In Situ Hybridization with Tyramide Signal Amplification

This protocol describes standard non-radioactive in situ hybridization (ISH) of (para)formaldehyde-fixed paraffin-embedded tissue sections (FFPE) or frozen sections (FS) using fluorescein-labeled probes and HRP-DAB or fluorescence detection with the Tyramide Signal Amplification (TSA) System from NE\textsuperscript{N} Life Science Products.

1. Tissue fixation, embedding, and sectioning

- Remove piece of tissue using forceps, scissors, scalpel. Briefly rinse or wash with 0.9% NaCl and transfer to fixation solution.
- For ISH fix tissue in 4% PFA in TBS at 4°C for 2-24 h. Fixation time depends on the size of the sample and should be long enough for complete penetration of the sample by the fixation solution. Over-fixation should be avoided because this leads to poor hybridization results.
- Use automatic dehydration and embed the sample in paraffin. Paraffin blocks can be stored at room temperature.
- Using a microtome cut sections of 4-6 µm and transfer sections to slides with adherent coating. Label slides and dry slides for 2-16 h at 50°C in an incubator.
- Alternatively, transfer tissue to Tissue-Tek and freeze in liquid N\textsubscript{2}. Store at -75°C.
- Using a cryo-microtome cut sections of 4-6 µm and transfer sections to labeled slides with adherent coating. If not used immediately, store slides at -20°C.

2. Preparation of labeled probes

- Prepare fluorescein-labeled dsDNA probes using the Gene Images Random Prime Labeling Kit (Amersham)
- Prepare fluorescein-labeled ssDNA probes by one-side PCR (thermal cycling with only one primer and labeled nucleotides)
- Prepare fluorescein-labeled ssRNA probes (sense and antisense) by in vitro transcription using the RNA Colour Kit (Amersham)
- Order fluorescein-labeled Oligonucleotide probe from commercial supplier

3. Hybridization

a. Paraffin-embedded sections

- Remove paraffin by incubating slides for 3 min each in: 4x Xylol - 100% EtOH - 96% EtOH - 80% EtOH.
- Transfer slides to a tray and wash 5 min in H\textsubscript{2}O and 5 min in TBS.
- Add 500 µl 20 mM HCl and incubate 10 min at RT.
- When the slides have cooled so you can hold them transfer slides to a tray filled with TBS and incubate for 5 min.

b. Frozen sections

- Fix frozen sections in 4% PFA in TBS for 20 min at RT.
- Wash slides 30 min in TBS.

c. Pretreatment

- Remove slides from the tray and carefully remove liquid.
- Add 500 µl 20 mM HCl and incubate 10 min at RT.
- Add 500 µl 0.1% Triton X-100 in TBS and incubate 2 min at RT.
- Wash slides 2 x 3 min in TBS in a tray, then carefully remove liquid.
- Add 100-200 µl Proteinase K (10/100 µg/ml in TE) and incubate 5-20 min at RT.
- Add 500 µl 1% H\textsubscript{2}O\textsubscript{2} and incubate 15 min at RT.
- Wash 2 x 5 min in H\textsubscript{2}O in a tray, carefully remove liquid and air dry slides for ca. 5 min.

b. Hybridization and washing

- Firmly attach In situ Frame of appropriate size to completely enclose the section, heat 2 min 95°C to improve adhesion.
- Add the Hybridization Solution and attach the cover trying not to trap air bubbles. Firmly press cover on the frame using another glass slide and place the slide on the In situ Adapter mounted on the Thermocycler.
- Incubate 5 min at 95°C for initial denaturation and then o/n (ca. 16h) at 37-55°C for hybridization.
- Wash 2 x 5 min at RT in 1xSSC/0.1% SDS in a tray.
- Wash 2 x 10 min at 50°C in 0.5xSSC/0.1% SDS in a tray (prewarmed!).
4. Detection of bound probe

- Wash 5 min in TBS in a tray.
- Remove slides from tray and mount them on Coverplates for subsequent incubations.
- Wash 3 x with TNT.
- For blocking non-specific sites add 150 μl TNB and incubate 30 min at RT.
- Add 150 μl α-Fluorescein-HRP, incubate 60 min at RT.
- Wash 3 x with TNT.

a. Chromogenic detection:

- Add 150 μl BT Working Solution and incubate 10 min at RT.
- Wash 3 x with TNT.
- Add 150 μl SA-HRP and incubate 30 min at RT.
- Wash 3 x with TNT.
- Disassemble slides from Coverplates, carefully remove liquid.
- Add 100-200 μl DAB-Substrate to completely cover the section and incubate 5 min at RT.
- Transfer slides to a Tray and rinse for 5 min in running tap water.
- Counterstain slides for 1 min in a tray containing Mayer's Hämalaun.
- Transfer slides to a Tray and rinse for 5 min in running tap water.
- Dehydrate slides by incubating for 3 min each in: 70% EtOH - 80% EtOH - 96% EtOH - 2x 100% EtOH - 2x Xylol
- Mount coverslips using a Xylol-based Fast Mounting Medium.

b. Fluorescence detection:

- Add 150 μl FT Working Solution and incubate 10 min at RT.
- Wash 3 x with TNT.
- Disassemble slides from Coverplates, carefully remove liquid.
- Add 100 μl PI (or DAPI) (1µg/ml) to completely cover the section and incubate 5 min at RT.
- Remove PI (DAPI) (save for reuse) and incubate slides 5 min in a Tray containing TBS.
- Mount coverslips using an aqueous-based Fluorescence Mounting Medium and store slides in the dark.

5. Materials and reagents

- **Slides** with adhesive coating (Polysine von Menzel)
- **Glass Trays** and **PP Trays** for slides and suitable slide holders
- **Coverplates** and **Coverplate Racks** (Shandon, plastic incubation holders for slides, incubation volume ca. 100 μl)
- **In situ Frames** (Eppendorf, self-adhesive frames and covers for incubation volumes 25μl, 65μl, 125μl, 300μl)
- **Thermocycler** with **In situ Adapter** (Eppendorf)

### 4% PFA in TBS:
Dissolve 20g Paraformaldehyde (Merck) in 500ml TBS by heating and stirring for several hours, filter and store at 4°C

### Xylol, 100% Ethanol, 96% Ethanol, 80% Ethanol, 70% Ethanol
These can be reused for several weeks, use separate series of solutions for paraffin removal and dehydration

### 10xTBS:
500mM Tris (60.55g/l), 1500mM NaCl (87.66g/l), pH=7.5 (HCl)

### TBS:
100ml 10xTBS + 900ml H₂O

### 20 mM HCl:
1ml/50ml 1M HCl

### 0.1% Triton X-100:
500µl/50ml 10% Triton X-100 in TBS

### Proteinase K:
Molecular biology grade lacking DNase and RNase activity (Amersham, Dako, Roche)
Stock = 10 mg/ml in H₂O
Working 1 = 100 μg/ml in TE (10mM Tris.Cl, 1 mM EDTA, pH=8.0)
Working 2 = 10 μg/ml in TE (10mM Tris.Cl, 1 mM EDTA, pH=8.0)
Store at -20°C, avoid repeated freezing and thawing

### 2 mg/ml Glycine:
1g/500ml Glycine in TBS, store at 4°C

### 1% H₂O₂:
33μl 30% H₂O₂ (Merck) + 967μl TBS (prepare fresh, store at 4°C not longer than a week)

### Probe:
dsDNA, ssDNA, ssRNA, or Oligonucleotide probe labeled with fluorescein (store at -20°C)
Hybridization Buffer: Hybridization Buffer with 50% Formamide from RNA Colour Kit (Amersham)
final: 2xSSC, 1x Denhardt's solution, 300µg/ml herring testes DNA, unspecified rate enhancement compound, 50% formamide, store aliquots at -20°C

Hybridization Solution: Probe diluted appropriately in Hybridization Buffer

20xSSC: 3M NaCl (175.3g/l), 0.3M Na-citrate.2H2O (88.2g/l), pH=7.0 (HCl), autoclave, store at RT
20% SDS: 20g/100ml Sodium dodecyl sulfate, autoclave, store at RT
1xSSC/0.1% SDS: 50ml/l 20xSSC, 5ml/l 20% SDS
0.5xSSC/0.1% SDS: 25ml/l 20xSSC, 5ml/l 20% SDS
TNT: TBS + 0.05% Tween 20 (500µl/l)
TNB: TBS + 0.5% Blocking Reagent (500mg/100ml, from TSA-Kit (NEN))
dissolve by continuous stirring while heating up to 60°C, store aliquots at -20°C

α-Fluorescein-HRP: Horseradish Peroxidase-conjugate specific for Fluorescein (NEN NEF710)
diluted appropriately in TNB before use

BT Working Solution: 3µl BT Stock + 147µl Amplification Diluent per slide (1:50, prepare fresh before use)
BT = Biotinyl Tyramide in DMSO

FT Working Solution: 3µl FT Stock + 147µl Amplification Diluent per slide (1:50, prepare fresh before use)
FT = Fluorescein Tyramide in DMSO

SA-HRP: Streptavidin-horseradish peroxidase-conjugate, diluted appropriately in TNB before use

DAB-Buffer: 50mM Tris (6.05g/l), pH=7.6 (HCl)
2% DAB: 3,3'-Diaminobenzidine.4 HCl (2g/100ml H2O, Sigma), store aliquots at -20°C

DAB-Substrate: 1000µl DAB-Buffer + 25µl 2% DAB + 10µl 2% H2O2 (prepare immediately before use)

Mayer's Hämalaun: Merck, can be reused for several months

PI: Propidium iodide, Stock = 50µg/ml H2O, Working solution = 1µg/ml H2O
DNA stain that produces red fluorescence with green illumination (Ex/Em=535/617nm)

DAPI: 4',6-Diamidino-2-phenylindole, Stock = 50µg/ml H2O, Working solution = 1µg/ml H2O
DNA stain that produces blue fluorescence with UV illumination (Ex/Em=358/461nm)

Fast Mounting Medium: Eukitt (EMS), Entellan (Merck), Pertex (Medite)

Mounting Medium: Fluorescence Mounting Medium (Dako S3023), reduces fading of fluorescence

CONTRIBUTED BY: Hubert Schwelberger (hubert.schwelberger@i-med.ac.at)
LAST MODIFIED: 2012-06-20