RNA in situ hybridization

Nature Communications

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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

EXTERNAL LINK

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Leigh ND, Dunlap GS, Johnson K, Mariano R, Oshiro R, Wong AY, Bryant DM, Miller BM, Ratner A, Chen A, Ye WW, Haas BJ, Whited JL, Transcriptomic landscape of the blastema niche in regenerating adult axolotl limbs at single-cell resolution. Nature Communications doi: 10.1038/s41467-018-07604-0

PROTOCOL STATUS

Working

GUIDELINES

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

MATERIALS

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

STEPS MATERIALS

NAME	CATALOG #	VENDOR
UltraPure™ DNase/RNase-Free Distilled Water	10977023	Thermo Fisher Scientific
Deoxynucleotide Solution Set - 25 umol of each	N0446S	New England Biolabs
OneTaq DNA Polymerase - 1,000 units	M0480L	New England Biolabs
QIAquick Gel Extraction Kit	28704	Qiagen
Protector RNase Inhibitor	3335402001	Sigma – Aldrich
T7 RNA Polymerase	10881767001	Sigma – Aldrich
SP6 RNA Polymerase	10810274001	Sigma – Aldrich
DIG RNA Labeling Mix	11277073910	Sigma – Aldrich
RNase Zap	R2020-250ML	Sigma Aldrich
RNeasy Mini Kit	74104	Qiagen
RNase-Free DNase Set	79254	Qiagen
UltraPure™ DNase/RNase-Free Distilled Water	10977023	Thermo Fisher Scientific
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UltraPure™ DNase/RNase-Free Distilled Water	10977023	Thermo Fisher Scientific
Tricaine methanesulfonate MS222		Sigma Aldrich
Tissue-Tek® O.C.T. Compound, Sakura® Finetek	25608-930	Vwr
RNase Zap	R2020-250ML	Sigma Aldrich
Tissue-Tek® O.C.T. Compound, Sakura® Finetek	25608-930	Vwr
Superfrost Plus Microscope Slies	4951PLUS4	Thermo Fisher Scientific
Proteinase K, recombinant, PCR grade	3115836001	Sigma Aldrich
Acetic anhydride	320102-1L	Sigma – Aldrich
Triethanolamine hydrochloride	T1502	Sigma – Aldrich
Formamide, deionized	AB00600-00500	American Bio
Bel-Art™ Polyethylene Utility Bags	22-260173	Fisher Scientific
Texwipe™ KOGO™ TPR Cleanroom Adhesive Tapes	18-366-432	Fisher Scientific
Plastic staining dish	25608-904	VWR international Ltd
RNase A	10109142001	Sigma – Aldrich
Lamb Serum, New Zealand origin	16070096	Thermo Fisher Scientific
Blocking Reagent	11096176001	Sigma – Aldrich
Anti-Digoxigenin-AP, Fab fragments	11093274910	Sigma – Aldrich
5-Bromo-4-chloro-3-indolyl phosphate disodium salt	B6149	Sigma – Aldrich
Nitrotetrazolium Blue chloride	N6639	Sigma – Aldrich
Polyvinyl alcohol mounting medium with DABCO®, antifading	10981	Sigma – Aldrich
Thermo Scientific™ Richard-Allan Scientific™ Cover Glass	102460	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
Depc (Diethyl Pyrocarbonate)	80730-888	VWR international Ltd
Lamb Serum, New Zealand origin	16070096	Thermo Fisher Scientific
Dextran Sulfate, 50% Solution	AB00426-00200	American Bio
Yeast tRNA	15401029	Thermo Fisher Scientific
Formamide, deionized	AB00600-00500	American Bio
Ficoll® 400	F2637	Sigma – Aldrich
Polyvinylpyrrolidone	PVP40	Sigma – Aldrich
Triethanolamine hydrochloride	T1502	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
by 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
by Vwr
Catalog #: 25608-930

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

RNase Zap
by 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
by Vwr
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
by Thermo Fisher Scientific
Catalog #: 4951PLUS4

11 Air-dry the sections 30 min-2 hrs. Store at -80^{O}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 Standard Buffer	10
10mM dNTP	1
10µm T7 primer (TAATACGACTCACTATAGGG)	1
10µm Sp6 primer (ATTTAGGTGACACTATAG)	1
OneTaq	0.25
2ng/µL pGEM-T Easy w/ probe inserted	1
Ultrapure H_20	35.75
Reaction Volume	50

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

Deoxynucleotide Solution Set - 25 umol of each
by New England Biolabs
Catalog #: N0446S

OneTaq DNA Polymerase - 1,000 units
by New England Biolabs
Catalog #: M0480L

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

QIAquick Gel Extraction Kit
by 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_20	to 20µL

Protector RNase Inhibitor
by Sigma – Aldrich
Catalog #: 3335402001

T7 RNA Polymerase
by Sigma – Aldrich
Catalog #: 10881767001

SP6 RNA Polymerase
by Sigma – Aldrich
Catalog #: 10810274001

DIG RNA Labeling Mix
by 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
by Sigma Aldrich
Catalog #: R2020-250ML

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

RNeasy Mini Kit
by Qiagen
Catalog #: 74104

RNase-Free DNase Set
by Qiagen
Catalog #: 79254

17 Combine all three reactions and volume up to 100µL with Ultrapure H_20.

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

18 Add 350µL RLT buffer and mix well.

19 Add 250µL100% 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 centrigue 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 H_20 directly to the column membrane. Close the lid and centrifuge for 1 min at 10,000 rpm.

UltraPure™ DNase/RNase-Free Distilled Water
by 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 H_20 and incubate at 60°C for 10 minutes.

00:10:00 incubation

60 °C incubation

UltraPure™ DNase/RNase-Free Distilled Water
by 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)
by VWR international Ltd
Catalog #: 80730-888

Lamb Serum, New Zealand origin
by 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 Na_2PO_4
2.4 g KH_2PO_4

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^{o}C (in aliquots)

100X Denhardt's solution (100 mL)

2g BSA
2g Ficoll (MW 400,000)
2g Polyvinylpyrolidone (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 $MgCl_2$

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
by American Bio
Catalog #: AB00426-00200

Yeast tRNA
by Thermo Fisher Scientific
Catalog #: 15401029

Formamide, deionized
by American Bio
Catalog #: AB00600-00500

Ficoll® 400
by Sigma – Aldrich
Catalog #: F2637

Polyvinylpyrrolidone
by Sigma – Aldrich
Catalog #: PVP40

Triethanolamine hydrochloride
by 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
by 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
by Sigma – Aldrich
Catalog #: 320102-1L

Triethanolamine hydrochloride
by Sigma – Aldrich
Catalog #: T1502

45 Rinse in DEPC PBT.

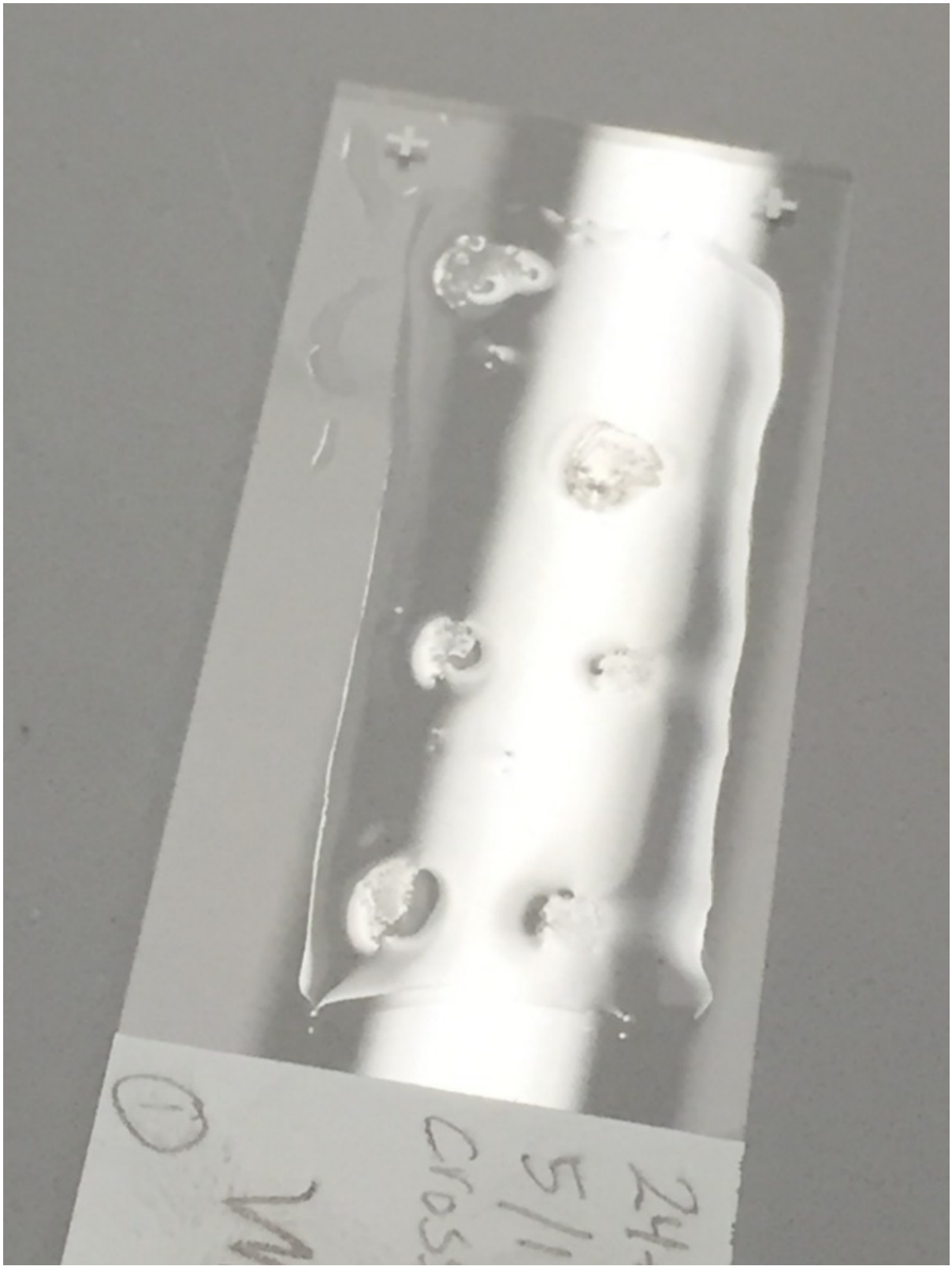
46 Wash twice in DEPC PBT, 5 min each.

47 Rinse in DEPC H_2O.

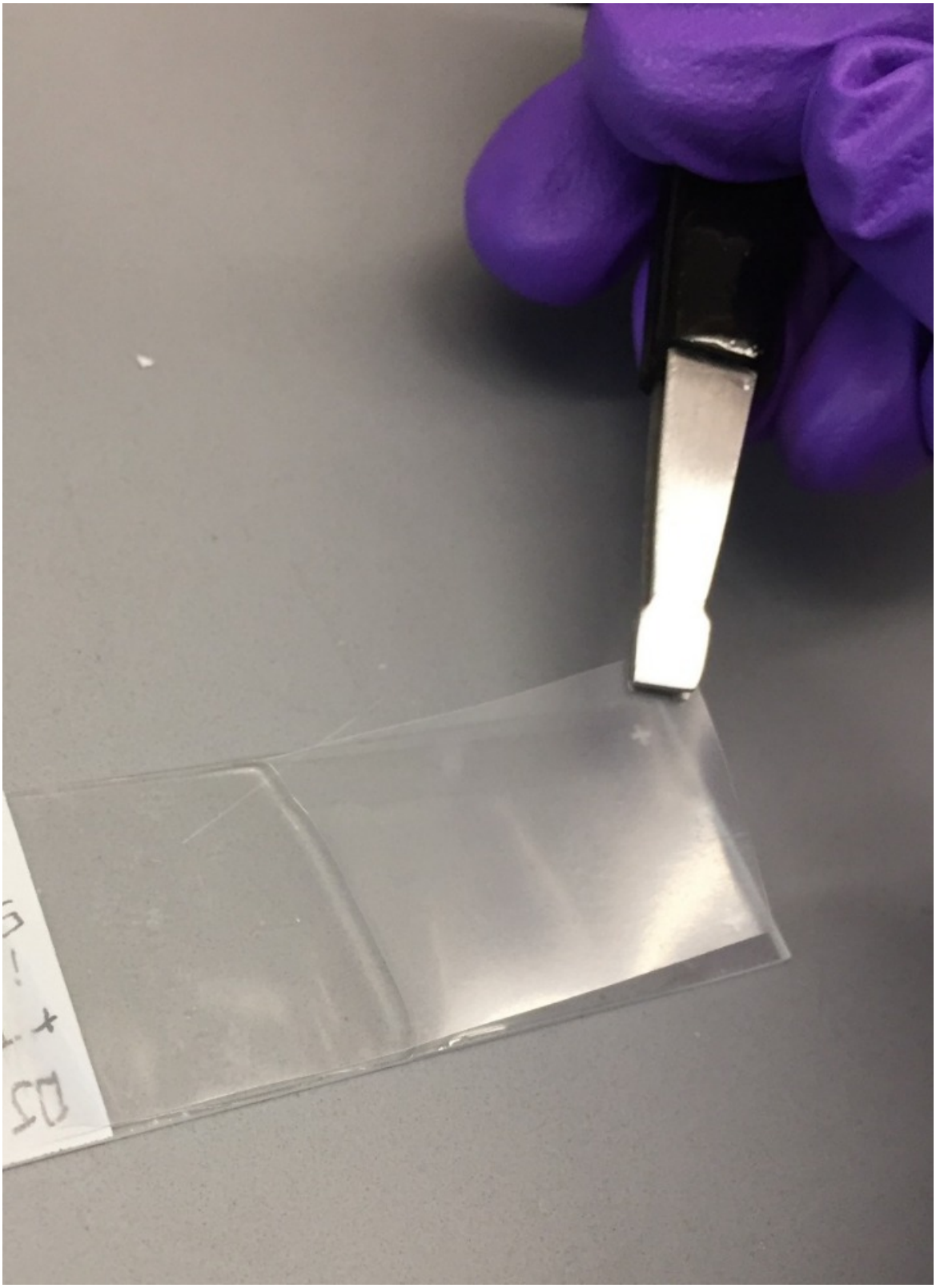
48 Prepare a humidified slide box. To do this, stand a small slide box on its end and roll a cylinder of paper towels that can fit into the bottom of the box (this will take up about a fifth of the slots). Soak the paper towels in ~25mL of 5X SSC/50% formamide. Set this on your bench with the remaining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

Formamide, deionized
by 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.



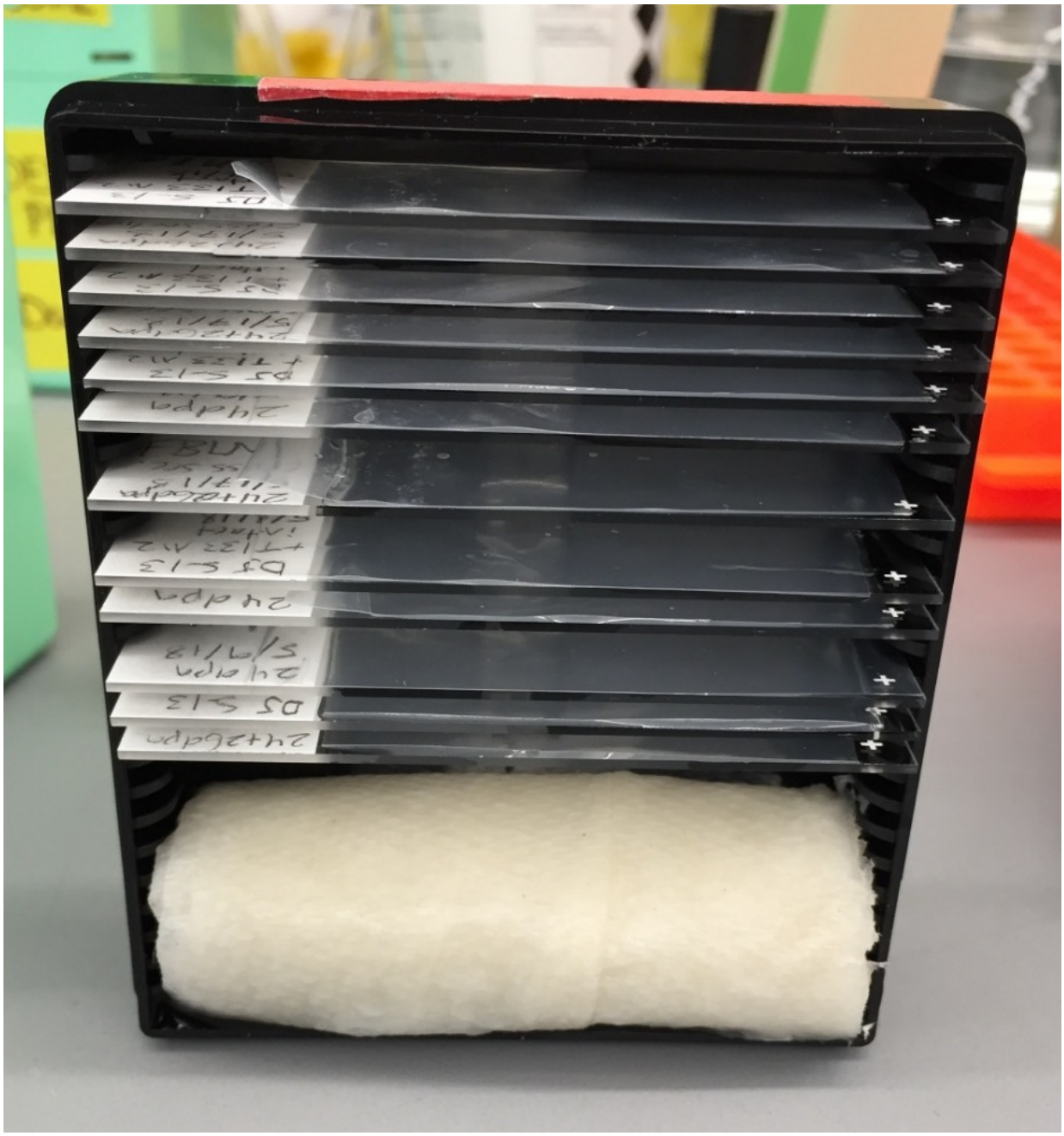
50 Apply the probe. Use 150µl of probe/hybridization buffer for each slide. Gently apply a coverslip-sized piece of plastic bag material (polyethylene) on each slide. Use 2 µl of probe for every 100 µl of hybridization buffer, so here use 3 µl, which should make about 150µl probe. Insert slide into humidified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



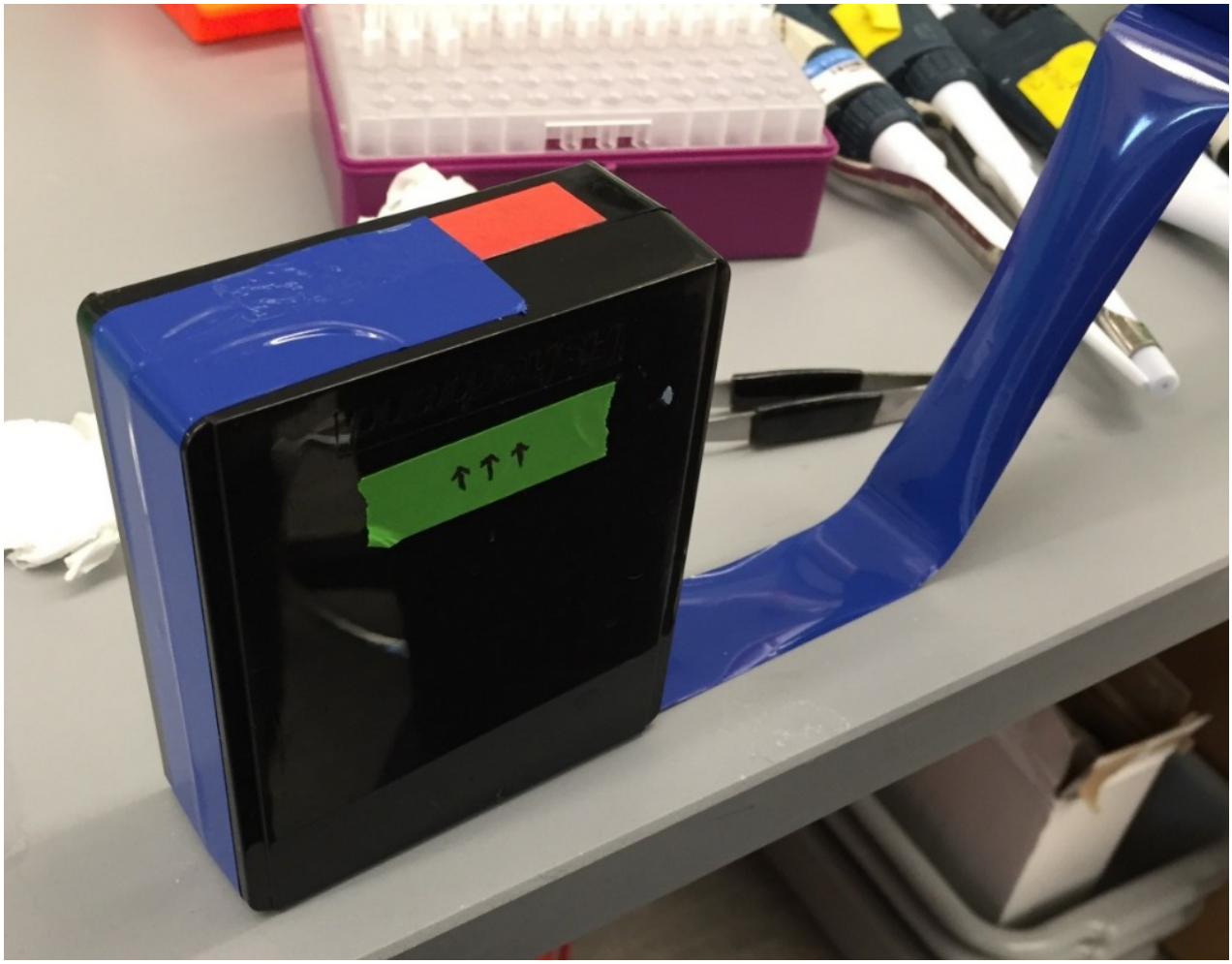
Bel-Art™ Polyethylene Utility Bags
by Fisher Scientific
Catalog #: 22-260173

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
by 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
by Thermo Fisher Scientific
Catalog #: 16070096

Blocking Reagent
by 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
by VWR international Ltd
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
by 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
by 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
by Sigma – Aldrich
Catalog #: B6149

Nitrotetrazolium Blue chloride
by 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
by Sigma – Aldrich
Catalog #: 10981

Thermo Scientific™ Richard-Allan Scientific™ Cover Glass
by Thermo Fisher Scientific
Catalog #: 102460

75 Cure the slides in a dry place overnight.