°C.

🔥 65 °C incubation

🕒 00:20:00 incubation

V. Antibody Incubation and Detection

- 63 Wash twice in MABT for 5 min. each at room temperature.

- 64 Move slides to a staining tray and add 1mL block/ slide. Block must be left on for a minimum of 1 hour.

- 65 Incubate in a-DIG-AP (1:2000) in 20% HISS/2% blocking reagent(w/v) in MABT (i.e. blocking solution made in step 53), 1mL/slide, overnight, 4°C. Do this in a humidified chamber.

☒ Anti-Digoxigenin-AP, Fab fragments **Merck MilliporeSigma (Sigma-Aldrich) Catalog #11093274910**

- 66 The next day, rinse once in MABT, then wash three times for 5 min. each in MABT (all room temperature).

- 67 Wash in NTM pH 9.5 for 10 min., room temperature.
00:10:00
- 68 Develop in NTM pH 9.5 + NBT + BCIP. To each mL NTM, add 4.5 µL NBT and 7 µL BCIP. Prepare 1mL/ slide. Once developing keep slides out of direct light.
5-Bromo-4-chloro-3-indolyl phosphate disodium salt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #B6149**
Nitrotetrazolium Blue chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #N6639**
- 69 Develop until you see the signal without background coming up (different times for different probes). If necessary to develop overnight, keep covered from light in a humidified chamber in the at 4°C. Developing in the cold can help reduce background even when the probe is strong enough to show good signal after a few hours. Prior to moving to 4°C and slides should be washed with NTM. New developing solution should be prepared when incubating at 4°C. This same wash and re-applying new developing solution should be performed if developing continues at room temperature the following day. This can go out for a 2-3 days as long as background doesn't appear.

VI. Post-detection

- 70 Wash in NTM pH 9.5 for 5 min.
00:05:00 wash
- 71 Wash in PBS for 5 min.
00:05:00 wash
- 72 Fix in 4%PFA/ PBS for 30 min.
00:30:00 fix
- 73 Wash: 1X PBS, 1X PBS, water (5 min. each).
- 74 Dry each slide slightly as described earlier in the protocol (step 48). While sections are still slightly wet, apply mounting media (warmed to 65°C) and coverslip. Avoid bubbles.
Polyvinyl alcohol mounting medium with DABCO®, antifading **Merck MilliporeSigma (Sigma-Aldrich) Catalog #10981**
Thermo Scientific™ Richard-Allan Scientific™ Cover Glass **Thermo Fisher Scientific Catalog #102460**
- 75 Cure the slides in a dry place overnight.