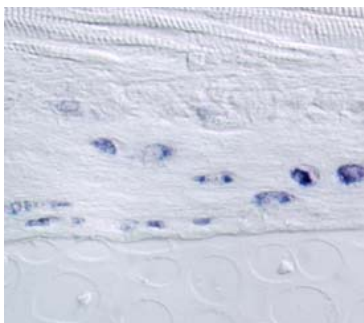




***in situ* hybridization protocol for paraffin sections of *Xenopus tropicalis* tadpole skeletal elements**

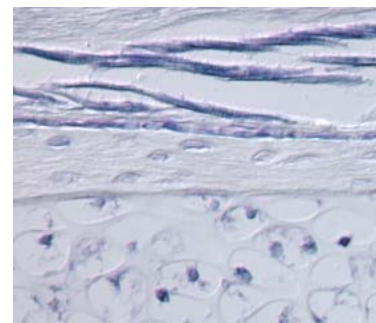
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Dentin Matrix Protein1
(Osteocyte-specific)



Osteocalcin
(Osteoblast-specific)



Collagen 8a2
(Osteoblasts, osteocytes, chondrocytes
and striated muscle fibers)

Getting started:

Prepare DEPC-treated water:

Add 1 mL of DEPC to 1 liter of nanopure water. Incubate O/N at 37°C and autoclave.

Prepare the following solutions in DEPC-treated water:

PBS 20X:

SALT	MW (g/mol)	Final concentration	g. for 500mL
NaCl	58.44	2.74 M	80.1
KCl	74.55	0.054 M	2
Na ₂ HPO ₄	141.96	0.2 M	12.6
KH ₂ PO ₄	136.09	0.04 M	2
Adjust to pH 7.4 and fill to 500 mL with DEPC-treated water. Autoclave.			

MORSE: 10% sodium citrate; 20% formic acid in DEPC-treated water. (See Shibata et al. 2000, Assessment of decalcifying protocols for detection of specific RNA by non-radioactive in situ hybridization in calcified tissues. *Histochem Cell Biol* 113(3): 153-159)

PFA 4%: See page 8!

MEM 10x: 1M MOPS; 20mM EGTA; 10mM MgSO₄

MEMFA: 1 volume of MEM 10x; 1 volume of Formaldehyde 37%; 8 volumes of DEPC-treated water.

TNE buffer (Tris/NaCl/EDTA): 10mM Tris pH7.5; 500mM NaCl; 1mM EDTA.

0.1M TEA and 0.25% acetic acid: See page 9!

HYBRIDIZATION BUFFER:

0.4 g	Roche Blocking reagent
20 mL	Formamide
10 mL	SSC 20 X
Heat for 1 hour at 65°C until dissolved, then add:	
4.6 mL	DEPC-treated water
4 mL	Yeast RNA (10 mg/mL)
80 µL	Heparin (50 mg /mL)
0.4 mL	10 % Tween
0.4 mL	CHAPS 10 %
0.4 mL	EDTA 0.5 M pH 8
Store at -20° C	

Sample preparation.

1. Anaesthetize tadpoles following bioethical rules recommended for amphibians (*See Close, Banister, Baumans, Bernoth, Bromage, Bunyan, Erhardt, Flecknell, Gregory, Hackbarth, Morton and Warwick. Recommendations for euthanasia of experimental animals: Part 1, 1996, Laboratory animals, Vol 30, pp 293-316*).
2. Dissect the tissue in 0.6X HBSS (correct osmolarity for *Xenopus tropicalis* live tissues).
3. Fix the samples in PFA/PBS 4% or in MEMFA (O/N) at 4°C.
4. Remove the fixative; then add 500 µL of MORSE solution and gently shake for 1 to 3 hours at RT (**IMPORTANT:** this step is to demineralize skeletal elements, and should be omitted for soft tissues).
5. Remove the MORSE solution and progressively dehydrate in EtOH as follows: 50% EtOH in DEPC-treated water for 5min; 100% EtOH for 5min; EtOH 100% 20min, and store at -20°C in EtOH 100%.

Paraffin inclusion.

1. Remove EtOH 100%. Add 500 µL of Xylene and gently shake for 15' (perform this step twice).
2. Replace with a 1V/1V solution of Xylene/paraffin and incubate at 60°C for 15min.
3. Replace with 100% paraffin, incubate 1 hour at 60°C (repeat 3 times).
4. Place the sample in a paraffin block (cassette). Leave O/N at 4°C.
5. Cut the sample at 7 µM thickness with a microtome. For bone sections it is recommended to change the microtome blade regularly.

Sample mounting:

1. Place a drop of DEPC-treated water on a slide, at 50-55 °C on a heating block.
2. Place the samples on top of the drop, then wait until the sample is well stretched, and gently remove the water.
3. Incubate the slide at 60°C O/N.

Materials preparation:

All materials (slide racks, moist chamber, etc) **must to be treated in NaOH 0,5%** to eliminate RNAses. Immerse the materials in NaOH 0,5% the day before starting your hybridization *in situ*, extensively rinse in DEPC-treated water before use.

Solutions that can be prepared in normal nanopure water (i.e. non-DEPC treated).

Buffer 1: For 300 ml, mix 1M Tris-pH7.5 (30 ml), 3M NaCl (15 ml) and 255 ml of nanopure water.

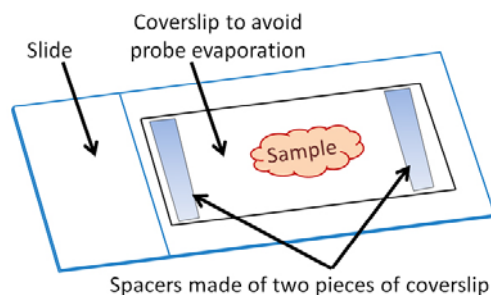
Alkaline phosphatase buffer (pH 9,5) : 0.1M Tris pH 9.5 (5ml 1M Tris); 0.1M NaCl (1.66ml 3M NaCl); 50mM MgCl₂ (2.5ml 1M MgCl₂); 0.1% Tween (500µl Tween 10%) and fill to 50ml with nanopure water.

Day 1

We perform the washes and incubations in slide racks of 50 mL.

All the solutions are prepared with DEPC-treated water

1. Directly place the slide from the 60°C incubator (without cooling) in Xylene for 10 min.
2. Wash in Xylene for 10 min (Use a different Xylene slide rack).
3. Rehydrate as follows:
 - 5min in EtOH 100%
 - 5min in EtOH 95% / PBS
 - 5min in EtOH 75% / PBS
 - 5min in EtOH 50% / PBS
 - 5min in EtOH 25% / PBS
 - 5min in PBS 100% (twice)
4. Post-fix in PFA 4%/PBS for 20 min at RT.
5. Wash the samples with PBS/Tween 0.1% for 5 min (twice).
6. Treat slides with Proteinase K (5 to 15ug/mL) / PBS at 37 °C for 30min. (**IMPORTANT:** it is crucial to optimize this step, as weak PK treatments will result in a drastic decrease of signal, and excessive treatments will damage your sample. We recommend to calibrate the concentration and duration of PK treatments)
7. Post-fix with PFA 4%/PBS for 5 minutes at RT (Reuse fixative from step 5).
8. Wash with PBS/Tween 0.1% for 5 min (twice).
9. Acetylate with Trietanolamine (TEA) 0.1M pH 7-8 and 0,25% acetic anhydride for 5 min at RT. (**IMPORTANT:** This treatment removes electric charges on the slide and tissue to avoid probe binding. Acetylation of the positively charged amino groups in the proteins of the tissue decreases background binding of the negatively charged RNA probe. It also appears to inactivate RNases and may help producing a strong signal)
10. Wash in PBS/Tween 0.1% at RT (twice)
11. Rinse in PBS for 10 seconds.
12. Prehybridize with hybridization buffer for 2-3 hr at 60°C.
13. Hybridization: denature the probes (100 to 400 ng) in 150 µl hybridization buffer by heating at 80°C for 5 min, then quickly chill on ice for 5 min.
14. Incubate the samples with hybridization mix (probe + hybridization buffer) at 60°C O/N. Place your slides in a moist chamber and cover your samples with a coverslip to prevent evaporation as follows:



Day 2

All the solutions are prepared in normal nanopure water (*i.e.* non-DEPC treated).

1. Remove the coverslips dipping the slide in SSC 4X-0.1% CHAPS at 60°C.
2. Wash in SSC 2X-0.1% CHAPS/ 50% formamide for 30 min (immerse the slides in a rack containing 40ml of solution). Incubate at 60°C.
3. Wash in TNE buffer for 10 min at 37 °C.
4. Wash in TNE + RNase A (20ug/mL) for 30 min at 37°C.
5. Wash in TNE for 10 min at 37°C.
6. Wash in SSC 2X 0,1% CHAPS for 20 min at 60°C.
7. Wash in SSC 0.2X 0,1% CHAPS for 20 min at 60°C (twice).
8. Wash in 50% (SSC 0.2X-0.1% CHAPS)/50% buffer1 for 5 min at RT.
9. Wash with Buffer 1 for 5 min at RT.
10. Block for 1 hr at RT with Buffer 1 + 20% goat serum.
11. Incubate with antibody (anti-DIG-AP) 1:2000 in Buffer 1 + 20% goat serum O/N at 4°C, in a moist chamber.

Day 3

1. Wash in Buffer 1 + Tween 0,05% in deionized water for 10 min at RT (4 times).
2. Wash in alkaline phosphatase buffer (pH 9,5) for 5 min at RT (twice).
3. Replace with coloration solution (1 tablet of NBT/BCIP dissolved in 10 ml of nanopure water). To avoid sample dying or evaporation, add more coloration solution regularly.
4. When appropriate, stop the reaction by washing for 10 min in PBS at RT (3 times).
5. Fix in MEMFA for 1hr at RT.
6. Dehydrate samples progressively by washing your slides as follows:
 - 5 min in EtOH 100% I
 - 5 min in EtOH 100% II
 - 5 min in Xylol
 - 5 min in Xylol II
 - 5 min in Xylol III
 - 5 min in Xylol IV
7. Mount in Entellan.

Reagents

	Supplier	Reference number
Acetic anhydride	Sigma-Aldrich	33214
Anti-Digoxigenin AP conjugate	ROCHE	11093274
CHAPS	Merck-Calbiochem	220201-10GM
DEPC	Sigma-Aldrich	159220-100G
Entellan	Merck	1079610100
Formaldehyde 37%	Sigma-Aldrich	F8775
Formamide	Merck-Calbiochem	344206
Goat serum	USBiological	S1003-45
HBSS	Thermo Scientific Hyclone	SH30031.03
Heparin	Merck-Calbiochem	375095-100KU
Microtome Blades for bone	Sakura	4685
MOPS	Merck-Calbiochem	475922
NBT/BCIP tablet	Roche	11697471001
Paraffin	Merck	111609
Proteinase K	Life Technologies-Ambion	AM2542
RNaseA	USBiologicals	R2011
Roche Blocking Reagent	Roche	11096176001
Slides	Fisher-Scientific	12-550-15
SSC 20 X	Life Technologies-Invitrogen	15557044
Triethanolamine (TEA)	Merck	1083790250
Yeast RNA	Sigma-Aldrich	R6625-25G

General Comments - Working with RNA

RNA is sensitive to degradation by RNases. As well as being found on human skin and produced by a micro-organisms, RNases can be very hard to destroy: They are for instance resistant to temperatures well over 100°C. For these reasons a number of precautions to avoid contamination are usually used.

1. Wear gloves
2. Keep special stocks of tips and tubes for RNA work.
3. Treat solutions with diethylpyrocarbonate (DEPC). Added to 0.1% and left overnight, this destroys RNases. Autoclaving then breaks down DEPC to H₂O and CO₂. Be careful: **TRIS is incompatible with DEPC** so you should always prepare Tris solutions with DEPC treated H₂O. When you weigh powder don't use spatula, use either disposable scalpel or pour the powder.

The solutions you can treat with DEPC are (you make the solutions with bi-distilled H₂O and then you add 0.1% DEPC, you leave them stirring overnight and the next morning you autoclave them):

PBS	H ₂ O
0.5M EDTA	3M CH ₃ COONa

Solutions to make with 0.1% DEPC water (you have treated the H₂O with DEPC and then you use it for making the solutions):

	70% ethanol
4M LiCl	Hydrolysis mix
Heparin	10mg/ml yeast RNA
10% CHAPS	10% Tween 20

Solutions to keep specially for RNA work:
100% ethanol

Fixatives

For optimal fixation it may be critical to use fresh formaldehyde solutions. Fresh 4% solutions can be made from 16%, methanol free, formaldehyde or from solid paraformaldehyde (4% w/v).

MEMFA Fix: 1 part 10x MEMFA salts + 1 part 37% formaldehyde + 8 parts water

10x MEMFA Salts: 1 M MOPS + 20mM EGTA + 10mM MgSO₄ (10x salts can be autoclaved and stored. MEMFA turns yellow after autoclaving).

For *in situ*: fix for 2 hours or O/N @ 4°C.

4% Paraformaldehyde (PFA) Fixative

We recommend that use Fresh Solution of fixative per each *in situ*:

1. Prepare 10ml of 1X PBS then add 0.4 g of paraformaldehyde powder.
2. Add 100ul of NaOH 10N. Mix and maintain at 60°C per 10'.
3. Once again that Fixative solubilize, add 75ul of HCl 37%. Mix and cool a TA.
4. Use immediately.

How to prepare TEA - Acetic anhydride?

The TEA (Triethanolamine) molecular weight is FW 149.2

Therefore to prepare 50 mls of 1M TEA, one should weight:

$$0.05 \text{ l} \times 1 \text{ mol.l}^{-1} \times 149.2 \text{ g.mol}^{-1} = 7.46 \text{ g}$$

TEA is a liquid. But because it is extremely viscous, there is no way we can measure the volume corresponding to 7.46 g. Rather, it is much more convenient to place a 50 ml FALCON tube on the balance, calibrate to zero, and **transfer 7.46 g with a pipette**.

Then, add DEPC-treated nanopure water to 30 ml, mix well. Fill the tube to 50 ml, mix.

Preparing [TEA – Acetic anhydride] for *in situ* hybridization (0.1M TEA and 0.25% acetic acid):

For a 50 ml tube:

- 5ml of TEA 1M
- 45 ml of DEPC-treated nanopure water
- Adjust pH to 7 with “pH paper”: (add approximately 300 µl of HCl 37%, work under the fume hood)
- Add Acetic anhydride (125 µl)

Shake well and use immediately!

Bench table – Day 1

T°C	Time		Solution																
RT	10'	2x	Xylene																
	5'		EtOH 100%																
	5'		95% EtOH in PBS																
	5'		75% EtOH in PBS																
	5'		50% EtOH in PBS																
	5'		25% EtOH in PBS																
	5'	2x	PBS																
	20'		PFA4%-PBS																
	5'	2x	PBS-Tween 0.1%																
37°C	30'		PK 10 µg/ml																
RT	5'		PFA4%-PBS																
	5'	2x	PBS-Tween0.1%																
	5'		TEA + anhídrido acético																
	5'	2x	PBS-Tween 0.1%																
	10''		PBS 1X																
60°C	2-3 h		Prehybridization buffer																
80°C	5'		Probe denaturation																
4°C	5'		Chill on ice																
60°C	O/N		Hybridization																

Bench table – Day 2

T°C	Time		Solution																
60°C	10''		4 x SSC 0.1% CHAPS																
	30'		2 x SSC 0.1% CHAPS/ 50% formamide																
37°C	10'		TNE																
	30'		TNE+RNase A (20ug/mL)																
	10'		TNE																
60°C	20'		SSC2X 0.1% CHAPS																
	20'	2X	SSC 0.2X 0.1% CHAPS																
RT	5'		50% Buffer1 / 50% [0.2x SSC – 0.1% CHAPS]																
	5'		Buffer 1																
	1hr.		Buffer 1 + 20% serum																
4°C	O/N		anti-DIG-AP 1:2000																

Bench table – Day 3

T°C	Time		Solution																	
RT	10'	4x	Buffer 1 + Tween0.05																	
	5'	2x	Phosphatase alkaline buffer																	
			NBT/BCIP																	
	10'	3x	PBS																	
	1hr.		MEMFA																	
	5'	2X	EtOH 100%																	
	5'		Xylene I																	
	5'		Xylene II																	
	5'		Xylene III																	
	5'		Xylene IV																	
Mount in Entellan																				