In-situ hybridization using GeneDetect™ oligonucleotide probes

^{35}S-labeled probe, frozen tissue sections. Detection by emulsion or film autoradiography.

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Introduction:

Non-radioactive oligonucleotide probe labeling methods are becoming increasingly popular with in situ hybridization. Nevertheless the use of radioactive oligonucleotide probes remains strong due to the fact that they provide excellent sensitivity and allow detection of low abundance mRNAs in tissue sections. Many investigators have achieved excellent results with radioactive in situ hybridization and are therefore loath to change. Oligonucleotide probes are most commonly labeled using the enzyme terminal deoxynucleotidyl transferase (TdT) to add a "tail" of 25-50 deoxynucleotides labeled with ^{35}Sulphur (^{35}S) to the 3′ end of the oligonucleotide. This protocol for example uses [^{35}S]-labeled deoxyadenosine 5'-(α-thio) triphosphate to tail GeneDetect™ oligonucleotide probes. One advantage of radioactive in situ hybridization is that it allows for the intensification of probe signal simply by re-exposing tissue sections to film or emulsion for longer periods.

Other Materials/Kits required:

For best results with GeneDetect™ oligonucleotide probes and in-situ hybridization we recommend using combinations of the following reagents and kits.

1. NEN/Perkin Elmer, Deoxyadenosine 5'-(α-thio) triphosphate, [^{35}S], 250µCi in 20µl (9.25MBq), HIGH specific activity of 1250Ci/mmol. Cat# NEG 034H

NEN/Perkin Elmer, http://lifesciences.perkinelmer.com/

2. Invitrogen, Terminal deoxynucleotidyl transferase (rTdT) 500U @ 15U/µl, Cat#10533-016
3. Invitrogen, 5X Terminal deoxynucleotidyl transferase (rTdT) buffer, Cat#16314-015

Invitrogen, http://www.invitrogen.com

4. G-50 Sephadex columns (various suppliers). Amersham Pharmacia Biotech (APB), ProbeQuant G-50 Micro Columns, Cat# 27-5335-01. Qiagen, QIAquick Nucleotide Removal Kit, Cat# 28304. Ambion, NucAway™ Spin Columns, Cat# 10070.

APB, http://www.apbiotech.com
Qiagen, http://www.qiagen.com
Ambion, http://www.ambion.com

5. Emulsion or Film autoradiography. Amersham Pharmacia Biotech (APB), Hyperfilm βmax, Cat# depends on sheet size, Hypercoat Nuclear Emulsion LM-1, Cat# RPN40.

APB, http://www.apbiotech.com

6. Sigma BSA (Molecular Biology grade) acetylated, Cat#B8894.

Sigma, http://www.sigma.com

Abbreviations/Codes:

RT = room temp
4°C, standard refrigerator
-20°C, non-cycling standard freezer (i.e. should not be auto-defrost type)
-70°C, ultra low temperature freezer for long term storage of biological materials
2.5pmol of a 48mer oligonucleotide ~ 35ng
x = times, as in wash 2 times
X = times (concentration), as in 2 X SSC

Protocol Summary (3 steps)

A. Labeling your GeneDetect™ oligonucleotide probe with ^{35}S-dATP.
Labeling (2 hours).
Purification/quantification (1 hour)

B. **Tissue Preparation** (30 mins)
Post Fix tissue sections.

C. In-situ hybridization of ^{35}S-labeled GeneDetect™ oligonucleotide probe to tissue
Hybridization with probe (18-40 hours).
Post-hybridization washes (2 hours).

D. **Detection steps**

1. Film autoradiography (5-7 days)
2. Emulsion autoradiography (several weeks)

This protocol can easily be completed over 2 days when solutions are pre-made*. Exposure times required for probe detection will depend on detection method used (film versus emulsion autoradiography) and signal intensity.*

FOLLOW YOUR INSTITUTIONAL SAFETY AND REGULATORY GUIDELINES WHEN USING RADIONUCLEOTIDES.

A. Labeling your GeneDetect™ oligonucleotide probe with ^{35}S-dATP.

Labeling

The aim of this step is to tail your probe at the 3′ end with multiple ^{35}S-labeled nucleotides (~25-50 nucleotides long) that can be detected by film or emulsion autoradiography later on once probe has bound to target mRNA within the tissue section. Note that tailing is quite different from 3′-end labeling which only adds a single nucleotide to the probe. For in-situ hybridization 3′ tailing is preferred.

Briefly, add IN ORDER the following together in an autoclaved aliquot tube:

GeneDetect™ probe (2.5-5pmol)	1µl
dH_20 (sterile, autoclaved)	6.5µl
5X TdT reaction buffer	4µl
BSA (2mg/ml)	0.5µl
^{35}S-dATP	5µl
Enzyme (TdT, 45U)	3µl

Keep TdT on ice at all times and return to freezer as soon as possible. The most common reason labeling reactions fail is poor/inactive enzyme. Therefore use new enzyme if possible. Total reaction volume ~ 20µl. Mix the reagents together by gently tapping the tube. Briefly (5 sec) spin in a TOMY spinner or equivalent, see http://www.tomytech.com (5000rpm, 2000g, Cat# PMC-060) to ensure all of the reaction mixture is at the bottom of the tube. Alternatively tap the tube on a bench to achieve the same result.

Allow the tailing reaction to proceed for 1-2 hrs at 37°C. Stop by incubating reaction mixture at 70°C for 15 mins.

Purification/quantification

With in-situ hybridization it will be necessary to purify your ^{35}S-labeled probe.

Use one of the commercial spin columns to remove unincorporated ^{35}S-nucleotides from your probe mixture. Follow the manufacturers recommended procedures and purify the probe.

It will then be necessary to calculate % incorporation and the specific activity of your radiolabeled oligonucleotide probe to ensure that the labeling reaction has been successful.

From a successful labeling reaction you might expect 25-90% incorporation of ^{35}S-dATP into probe and the generation of a labeled oligonucleotide probe with a specific activity within the range 5 - 40 x 10^8 cpm/µg GeneDetect™ probe.

Calculating % incorporation of label:

Total label: 5µl of ^{35}S-dATP added to the labeling reaction contains 62.5µCi (supplied at 250µCi in 20µl). Given that 1 µCi = $2.2x10^6$ cpm, 62.5µCi = $1.38x10^8$ cpm

Percentage incorporated: Take a small quantity of the solution that emerges from the spin column. Add into scintillation fluid and count cpms. From this calculate the total cpms eluted from the spin column.

(Total eluted (cpm) / $1.38x10^8$) X 100 = % incorp.

Calculating specific activity:

Specific activity may be broadly defined as the level of radioactivity (in counts per minute or "cpm") exhibited by the products of a reaction in relation to the total mass of those products. Excellent in situ hybridization will usually result if probes are labeled to specific activities in the range:

5 - 40 x 10^8 cpm/µg GeneDetect™ probe.

Calculate the specific activity using the total cpms eluted from spin column and the fact that 35-70ng of probe (2.5-5pmol) was labeled (assume 100% elution of the oligonucleotide or adjust if specific % recovery is known).

Store probes at -20°C until use. Half-life of ^{35}S = 87.9 days. ^{35}S-radiolabeled probes then are good for at least 2-3 months.

B. Tissue Preparation:

Fresh tissue should be rapidly removed from the animal and placed on aluminum foil. Snap freeze the tissue by immediately placing it into a –70°C freezer for several hours. Tissue can be stored this way (if subsequently fully wrapped in foil and placed in sealed plastic bags) for several years with little or no tissue RNA degradation. For in-situ hybridization, 16µm frozen tissue sections are cut and mounted onto poly-L-lysine coated (or equivalent) slides. Sections can be cut using a cryostat and tissue must be kept frozen during section preparation. Tissue sections (mounted on slides and stored in plastic slide boxes) can also be stored long term in a –70°C freezer. We have successfully performed in-situ hybridization on frozen tissue sections stored for over 5 years at –70°C.

All of the following steps in the protocol are performed by incubating slides containing tissue specimens (held in a slide rack or Coplin jar) in the indicated solutions. All solutions are made up fresh and are used only once unless indicated otherwise. All solutions are presumed to be at RT unless otherwise indicated.

Post-Fix:

Remove your slides (containing the tissue sections) from the -70°C freezer and immediately place sections into cold (4°C) 4% PFA in 0.1M PB solution (made up within the last 7 days and stored at 4°C). DO NOT let your tissue sections come to room temperature or defrost before fixing. Fix for 10-15 mins.

Wash slides 3 x 5 mins with 0.1M PB.

Incubate slides with 100% ETOH for 5 mins. Air dry slides.

At this point your tissue RNA is "reasonably" safe from RNases and since GeneDetect™ oligonucleotide probes are resistant to degradation by RNases we have found that the following steps only require that sterile, autoclaved solutions be used. DEPC-treatment of solutions is not required.

C. In-situ hybridization of ^{35}S-labeled GeneDetect™ oligonucleotide probe to tissue:

Prepare Hybridization Buffer:

Hybridization Buffer (prepare in 50 ml Falcon tube with screw cap, store @ -20°C)

To make 20 mls.

20 X SSC	4 ml
Dextran sulphate	4 g
Formamide (deionized)	10 ml

Add these all together then sonicate (with cap on) for about 3-4 hrs.

Then add aliquots of the following (stored at -20°C)

PolyA (10mg/ml)	0.5 ml
ssDNA (10mg/ml)	0.5 ml
tRNA (10mg/ml)	0.5 ml
DTT (of 1M solution)	2 ml
50 x Denhardts	0.2 ml

Mix VERY well before use. Hybridization buffer can be pre-made and stored long-term at -20°C. Bring to 37°C on the day of use.

Hint: Hybridization buffer is generally quite viscous at RT. However when it is heated to 37°C it loses some of its viscosity and becomes "runny" allowing for easier pipeting and more homogenous mixing of probe with hybridization buffer. Therefore heat to 37°C before addition of probe.

Add probe to hybridization buffer (brought to 37°C). **Add 0.5 x 10^6 cpm of labeled probe per 50µl of hybridization buffer.** Mix well BY HAND ONLY (no vortexing) to ensure even probe dispersal.

Hybridization of tissue sections with probe:

Place air-dried slides into the hybridization chamber.

Hint: Plastic tupperware containers can be used as good in-situ hybridization chambers. We balance slides on an overturned plastic 1.5ml microfuge tube rack placed into a tupperware container which is then partially filled with dH_20 to keep the chamber humid and the sections from drying out.

Carefully overlay each tissue section with well mixed hybridization buffer containing probe. The volume of hybridization buffer you will need to add to each tissue section will depend upon the size of the tissue. 50µl is a normal volume for a tissue section 2cm x 2cm. With a large block of human tissue you may require a higher volume.

Cover each section with a piece of Parafilm about the same size as the tissue. Make sure you don't get any air bubbles trapped between the section and the Parafilm. To stop hybridization buffer running off the section with the overnight hybridization carefully ensure all of your slides are kept level. It is important not to let tissue sections dry out. If your slides dry out they have a higher chance of having high background staining. Further, not having your slides level could lead to uneven hybridization of the probe across the tissue section.

Incubate your slides in the sealed humid hybridization chamber (i.e. water filled tupperware container) overnight at 37°C (approx 18 hrs) by putting the lid on the container and carefully placing it in an oven set at 37°C. Hybridization can be left for up to 40 hrs to increase signal intensity as long as tissue sections DO NOT dry out.

Post-hybridization washes:

For washes, prepare 0.5 and 1 X SSC solutions from 20 X SSC and add DTT on the day of use. (Note: make sure stock 0.5 and 1 x SSC solution are at 55°C before incubating slides.) Note: 1.2g DTT into 800mls SSC = 10mM

At the end of the hybridization remove the parafilm from sections by using forceps/tweezers before tipping off the hybridization buffer and putting the slides into wash solutions. Using a shaking water bath at 55°C, give slides the following washes

Quick wash	1 X SSC (10mM DTT) RT
2 x 15 mins	1 X SSC (10mM DTT) 55C
2 x 15 mins	0.5XSSC (10mM DTT) 55C
1 x 10 mins	0.5XSSC (10mM DTT) RT

Quick dip slides in dH_2O (to remove salts)

Dehydrate 1 x 10 min in 100% ETOH at RT. Allow slides to air dry.

C. Detection steps

At this point we offer the option of two different detection methods. Both methods have advantages and disadvantages depending on your requirements. Autoradiography is used to detect and quantitate radioactive probe hybridized to your tissue section. Autoradiographic film detection is most useful for rapid detection in experiments. Emulsion autoradiography is required to obtain measurement of gene expression (mRNA levels) at the single cell level but often requires exposure times significantly longer than for film autoradiography. Commonly both are used in parallel in the same experiment.

1. Film autoradiography

Tape slides into the back of an autoradiography casette (for example, use a Hyper-cassette available from Amersham Pharmacia Biotech Cat# RPN 11642-11650) with tissue sections facing up. In a dark room (with safe light) place film (for example Hyperfilm βmax also from APB) directly onto the slides. The "generally" duller "emulsion" side of the film should go towards the tissue sections. You may want to "pack" the cassette with a few pieces of cardboard to ensure a tight fit between slides and film so as to gain a sharp image. Close the cassette and place at 4°C initially for 5-7 days. Develop the film manually using the manufacturers recommended procedures. If the signal is weak on developing the film, then expose sections to film for longer periods. As an alternative to film autoradiography, researchers with access may decide to "develop" slides using a PhosphoImager or comparably sensitive imaging system for ^{35}S.

2. Emulsion autoradiography

Single-cell resolution can be obtained by coating slides with autoradiographic emulsion. Follow the manufacturers recommended procedures to coat, expose and develop slides.

Controls:

Of course the most important part of any experimental procedure is the inclusion of controls. However often with in situ hybridization experiments controls not used properly, if at all. In carrying out an in situ hybridization experiment one has to be confident that the hybridization reaction is specific and that the probe is in fact binding selectively to the target mRNA sequence and not to other components of the cell or other closely related mRNA sequences. In addition if no staining is observed with the probe does this mean that there really is no expression of that mRNA in the tissue or does it mean that there may be a problem with tissue preparation or the tissue itself or your technique?

If the correct controls are included in the experiment we can, with high certainty, answer these questions. Note that the polyd(T) probe is included with all orders and that the nonsense probe and pan-species actin probe are contained within our Control Probes product. Both sense and antisense probes are sent when you order a probe from us in amounts that allow for 10X competition studies to be performed as mentioned below. RNase enzyme should be purchased from a trusted supplier.

Controls for tissue mRNA quality and the efficacy of your protocol.

If the quality of your tissue is poor and/or your RNA is degraded it will be very hard to get good results with in situ hybridization. There are however a number of controls you can add to your experiment to verify the status of your tissue and mRNA within the tissue. If you are using fresh tissue and these controls are negative, then this suggests a problem with your technique or protocol.

Poly d(T) probe.

The poly d(T) probe we supply will detect total mRNA polyA tails. If a very weak signal is obtained using this probe then it is likely your tissue RNA is degraded. The chance of detecting a specific mRNA in this tissue is therefore unlikely.

Probes against house keeping sequences.

Some genes are always expressed constitutively such as Actin or beta-tubulin. We offer probes to detect these mRNAs. A low signal once again suggests tissue RNA degradation.

Positive control.

Perform in situ hybridization using the correct oligonucleotide probe on a fresh, positive control tissue known to have the sequence of interest (not always possible). If you detect no signal then this suggests the problem exists within your technique or protocol.

Specificity controls.

Determine that your probe is only binding to RNA.

When probing for mRNAs one can determine that the binding is specific to RNA by digesting the tissue with RNases prior to hybridization with the oligonucleotide probe. The absence of binding after RNase treatment indicates that binding was indeed to RNA within the tissue.

RNase solution (200ml) can be made up as follows.

0.8ml 10mg/ml RNase (Sigma)
4ml 1M Tris buffer (pH 7.5)
0.4ml 0.5M EDTA
Add dH_2O to 200ml (i.e. add 194.8ml).

Sections should be incubated in RNase solution for 1 hour at 37°C immediately after the 3 x 5 mins washes with PB that follow post fixation. These RNase-treated sections should be compared with sections also incubated with RNase solution (minus the RNase) for 1 hr at 37°C. Following RNase treatment, sections should be washed 3 x 5 min in PB before entering the main protocol again.

Specific versus non-specific binding.

The first control involves hybridization of the tissue with both labeled sense and antisense probes in parallel. The antisense probe in theory detects both the target mRNA and any non-specific targets it can bind to due to the chemical properties of the probe (but not due to the probe sequence). The sense control probe gives a measure of non-specific probe binding only due to the chemical properties of the probe. In essence if your sense probe detects nothing, then you can be sure that any signal detected by your antisense probe is due to sequence-specific binding to mRNA and not due to binding to other targets within the cell.

Competition studies with labeled and excess unlabeled probes can also help distinguish between specific versus non-specific binding. This is because by definition specific binding is saturable (i.e. there are finite target mRNA molecules to which the probe can bind) while non-specific binding is not (there are infinite non-specific targets). Therefore excess unlabeled probe can displace (by competition for binding sites) the specific binding of the labeled probe (i.e. to the target mRNA) but not non-specific binding of the labeled probe.

We recommend co-hybridizing tissue with:

1. Excess 10 X molar concentration of unlabeled antisense probe plus the usual concentration of labeled antisense probe

2. Excess 10 X molar concentration of unlabeled nonsense probe plus the usual concentration of labeled antisense probe

The nonsense probe should preferably have a similar CG content, a similar length and have no homology to the sequence of interest.

It is important to note however that competition studies do not verify the identity of the mRNA to which the labeled probe is binding since both the labeled and unlabeled probes have the same sequence.

Determine that your probe is binding to the correct target sequence.

The best way to ensure that your probe is binding to the correct target sequence is by choosing a correct probe sequence from the start and having high stringency hybridization and wash conditions in your experiment.

In summary we recommend that the following controls are performed in parallel with your in situ experiments.

1. polyd(T) probe is hybridized to sections. What is the quality of the mRNA in your tissue sample?

2. RNase treatment of sections before labeled antisense probe hybridized. Is probe binding to mRNA?

3. Hybridize in parallel labeled sense and labeled antisense probes. Is the probe binding to the tissue in a sequence-specific fashion?

4. Can sequence-specific binding be displaced? Hybridize labeled antisense probe in presence of

    a) 10X unlabeled antisense probe and separately in presence of
    b) 10X unlabeled nonsense probe.

Solutions and chemicals required:

If the purchases of salts, buffers and the like are made from Sigma or another similar supplier we suggest you use Molecular Biology grade products.

All glassware, plasticware, pipette tips etc should be autoclaved.

dH_20 (distilled water). Must also be autoclaved.

Standard buffers. SSC, PBS, TE, PB (Any protocols book will have the recipes for these).

0.1 and 0.2M phosphate buffer (PB, store on lab shelves @ RT)

20 x SSC (stock, store on lab shelves @ RT)

100% Ethanol

Parafilm

Hybridization chambers (fill with dH_20 before overnight hybridization)

Oven @ 37°C, for overnight hybridizations.

Shaking water bath @ 55°C

Wash containers that will hold slides and SSC wash solutions (Plastic Coplin Jars work well)

Dextran sulphate (Sigma, 50g D8906, store @ 4°C)

Deionized formamide (use as bought, Sigma, 100ml F9037, store 10ml aliquots @ -20°C)

PolyA (Sigma, 25mg, P9403, dissolve to 10 mg/ml in dH_2O, store 0.5ml aliquots @ -20°C)

ssDNA (Life Technologies, 10 mg/ml, use as bought, store 0.5ml aliquots @ -20°C)

tRNA (use as bought Sigma, 10 mg/ml, store 0.5ml aliquots @ -20°C)

DTT (various sources, 154mg/ml in dH_2O sterile = 1M solution, store as 1ml aliquots @ -20°C)

50XDenhardts (use as bought, if required dissolve with dH_20, store 0.2ml aliquots @ -20°C)

BSA (dilute stock 20mg/ml Molecular Biological grade from Sigma Cat#B8894 to 2 mg/ml using dH_2O, store @ -20°C)

4% PFA (in 0.1M PB, phosphate buffer): 8g PFA dissolved in 100ml of H_2O. Heat to partially dissolve then add 1M NaOH dropwise until solution clears. Add 100ml of 0.2M PB, mix. Filter. Cool to 4°C before use. Store at 4°C. Prolonged storage (>1 week) may require that pH is checked. pH should be around 7.4, DO NOT autoclave.

0.5XSSC and 1XSSC buffers + 10mM DTT for washes. (Make 0.5XSSC and 1XSSC buffers by diluting 20XSSC with dH_20. Into 200ml SSC (0.5X or 1X) buffer add 0.31g DTT powder and mix well. We usually make up 200ml 1XSSC (0.31g DTT) and 200 ml 0.5XSSC (0.31g DTT) on the day of washes because DTT inactivates in solution.

Questions? For our customers we guarantee a response within 24 hrs.

Email us at Scientific@GeneDetect.com