Preparation of Paraffin Sections and Frozen Tissue for FISH

Section of Cancer Genomics, Genetics Branch, NCI
National Institutes of Health

Reagents

Acetic acid, glacial
Mallinckrodt, Cat. V193
Detergent, mild (Triton X-100 or Tween 20)
Ethyl alcohol, anhydrous
Formaldehyde (37%)
Magnesium Chloride ($MgCl_2$) 2M
Methyl alcohol, anhydrous
Mallinckrodt, Cat. 6858
Pepsin
Sigma, Cat. P6887, 5g
Phosphate Buffered Saline (PBS), 1X
RNase
Sodium Chloride/Sodium Citrate (SSC), 20X
Sodium Isothiocyanate (NaSCN) 1M
Xylene, 100%
Water, distilled

Preparation

Ethyl alcohol (ethanol, EtOH)
100%, and diluted with dH_2O to 70% and 90%.

70% Formamide/2XSSC (FA/SSC)

20X SSC	10 ml
dH_2O	20 ml
deionized formamide	70 ml

Adjust to pH 7.25 with IV HCl
Aliquot and store at -20˚C

4X SSC/detergent

20X SSC	100 ml
H_2O	400 ml

0.5% Tween 20 or 0.1% Triton-X 100
Room temperature

Pepsin, 10% stock solution
Dissolve 100 mg/ml in dH20,
Make 50 µl aliquots and store at –20˚C

Pepsin, working solution
Immediately prior to slide treatment step, place 5-30 µl pepsin dilution into 37˚C pre-warmed diluted HCl (99 ml dH20 + 1 ml 1N HCl). Repeated thawing and usage of pepsin over time will weaken catalytic activity. One may consider using a new aliquot after a half dozen uses.

PBS/$MgCl_2$

2 M $MgCl_2$	25 ml
1X PBS	950 ml

Formaldehyde/PBS/$MgCl_2$

37% formaldehyde	2.7 ml
PBS/$MgCl_2$	100 ml

Procedure

For Paraffinized sections only

A. Deparaffinization, pre-fixation

1. Place slides with paraffin sections into 100% xylene for 5 min, change xylene and repeat for another 5 min.

2. Rehydrate for 5 min each in 100% EtOH, 90% EtOH, and 70% EtOH.

3. Wash in 4X SSC/detergent at RT for 30 min in rotating shaker.

4. For formalin-fixed slides, place in coplin jar with 1M NaSCN at room temperature overnight. Wear gloves when handling NaSCN! For ethanol-fixed slides, you can consider skipping NaSCN step but it may improve hybridization if necessary.

5. Wash slides in distilled water x 5 min.

6. Skip to Pepsin treatment step.

For Frozen sections only

A. Pre-fixation.

1. Thaw slides at room temperature and equilibrate in 2X SSC for a couple of min. (For freshly harvested, frozen and sectioned slides, can also apply 100 µl of 1:200 RNase in 2X SSC to slide under a cover slip and incubate at 37˚C for 30 min. For tissues snap frozen in liquid nitrogen, thawed and sectioned, can skip RNase treatment.)

B. Pepsin treatment, fixation (for both paraffin-embedded or frozen sections)

1. After Pre-fixation steps above, place slides in dilute pepsin suspension containing 5-30 µl pepsin for fresh material and 20-500 µl pepsin for paraffin material for 2-5 min. (*Critical step*! See Note below)

2. Wash in PBS x 2 at RT, 5 min each.

3. Wash in PBS/$MgCl_2$ x 1 at RT, for 5 min.

4. Wash in Formaldehyde/PBS/$MgCl_2$ for 10 min.

5. Wash in PBS for 5 min.

6. Dehydrate in 70%, 90%, and 100% ethanol for 3 min each and air dry.

C. Denaturation

1. Apply FA/SSC to slides and denature at 80˚C for 2 min for freshly sectioned tissues and 3 min for older or paraffinized tissues.

2. Plunge into ice cold 70% ethanol for 3 min and dehydrate with 90% and 100% ethanol, three min each, and air dry.

3. Once dry, may add denatured/preannealed probe, coverslip, seal, and hybridize.

Notes

Sodium Isothiocyanate (1M = 400 mg/4.9 mls). Disrupts protein:DNA complexes which can improve DNA:DNA hybridization. Warning, NaSCN is highly toxic and requires the user to wear gloves at all times!

Xylenes. Dissolve complex organics such as paraffin. Used at full concentration (from supplier) but only in fume hood! A 100 ml Coplin jar with glass cover may be used to store 70 ml xylene for repeated use over two-three days. Otherwise, discard in appropriate biochemical waste container.

Enumeration in tissues. The challenge of tissue FISH is to allow sufficient specificity and sensitivity for probe enumeration while also preserving tissue architecture and nuclear integrity. Tissue FISH, unlike interphase cytogenetics of tissue imprints, requires the enumerator to take into account sectioning artifacts, overlapping cells, and negative hybridization rates (Siebert et al. 1999 J Urol 160: 534). Enumeration is best done after co-hybridization with an experimental probe and an internal standard in a different color. Probes must first be tested on normal metaphase chromosomes to determine the presence of minor binding sites.

Pepsin requirements. Optimal amounts of pepsin will always require pilot hybridization but, generally, normal metaphase cells require only 2-5 µl /100ml pepsin for 90 seconds, frozen sections 5-10 µl for 1-2 minutes, and paraffin sections 10-20 µl for 2-4 minutes. Older slides, and tissues within a large stromal component (e.g., more protein) will require much more pepsin for optimization than tissues with, for example, free, unattached clusters of epithelial cells.

Correlation of treated sections with stained sections. Sometimes, a digestion adequate for excellent hybridization may destroy delicate cytoarchitecture. It is therefore essential that a reference slide, untreated, is kept for either H & E or DAPI-staining to correlate untreated with treated sections.

Overcoming problems. Age and formalin diminish the efficiency of hybrization. NaSCN treatment can help minimize formalin induced protein:DNA complexes but boiling in 0.1 M Tris/0.05 M EDTA pH 7.0 for 2 min x 4 can help as well. Varying the time of pepsin treatment and denaturation are the two most helpful strategies for inefficient hybridizations.