

In situ Hybridization

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Abstract

Hybridization is the formation of hybrid nucleic acid molecules with complementary nucleotide sequences in DNA:DNA, DNA:RNA, or RNA:RNA forms. In situ hybridization is a highly sensitive technique that allows detection and localization of specific DNA or RNA molecules in morphologically preserved isolated cells, histological tissue sections, or chromosome preparations. In situ hybridization has broad range of applications and has been used to (a) localize viral infection, (b) identify sites of gene expression, (c) analyze mRNA transcription and tissue distribution, and (d) map gene sequences in chromosomes. There are several advantages of the use of in situ hybridization including the fact that it can be applied to archival materials and frozen tissues and can be combined with immunohistochemistry to detect protein as well as mRNA of interest or phenotype of cells expressing the target genome, detecting more than one nucleic acid sequences using different labeling methods.

The major steps involved in in situ hybridization are as follows: probe preparation and labeling, tissue fixation, permeabilization, hybridization, and signal detection and these are described in detail in this chapter.

Key Words: In situ hybridization; DNA; RNA; blotting.

1. Introduction

1.1. Background

Hybridization is the formation of hybrid nucleic acid molecules with complementary nucleotide sequences in DNA:DNA, DNA:RNA, or RNA:RNA forms. In situ hybridization is a highly sensitive technique that allows detection and localization of specific DNA or RNA molecules in morphologically preserved isolated cells, histological tissue sections, or chromosome preparations (1). The technique was first described by Gall and Pardue (2) and independently by John et al. (3) in 1969. In situ hybridization is believed to have higher sensitivity than filter hybridization (Northern or Southern) where the nucleic acid sequence of interest is

present in low copies or in a small proportion of cells within tissue. The concept behind the in situ hybridization of nuclear DNA or cytoplasmic RNA is similar, although the techniques used in practice vary considerably. The hybridized sequences can subsequently be detected using autoradiography (4) or immunohistochemistry (5) depending on whether radiolabeled or chromogenic labeled probes have been used.

In situ hybridization has broad range of applications and has been used to (a) localize viral infection, (b) identify sites of gene expression, (c) analyze mRNA transcription and tissue distribution, and (d) map gene sequences in chromosomes.

Advantages of in situ hybridization include: (1) greater sensitivity than Northern blotting, (2) precise cellular or subcellular localization of DNA or RNA, (3) application to archival materials (formalin-fixed paraffin embedded tissues) as well as frozen tissues, (4) combination with immunohistochemistry to detect protein as well as mRNA of interest or (5) phenotype of cells expressing the target genome, (6) detecting more than one nucleic acid sequences using different labeling methods (5–9).

However, in situ hybridization is cumbersome, expensive, and time consuming. The major steps involved in in situ hybridization are as follows: *probe preparation and labeling* (see Fig. 1), *tissue fixation, permeabilization, hybridization*, and *signal detection* (see Fig. 2).

Probe preparation: Different types of probes (double-stranded DNA, single-stranded DNA, single-stranded RNA, and oligonucleotide) can be used for in situ hybridization (6,10–13). Oligonucleotides represent the least sensitive probes and can also bind electrostatically (nonspecifically) to genomic components of cells owing to their small size. In contrast, ribo-probes (RNA probes) are the most sensitive probes, binding specifically to the target mRNA using hydrogen bonds.

Probe labeling: A number of methods are available to label probes. These include nick translation and random primer labeling which is applied in labeling of cDNA probes; 3' or 5' end labeling for labeling oligonucleotides; and in vitro transcription to synthesize RNA probes (14–17).

Radioisotope and nonradioisotope labeling are used for probe labeling in in situ hybridization.

- (a) **Radioactive labels:** Tritium (^3H), sulfur (^{35}S), and phosphorus (^{32}P) have been widely used to label probes (18,19). ^3H has the lowest energy emission and gives the finest resolution. However, using ^3H requires the longest exposure time and in some studies several months of exposure have been reported. Therefore, despite high resolution (localizing signals at subcellular levels), ^3H is not commonly used in in situ hybridization. In contrast, ^{32}P , a highly energetic nuclide requires a very short exposure time (3–5 d), but provides poor resolution. Amongst radioisotopes, ^{35}S has been widely applied because of its relatively short exposure time (10–20 d) and good resolution. Radioisotope labels are highly sensitive, provide reproducible

Probe preparation

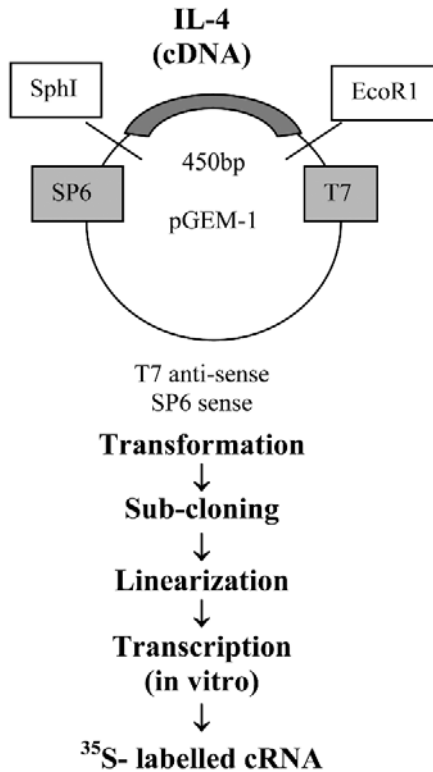


Fig. 1. Schematic representation of probe preparation and labelling. pGEM vector expressing human IL-4 genome cDNA is shown. RNA promoter sites and restriction enzyme sites for sense and anti senses are also illustrated.

results, and are easily incorporated into newly synthesized DNA or RNA. However, these labels are expensive, require long exposure, and their safety measures and waste disposal should all be taken into consideration before application.

- (b) *Nonradioactive labels:* A number of nonisotopic labels have been used in in situ hybridization. These include digoxigenin, biotin, and fluorescein. In contrast to radiolabeled probes with limited half-life (with the exception of ^3H), chromogenic labeled probes are stable, economical, and do not require long exposure. Furthermore, they provide excellent resolution and are safe to handle. However, because of their relatively low sensitivity they are not applicable where low copies of the target genome are present in a small proportion of cells within tissues (20–22).

This chapter focuses on in situ hybridization to detect mRNA using radio-labeled ribo-probes.

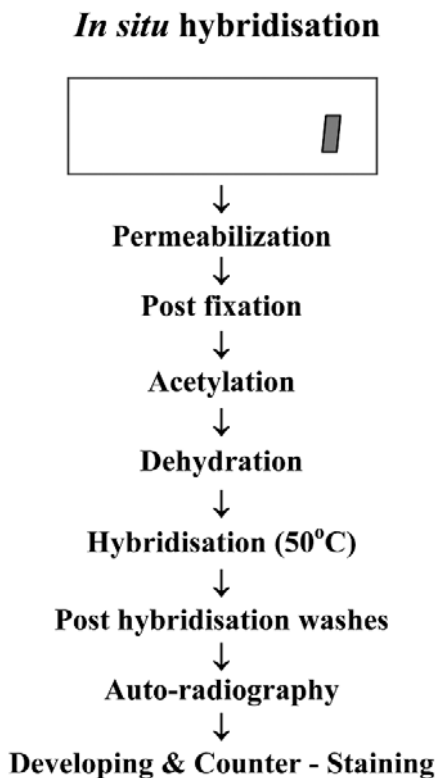


Fig. 2. Schematic representation and steps involved in in situ hybridization are illustrated.

Fixation: Tissue fixation is one of the most critical steps in in situ hybridization. This is used to maintain tissue architecture as well as to ensure the retention of the target RNA or DNA within the specimens. Fixation should be carried out with minimal delay from tissue separation particularly when the target nucleic acid is RNA. Tissue fixation also influences probe penetration to the target genome. A variety of fixatives have successfully been used for tissue fixation in in situ hybridization. These include acetone, ethanol, formalin, paraformaldehyde, and glutaraldehyde. The crosslinking fixatives such as formalin, paraformaldehyde, and glutaraldehyde are the most appropriate fixatives when RNA transcripts are the target genome, whereas precipitating fixatives such as acetone and ethanol are often used for cytospin preparations (23).

Permeabilization: This step is intended to remove proteins that surround nucleic acids, involves protease digestion, and increases signal intensity. Weak acid or detergent (Triton) treatment of sections prior to hybridization would also amplify signals by removing lipids within tissues.

Hybridization: Hybridization buffer consists of a number of high molecular weight chemicals such as dextran sulfate and Denhardt's solution which maintains a close contact between the labeled probe and target nucleic acid sequence of interest, hence formation of hybrid molecules. The use of formamide in hybridization buffer assists the formation of hybrids at lower temperature resulting in better specimen morphology (i.e., 1% formamide reduces hybridization temperature by 0.61°C for DNA:DNA and 0.35°C for RNA:RNA). The salt concentration and pH of the buffer determine the stringency conditions during hybridization. Hybridization temperatures of ~37°C for oligonucleotide, 40°C for cDNA, and 50°C for RNA probes are used and the procedure is normally carried out overnight (24).

Detection of hybridized signals: Autoradiography is the only means of detecting signals when radioactive probes are applied. Immunohistochemistry is the method of detecting hybridized signals when nonradioactive probes are used. Radioactive detection is more robust, sensitive, and reproducible, whereas nonradioactive probes provide more rapid results.

Controls for in situ hybridization: As many factors influence the outcome of in situ hybridization, several controls should be included in each run to ensure that the results are not because of experimental artifacts. The technique is controlled by including negative and positive control sections for each probe, RNase treatment of sections prior to hybridization (which should remove hybridization signals), and inclusion of a sense probe which should result in no hybridization signals. Similarly sections hybridized in the absence of the probe, but which have gone through all other steps including autoradiography serve as a further negative control.

1.2. Ribo-Probes and Their Application

Ribo-probes are also called cRNA (complementary RNA) or antisense probes which have sequences complementary to that of target mRNA. The binding between these probes and target mRNA are specific (hydrogen bonds) and not easily dissociated (25–26). To generate ribo-probes, the cDNA of interest must be inserted into specific vectors expressing RNA polymerase sites and restriction enzyme sites to allow synthesis of a sense (with a sequence identical to mRNA) and an antisense (with a sequence complementary to mRNA) probe (see Fig. 1).

Several RNA expressing vectors such as pGEM and Bluescript are commercially available. Ribo-probes have several advantages: (a) they have high affinity and thermal stability which are greater for RNA:RNA than DNA:DNA or DNA:RNA hybrids, (b) they have constant and defined size following labeling (directly) or indirectly can be made to a smaller size by alkaline hydrolysis following labeling, (c) they are produced without vector sequences, (d) they have higher specificity compared to oligoprobes, (e) unbound probe can be removed using RNase treatment post hybridization, (f) using a sense probe (with identical sequence homology with the RNA of interest), hybridization signals can be controlled.

2. Materials

All reagents should be of molecular biology grade.

2.1. Specimen Preparation

1. 0.1 M phosphate-buffered saline (PBS) (NaCl , KH_2PO_4 , Na_2HPO_4).
2. 4% paraformaldehyde/PBS.
3. 15% sucrose/PBS.
4. OCT (BDH, Lutterworth, Leicestershire, UK).
5. Isopentane (BDH).
6. Liquid nitrogen.
7. Polysine™ microscopic slides (BDH).

2.2. Probe Preparation

2.2.1. Transformation

1. Polypropylene test tube (10 mL).
2. Maxi Efficiency DH5 α ™ Competent cells (Life Technologies, Paisley, Scotland, UK).
3. LB broth base medium (Sigma, Dorset, UK).
4. LB agar medium (Sigma).
5. Ampicillin (Sigma).

2.2.2. Mini-Preparation

1. LB broth base medium (Sigma).
2. Ampicillin (Sigma).
3. Eppendorf tubes (1.5 mL).
4. Denaturing buffer: 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 5% Triton X-100.
5. 10 mg/mL lysozyme (Roche, Sussex, UK).
6. 10 mM Tris-HCl (pH 8.0).
7. Gene Clean II Kit (Strattech Scientific Ltd, Luton, Bedfordshire, UK).
8. Restriction enzymes (as required) (Life Technologies).
9. Reagents for gel electrophoresis.
 - a. Agarose (Sigma).
 - b. Ethidium bromide (10 mg/mL) (Promega, Southampton, UK).
 - c. TBE buffer: 45 mM Tris-borate, 10 mM EDTA.
 - d. Gel loading solution (6 $\times$ concentrate) (Sigma).
 - e. Molecular weight marker (100 bp and λ DNA Hind III) (Promega).

2.2.3. Maxi-Preparation

1. LB broth base (Sigma).
2. Ampicillin (Sigma).
3. Plasmid Maxi kit (Qiagen Ltd, Dorking, Surrey, UK).
4. Restriction enzymes (as required) (Life Technologies or Promega).
5. Reagents for gel electrophoresis (*see* **Section 2.2., 2.9.**).

2.2.4. Linearization

1. DNA template, e.g., human IL-4 plasmid (hIL-4).
2. Restriction enzyme (as required) (Life Technologies or Promega).
3. Phenol (pH 6.7) (Sigma).
4. Chloroform (BDH).
5. Isoamyl alcohol (BDH).
6. Reagents for electrophoresis (*see* Section 2.2., 2.9.).

2.3. In Vitro Transcription

1. 5X transcription buffer (Promega).
2. 100 mM DTT (Promega).
3. RNasin 20 U/ μ L (Promega).
4. ^{35} S – UTP 20 mCi/mL (Amersham, Buckinghamshire, UK).
5. cDNA template (1 μ g/ μ L).
6. ATP, GTP, CTP (7.25 mM each) (Promega).
7. RNA polymerase (SP6 or T7 or T3) (10 U/ μ L) (Promega).
8. 4 M NaCl.
9. RNase-free DNase (1 μ g/ μ L) (Promega).
10. tRNA (20 μ g/ μ L) (Sigma).
11. 7 M ammonium acetate.
12. Phenol:chloroform:isoamyl alcohol (25:24:1) (pH = 5.2) (BDH, UK).
13. Chloroform:isoamyl alcohol (24:1) (BDH).
14. Ethanol (70 and 100%).
15. Diethyl pyrocarbonate treated H₂O (DEPC, Sigma).
16. Filter paper #1 (Whatman).

2.4. Pretreatment of Specimen Prior to Hybridization

1. 0.1 M PBS (as previously described).
2. 0.75% glycine in 0.1 M PBS.
3. 0.3% Triton-X 100 in 0.1 M PBS.
4. 4% paraformaldehyde in 0.1 M PBS.
5. 100 mM Tris–HCl, 50 mM EDTA buffer.
6. Proteinase K (1 μ g/ μ L) in Tris–EDTA (pH 8.0) (Sigma).
7. 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0).
8. 50% formamide in 2 $\times$ saline sodium citrate (2 $\times$ SSC; 0.3 M NaCl, 0.03 M NaCitrate).
9. Ethanol (70, 90, and 100%).

2.5. Hybridization Mixture

1. 10% dextran sulfate (Sigma).
2. 5 $\times$ Denhardt's solution (Sigma).
3. 50% formamide.
4. 0.5% sodium dodecyl sulfate (SDS) (Promega).
5. 0.5% sodium pyrophosphate (Sigma).
6. 100 mM dithiothreitol (DTT) (Promega).

2.6. Post Hybridization

1. 20× SSC (3 M NaCl, 0.3 M NaCitrate, pH 7.0).
2. RNase-A (20 µg/µL) (Sigma).
3. 0.3 M ammonium acetate in 70% ethanol.
4. 0.3 M ammonium acetate in 90% ethanol.
5. 0.3 M ammonium acetate in 100% ethanol.

2.7. Autoradiography

1. Photography emulsion – Hypercoat (Amersham).
2. Dipping chamber (Amersham).
3. Kodak Safelight filter (Type GBX-2) (Sigma).
4. Slide rack (RA Lamb, London, UK).
5. Black box and black rack (RA Lamb).

2.8. Developing and Detection

1. Developer (Kodak D-19) (Sigma).
2. Rapid fixer (Sigma).
3. Hematoxylin (mercury free) (BDH).
4. Ethanol (90, 100, and 100%).
5. Xylene (BDH).
6. DPX mountant for microscopy (BDH).

3. Methods (see Notes 1–3)

3.1. Specimen Preparation

1. Place biopsy in 4% paraformaldehyde for 2 h at 4°C (*see Notes 4 and 5*).
2. Transfer the biopsy into 15% sucrose for 1 h at 4°C.
3. Transfer the biopsy into fresh 15% sucrose overnight at 4°C.
4. Embed the biopsy in OCT, mount on a piece of cork, snap freeze in isopentane precooled in liquid nitrogen, and store at –80°C.
5. Cut 6-µm sections and place on polysine™ microscope slides, dry overnight at 37°C, and store at –80°C.

3.2. Probe Preparation

3.2.1. Transformation

1. Make up LB medium agar plates in advance. Add 30.5 g LB agar base to 1 L distilled water or according to the manufacturer's instructions. Autoclave for 15 min at 121°C. Allow to cool to 55°C before adding 50 µg/mL ampicillin. Under sterile conditions, pour into plates (30–35 mL per 85-mm sterile Petri dish) and allow to solidify. Plates can be stored, covered by a lid, sealed, and inverted at 4°C prior to use.
2. Place 100 µL DH5α competent cells into a 10-mL polypropylene tube (*see Note 6*).
3. Add 10 ng plasmid (e.g., hIL-4) DNA to the above cells.

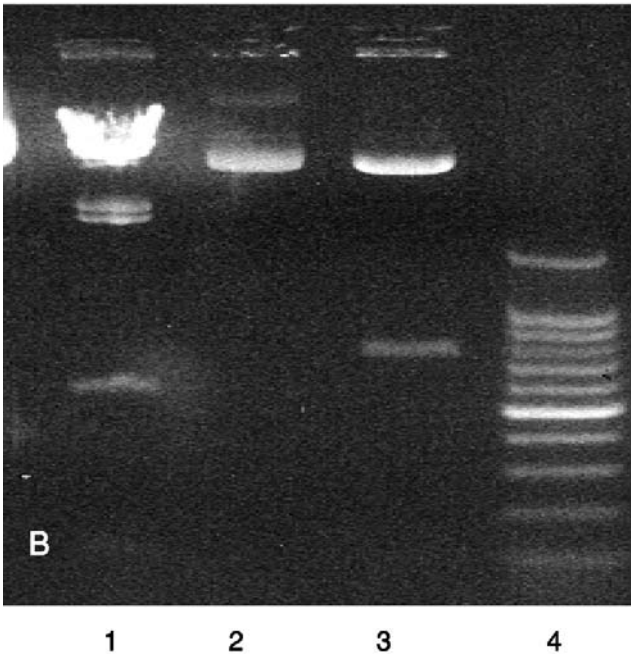
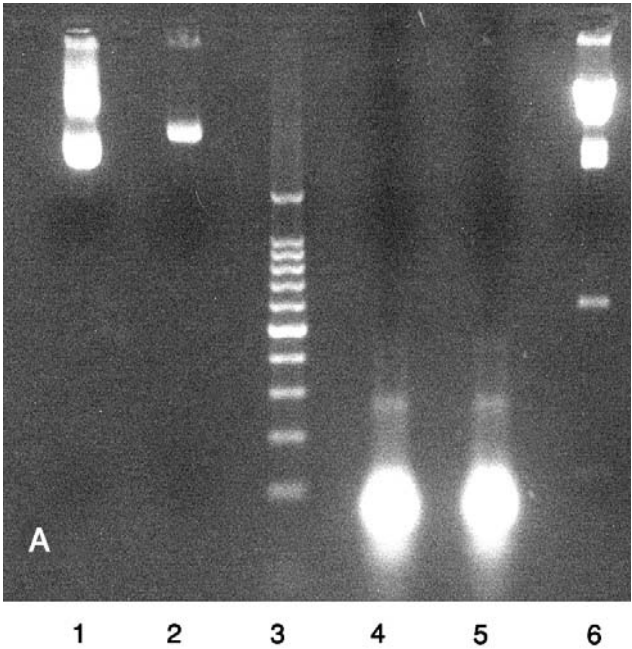
4. Place on ice for 30 min.
5. Incubate at 42°C for 45 s.
6. Place on ice for 2 min.
7. Add 900 μ L LB broth medium and incubate at 37°C for 1 h in an orbital shaker (shake vigorously).
8. Plate 100 μ L of the above mixture onto LB agar plates containing 50 μ g/ml ampicillin and incubate at 37°C for 16 h.

3.2.2. Mini-Preparation (see **Note 7**)

1. Select one single bacteria colony and resuspend in 5 mL LB medium (see **Note 8**) containing 50 μ g/mL ampicillin. Repeat this for two more colonies. Incubate at 37°C overnight in an orbital shaker with vigorous shaking.
2. Take 1.5 mL of bacteria cell suspension and spin at 12,000g for 30 s.
3. Resuspend the pellet in 350 μ L of denaturing buffer and add 25 μ L lysozyme.
4. Place the tube in boiling water for 40 s. Spin the bacteria lysate at 12,000g for 10 min.
5. Transfer supernatant to a new 1.5-mL tube and proceed using a Gene Clean Kit to isolate DNA.
6. Digest DNA using appropriate restriction enzymes. This is most commonly at 37°C for ~3 h but consult the manufacturer's instructions.
7. Prepare 1.5% agarose gel containing 0.5 μ g/mL ethidium bromide (see **Note 9**).
8. Run the digested DNA and M.W. markers in the agarose gel for 1 h at 80 mA.
9. Visualize bands under the UV light (see **Fig. 3**). Check the digested DNA has the correct size (see **Fig. 3b**) (see **Note 10**).

3.2.3. Maxi-Preparation

1. Prepare 250 mL LB broth medium in a conical flask. Autoclave the medium for 20 min at 121°C.
2. Allow to cool to 55°C, then add ampicillin at 50 μ g/mL.
3. Add 3 mL of bacteria from the mini-prep tube with confirmed insert size.
4. Incubate at 37°C overnight in an orbital shaker with vigorous shaking.
5. Perform DNA extraction using plasmid maxi kit according to the manufacturer's instruction. At the final step of the procedure check the purity of and quantify the DNA obtained:
Dilute plasmid DNA in H₂O (~500 μ L). Measure OD at 260 and 280 nm using spectrophotometry. The ratio 260:280 must be ≥ 1.8 (see **Note 11**). If the ratio is less than 1.6, discard the preparation.
Calculate the amount of DNA; e.g., OD reading at 260 nm $\times$ 50 $\times$ volume of solution = Concentration of DNA/ μ L.
6. Digest the DNA (~2 μ g) with appropriate restriction enzymes (i.e., those which will excise the probe from the plasmid) for about 3 h at 37°C or according to the manufacturer's instructions.
7. Run the digested DNA in agarose gel to confirm the correct size of the probe. Store at -20°C (see **Fig. 3b**).



3.2.4. Linearization

The plasmid DNA at this stage is in a circular form. To linearize the probe, plasmid DNA should be digested using appropriate enzymes to allow RNA polymerases to initiate at appropriate promoter sites.

1. Digest DNA (~10 µg) with an appropriate restriction enzyme for sense and another restriction enzyme for antisense probe labeling.
2. Incubate at 37°C for ~3 h.
3. Extract linearized DNA with equal volume phenol:chloroform:isoamyl alcohol (25:24:1).
4. Repeat step 3 using an equal volume of chloroform:isoamyl alcohol (24:1).
5. Remove the upper phase containing the DNA and precipitate it with 0.5 volumes 7 M ammonium acetate and 2.5 volumes of chilled ethanol.
6. Incubate at -20°C for a minimum of 3 h. Centrifuge at 13,000g for 10 min.
7. Wash the pellet with 70% ethanol. Dry the pellet and dissolve in ~10 µL of auto-claved H₂O.
8. Take 1 µL for electrophoresis (to confirm the linearization is complete) (see **Fig. 3a**).

3.3. In Vitro Transcription

Add the following to 1.5-mL Eppendorf tube:

1. 2 µL transcription buffer (5×).
2. 1 µL 100 mM DTT.
3. 0.5 µL RNasin.
4. 2 µL ATP, GTP, and CTP.
5. 1 µL linearized DNA template.
6. 2.5 µL ³⁵S UTP (see **Note 13**).
7. 1 µL SP6 or T7 or T3 RNA polymerases (as required). Incubate at 37°C for 1 h.
8. Add 1 µL of RNase-free DNase and incubate the mixture at 37°C for 10 min to digest template DNA.
9. Add 1 µL yeast tRNA, 5 µL 4 M NaCl, 175 µL DEPC-H₂O and equal volume (200 µL) of phenol:chloroform:isoamyl alcohol, vortex for 1 min. Centrifuge at 13,000g for 5 min.
10. Collect the upper phase and transfer to a new Eppendorf tube.
11. Add 0.5 volumes ammonium acetate and 2.5 volumes cooled ethanol. Incubate the mixture at -20°C for overnight or -80°C for at least 2 h.
12. Centrifuge at 12,000g at 4°C for 20 min.

Fig. 3. Agarose gel electrophoresis under UV light demonstrating (A) lane 1, plasmid DNA before linearization; lane 2, plasmid DNA after linearization. Lane 3, 100 bp molecular weight markers; lane 4, a ribo-probe sense (~300 bp); lane 5, a ribo-probe antisense (~300 bp); lane 6, Lambda DNA *Hind*III digested (B) lane 1, Lambda DNA *Hind*III digested; lane 2, plasmid DNA before digestion; lane 3, plasmid DNA after digestion with an insert of ~800 bp; lane 4, 100 bp molecular weight markers are shown.

13. Remove the supernatant and wash the pellet with 70% cold ethanol.
14. Dry the pellet for 3–5 min at room temperature. Resuspend the pellet in 20 μL H_2O (see **Fig. 3a**).
15. Take 1 μL and apply to a filter paper to determine ^{35}S incorporation. Place filter paper in scintillation fluid and count on β -counter.
16. Take 1 μL for gel electrophoresis to confirm the correct size of the newly synthesized radiolabeled ribo-probe (see **Fig. 3a**). Store the remaining at -80°C .

3.4. Pretreatment of Sections Prior to Hybridization

1. Rehydrate in PBS for 5 min at room temperature.
2. 0.75% glycine in PBS, for 5 min at room temperature.
3. Wash in $1\times$ PBS.
4. 0.3% Triton X-100 in PBS for 5 min at room temperature.
5. Wash in PBS.
6. Proteinase K in Tris buffer for 20 min at 37°C .
7. Postfix sections in 4% paraformaldehyde for 5 min at room temperature.
8. Wash $2\times$ in PBS at room temperature.
9. Block nonspecific binding using triethanolamine and acetic anhydride for 10 min at 37°C .
10. Prehybridize in 50% formamide in $2\times$ SSC for 15 min at 37°C .
11. Dehydrate for 5 min each in 70, 90, and 100% ethanol. Allow slides to dry at room temperature.

3.5. Hybridization (see Note 14)

1. Prepare a mixture of hybridization buffer and the probe (allow 1×10^6 cpm of ^{35}S -labeled probe per section and 14–20 μL buffer per slide) (see **Notes 15–18**).
2. Incubate the mixture at 60°C for 30 min in a shaking water bath (see **Note 19**).
3. Place the mixture on ice for 5 min.
4. Place slides on a staining rack. Apply 14–20 μL hybridization buffer containing the labeled probe onto each slide.
5. Cover the section with an appropriate size coverslip and incubate at 50°C for ~ 16 h.

3.6. Post Hybridization

Post hybridization washes are carried out as follows for 20 min at 42°C in shaking water bath (see **Note 21**):

1. 3 times in $4\times$ SSC.
2. Once in RNase in $2\times$ SSC at 37°C .
3. Once in $2\times$ SSC.
4. Once in $1\times$ SSC.
5. Once in $0.5\times$ SSC.
6. Once in $0.1\times$ SSC.

The following steps are performed at room temperature for 10 min:

1. 70% ethanol in 0.3 M ammonium acetate.

2. 90% ethanol in 0.3 M ammonium acetate.
3. 100% ethanol in 0.3 M ammonium acetate.

Allow slides to dry at room temperature.

3.7. Autoradiography

Perform the following in the dark room under safelight:

1. Melt emulsion in water bath at 42°C for ~30 min (*see Note 21*).
2. Pour some into a dipping chamber. Dip slides and allow to dry for at least 3 h at room temperature.
3. Place slides in a black box, seal, and store at 4°C for ~2–3 wk.

3.8. Developing and Detection

Prepare the fixer and the developer according to the manufacturer's instructions. Perform the following in the dark room under safelight:

1. Allow boxes containing slides to warm up to room temperature for ~30 min.
2. Place the rack containing slides in the developer's trough for 3.5 min.
3. Transfer the rack in a trough containing H₂O for 30 s.
4. Transfer the rack to a trough containing the fixer for 5 min.
5. Place the rack under running tap H₂O for ~20 min.

The remaining of experiment can be carried out in the laboratory under the normal light:

1. Scrape the excess of emulsion surrounding the section(s) from slides.
2. Counterstain with hematoxylin for 2 min. Wash slides thoroughly.
3. Dehydrate slides in 90, 100, and 100% ethanol for 2 min each.
4. Immerse slides in xylene 2 × 2 min (*see Note 22*).
5. Mount in DPX and cover section(s) with coverslip.
6. Observe signals using light or dark field microscopy (*see Fig. 4*).

4. Notes

All reagents should be molecular biology grade materials.

1. Gloves must be worn throughout the experiment and changed frequently to avoid RNase contamination.
2. Glassware must be baked at 220°C overnight.
3. All buffers must be treated with 0.1% DEPC, then autoclaved to ensure no traces of DEPC remain. Tris buffer is an exception, it should be prepared with autoclaved DEPC-treated H₂O.
4. Biopsies must be fixed immediately in freshly prepared 4% paraformaldehyde to prevent RNA degradation and to retain the tissue architecture. The length of fixation depends on the size of the biopsy, i.e., larger biopsies require longer fixation. In contrast, cytospin preparations do not require more than 20 min of fixation.

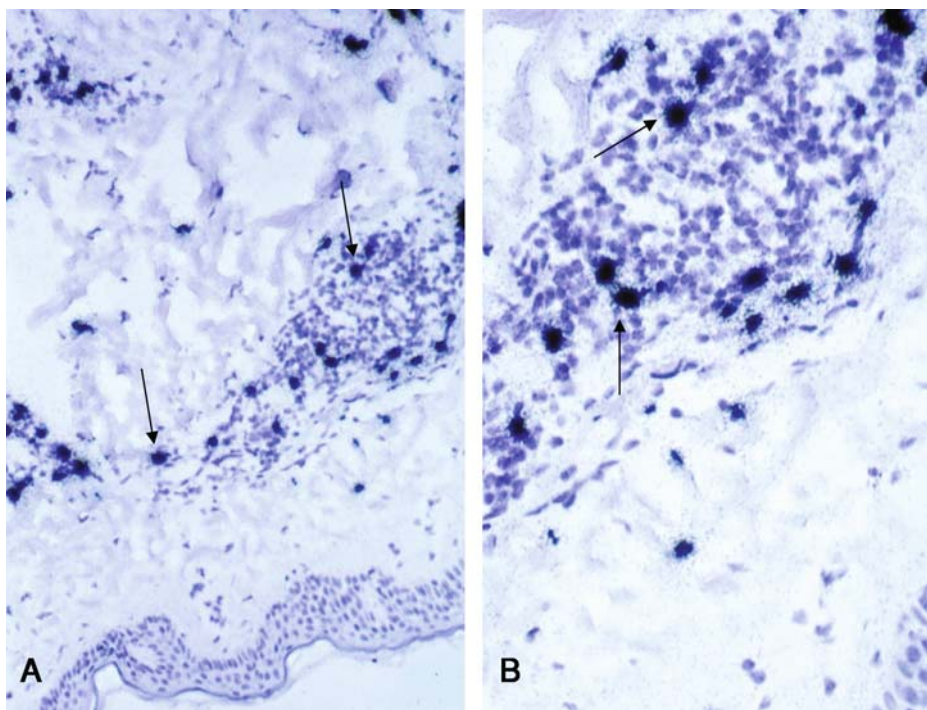


Fig. 4. Light microscopic examination of in situ hybridization performed on a skin biopsy (obtained 24 h after challenge with grass pollen extract) using ^{35}S -labelled IL-13 ribo-probe. Hybridization signals are shown as clusters of silver grains over nuclei of mononuclear cells (*a*) 20 $\times$ and (*b*) 40 $\times$ magnification.

5. Paraformaldehyde should be prepared in fume hood. Preheat PBS to 60°C. Add paraformaldehyde and monitor the temperature. Do not allow the temperature to exceed 60°C.
6. Extra care should be taken when handling DH5 α^{TM} Competent cells (*E. Coli*).
7. Mini-preparation can also be performed using Plasmid Mini Kit (Qiagen).
8. LB broth and LB agar are prepared according to the manufacturer's instructions.
9. Ethidium bromide is believed to be carcinogen and should not be discarded in the waste sink. It should be inactivated before discarding.
10. Protect eyes when analyzing gels under the UV light by viewing under perspex screen and/or wearing eye goggles.
11. Ratios below 1.8 for 260/280 nm indicate impurity of DNA preparation.
12. Phenol should be handled with care and not discarded in the waste sink.
13. When using ^{35}S , the procedure should be carried out behind the perspex screen.
14. All steps used in any in situ hybridization should be empirically determined as type of tissue, method of fixation used, choice of probe, type of and method of labeling,

concentration of the probe, the stringency of hybridization and post hybridization conditions, and type and level of expression of the target nucleic acid sequence of interest all influence the outcome of the results.

15. Formamide used in hybridization buffer should be deionized before use (using 10% (w:v) Mixed Bed Resin M-8032, Sigma).
16. Formamide can methylate DNA and is harmful to unborn babies and should therefore be handled with extreme care in the fume hood.
17. Acetylation of amino groups reduces nonspecific electrostatic binding of probes, particularly when ^{35}S -labeled probe is used.
18. Acetic anhydride should be used in the fume hood.
19. Heating the probe at 60°C is intended to unfold tertiary structure of ribo-probe and facilitate hybridization.
20. 20× SSC is prepared by 175.3 g sodium chloride and 88.2 g sodium citrate in 1.0 liter (pH = 7.0).
21. The stringency of post hybridization washes is critical for specific hybridization signals. Therefore high temperature and low salt concentration should be included in post hybridization washes to ensure hybridized signals are not artifacts.
22. Emulsion should not be stored in the same fridge as radioisotopes.
23. Xylene must be handled in the fume hood wearing nitrile gloves.

Acknowledgment

The author is grateful to Professor SR Durham and Dr MR Jacobson for their helpful comments on this chapter.

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