

In situ Hybridization on frozen sections using RNA probes labeled with DIG

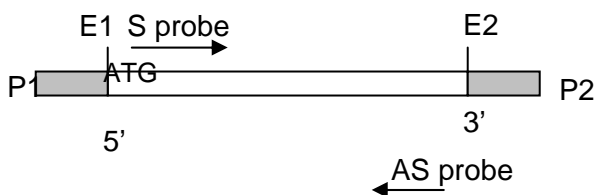
Preparation of sections

- Animal must be fixed by perfusion (see corresponding protocol) using Bouin's or DEPC-PB solution. Sections are cut and collected on RNase-free slides at 18-20 μ M.

Preparation of the probes

1. DNA template preparation

- The cDNA must be sub-cloned in a plasmid containing in each side promoter for the RNA polymerase (T3, T7 or SP6). Plasmid like Pbluescript is fine.
- The size of cDNA to make the RNA probe must be less than 1000 pb. If it's more, it needs to be hydrolyze with NaOH
- Linearize the plasmid with appropriate restriction enzyme (see graph) and resuspend the linearized cDNA in DEPC water.



AS Probe = E1+P2
S Probe = E2+P1
E: restriction enzyme
P: promoter

2. In vitro transcription of RNA probes

- In a 0.5 ml tube mix :
 - X μ l of DNA (usually 1 μ g)
 - 2 μ l Of 10 X DIG RNA labeling mix (Roche #1277073). To make the AS non-DIG for competition use NTP mix (Invitrogen #18109-017)
 - 2 or 4 μ l of Transcription buffer (either 10X or 5X)
 - 2 μ l of Rnase inhibitor
 - 2 μ l of Rnase polymerase (T3, T7, SP6)
 - - DEPC-water up to 20 μ l.
- Incubate at 37C for 2 H.
- Add 2 μ l of Dnasel and incubate at 37C for 15 min.
- Add 2 μ l 0.2 EDTAfree RNase (PH 8.0) to stop the reaction
- Precipitate with 2.5 μ l LiCl (0.1 volume) and 75 μ l of 100% Ethanol (2.5 volumes)
- Leave O.N at -20C.
- centrifuge at 4C for 15 min. at 12000xg
- Decant the ethanol, wash with EtOH 70% and vaccum for 2 min.
- dissolve pellet in 25 μ l of DEPC-water.
- Analyse RNA on a DNA gel (load 1 or 2 μ l)

3. Hydrolysis of Probe:

if the RNA probe is issued from a cDNA greater than 1000 pb, then hydrolysis should be performed at this step. You should suspend the RNA in 50 μ l of Hydrolysis buffer Rnase free (40mM NaHCO₃/ 60mM Na₂CO₃). Hydrolyze 10 to 12min.at 60°C (for fragment around 500pb). (the amount of time. t. is given by $t = (\text{starting length}-\text{desired length}) / (0.11 * \text{starting length} * \text{desired length})$). Re-precipitate probe as described above.

4. Assessing DIG incorporation using a DIG-Labeled Control RNA (Roche # 1585746). Standard dots can be choose between 50 to 0.5 ng. RNA is loaded on a nitrocellulose RNA blot, cross-linked for few second. Block the blot with 5% milk, incubate antibody anti-DIG Alcaline phophatase conjugated for 1hr at RT. Developed with NBT/BCIP (see part E).

-The RNA concentration used is generally 0.2-0.4 ng/ μ l final. It means that the RNA stock concentration must be at least 20 to 40 ng/ μ l due to the X100 dilution during hybridization.

- Conserve RNA probe at -80C.

Pre-hybridization of sections (day 1)

- The day before, incubate the slides at 42 C for ON.
- Work in **slide mailers**
- Wash slides in TBS for 5 min,
- Fix for 20 min. with bouin's
- permeabilize for 10 min. with 0.2M HCl
- wash 3X5 min with TBS
- Treat sections with Proteinase K at 1ug/ml (1.3ul/20ml) for 30 min.
- Post-fix 5 min with bouin's
- Wash 2X10 min. with TBS
- Treat for 10 min. with TEA pH8.0 added with 0.25% Ac. Anhydride (50ul/20ml TEA). This step can increase the signal. It can be omitted.
- Pre-hybridize sections in the hybridization buffer for at least 2 h. (generally 3 hours) at 58C.
- Prepare the probe in 100ul of hybridization buffer at a final concentration of 0.2-0.4ng/ul. Heat at 85C for 5 min, then put on ice directly. Load the probe on the coverslip and cover the slides.
- Incubate the slides O.N at 58C (Temperature is changing depending on the probe)

Hybridization of sections (day 2)

- Put the slides in a jar containing 2X SSC preheated at 58C. Incubate for 30 min. at 58C. This step is to remove the coverslip from the slides.
- Remove carefully the coverslip
- Work in **slide mailers**
- wash 2 times for 5 min. at RT.
- treat 30 min with Rnase (20ug/ml) at 37C
- Wash 3 times with 0.4XSSC (15 min at RT and 2 times 15 min at 58C)
- Wash 3 times 15 min. in TBS

Visualization of digoxigenin

- Block 1 H. in 10% FBS at RT
- Incubate Antibody (anti-DIG Apconjugated Fab fragment, Roche #1093274) at 1/1000 dilution in 10 % FBS at RT for 1H.
- Wash 2 times 10 min in TBS
- Develop in a slide mailer containing 20 ml of buffer 3, 5 mg levamisole, 66 ul NBT and 70ul of BCIP.
- Incubate O.N at 4 C
- The next day rinse the slides to stop the reactions and coverslip using aqua-polymount solution.

SOLUTIONS.

DEPC-Water (prepare 4 litter)

0.1% of DEPC in ddWater. Agitate at 37 C for 8-12Hours. Then autoclaved 20 min.

TBS

50mM Tris-HCL pH 7.5 (use autoclaved DEPC water)

150 mM NaCl

use DEPC-Water

autoclave

it is suggested to prepare a 10X solution of TBS (39.4gr of Tris, 44gr of NaCl and 500ml DEPC water)

TEA (prepare 1 l)

100mM triethanolamine pH8.0
use DEPC-Water
autoclave

Hybridization buffer (prepare 50 ml)

2X SSC
10% Dextran sulfate
0.01% (0.01mg/ml) sheared salmon sperm DNA
0.02% SDS
50% Formamide
use DEPC-Water
Stock in aliquots at -20C

Rnase Buffer (prepare 1 l)

500mM NaCl
10 mM Tris-HCL ph 7.5
5mMEDTA pH 8.0

Buffer 3 (prepare 500 ml)

100mM tris-base ph 9.5
100mM NaCl
50 mM MgCl₂