In-situ hybridization using GeneDetect® oligonucleotide probes

GreenStar* DIGOXIGENIN (DIG)-hyperlabeled probe, frozen tissue sections, Detection by direct fluorescence, AP (alkaline phosphatase) or tyramide signal amplification (TSA)

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Introduction:

Of the common non-radioactive methods used with in-situ hybridization, digoxigenin§ (DIG)-based detection remains one of the most sensitive. The methods described below allow for reproducible detection of high (direct fluorescence) medium (indirect amplified AP detection) to low abundance (detection incorporating tyramide signal amplification) target mRNAs in frozen tissue sections.

Other Materials/Kits required:

For best results with GreenStar* DIG-labeled GeneDetect® oligonucleotide probes and in-situ hybridization we recommend using combinations of the following probe detection reagents and kits.
For maximal detection sensitivity

1. DakoCytomation, GenPoint™ Catalyzed Signal Amplification System, Cat#K0620 with GenPoint™ Ancillary package, Cat#K0621.

DAKO, http://www.dakocytomation.com

Note: The DakoCytomation GenPoint™ Signal Amplification System (described by Dako for use with biotinylated probes) can be used successfully with any GreenStar* labeled probe as long as a HRP conjugate is included in the protocol immediately prior to the addition of the amplification reagent. Therefore if you choose to use the GenPoint™ kit you must also purchase the Anti-DIG/HRP coupled antibody (below) to replace the Streptavidin/HRP complex that comes in the kit.

2. Dako Anti-DIG/HRP Fab fragments, Cat#P5104

For medium abundance mRNAs

3. Anti-DIG-AP Fab fragments, Cat#1093274
4. NBT/BCIP Ready-to-use tablets, Cat#1697471

Roche, http://biochem.roche.com or

5. NBT/BCIP *FAST*™ tablets from Sigma.

Sigma, http://www.sigma.com

For high abundance mRNAs

6. Anti-DIG-fluorescein (FITC) Fab fragments, Cat#1207741
7. Anti-DIG-rhodamine Fab fragments, Cat#1207750

Roche, http://biochem.roche.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. **Tissue Preparation** (30 mins)
Post Fix tissue sections.

B. In-situ hybridization of GreenStar* DIG-labeled GeneDetect® oligonucleotide probe to tissue
Hybridization with probe (18-40 hours).
Post-hybridization washes (2 hours).

C. **Detection steps** (Several hours)

1. Direct fluorescent detection of GreenStar* DIG-labeled oligonucleotides using anti-DIG antibodies conjugated to either fluorescein (FITC) or rhodamine for highly expressed mRNAs.
2. Indirect detection using anti-DIG antibody conjugated to alkaline phosphatase for moderately expressed mRNAs.
3. Tyramide Signal Amplification (TSA) for low abundance mRNAs.

This protocol can easily be completed over 2 days when solutions are pre-made.

A. 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/defrost before fixing. Fix for 10-15 mins.

Wash slides 3 x 5 mins with 0.1M PB.

For detection method 3 it is necessary to inhibit endogenous peroxidase activity. Incubate sections in 0.3% H_2O_2 in PBS for 30 min at RT.

Wash slides 3 x 5 mins with 0.1M PB (remove these washes and the previous step if detection method 1 used).

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.

B. In-situ hybridization of GreenStar* DIG-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:

Add the GreenStar* DIG-labeled oligonucleotide probe to the hybridization buffer (brought to 37°C). The optimal amount of probe you will need to add will require a bit of trial-and-error, but is usually within the range 100-1000ng/ml of hybridization buffer with 200ng/ml a good starting concentration.

Mix well BY HAND to ensure even probe dispersal. Since each GreenStar* vial contains 5µg (5000ng) of antisense DIG-labeled probe you should be able to make 25mls of hybridization buffer if your probe is diluted to 200ng/ml.

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

Slides remain in the last wash solution until the next step.

C. Detection steps

At this point we offer the option of three different detection methods depending on the abundance of your target mRNAs/detection sensitivity required. DIG-labeled oligonucleotides can be detected after hybridization by an anti-DIG antibody conjugated to (1) either of the fluorescent molecules fluorescein (FITC) or rhodamine for direct fluorescent detection, (2) the enzyme alkaline phosphatase which catalyzes a chemical reaction for indirect probe detection or (3) horseradish peroxidase (HRP) that allows for incorporation of tyramide signal amplification into your detection step.

1. Direct fluorescence for highly expressed mRNAs.
2. Indirect detection using an anti-DIG antibody conjugated to alkaline phosphatase for moderately expressed mRNAs.
3. Tyramide Signal Amplification (TSA) for low abundance mRNAs (maximum sensitivity).

1. Direct fluorescent detection of GreenStar* DIG-labeled oligonucleotides using anti-DIG antibodies conjugated to either fluorescein (FITC) or rhodamine for highly expressed mRNAs.

Antibodies (anti-Digoxigenin-fluorescein (FITC) Fab fragments and anti-Digoxigenin-rhodamine Fab fragments both made in sheep) can be used for the direct fluorescent detection of DIG-labeled compounds. Both antibodies are available from Roche Molecular Biochemicals.

Hint: Small antibody aggregates in the Anti-DIG-FITC/rhodamine solutions may lead to higher background staining. It is therefore suggested that the vial(s) be centrifuged for 5 mins at 13,000 rpm before first use. Thereafter, it is sufficient to centrifuge each for 1 min directly before dilution.

Transfer slides to Tris-buffered saline (TBS, 100mM Tris HCl, 150mM NaCl, pH 7.5)

Wash sections 3 x 5 mins in TBS.

Optional. Cover sections for 30 mins with blocking solution (TBS + 0.1% Triton X-100 + 1% normal sheep serum from Sigma or 1% proprietary blocking agent from Roche).

Pour off blocking solution and incubate sections for 4 hrs minimum at RT with anti-DIG (FITC or rhodamine) diluted 1:50 to 1:500 (1:100 initial recommended dilution) in TBS + 0.1% Triton X-100 + 1% normal sheep serum from Sigma or 1% proprietary blocking agent from Roche).

Wash sections 3 x 5 mins in TBS.

Finally rinse sections several times in dH_2O to remove salts and then mount sections using a medium containing an anti-fading agent (for example, Vectashield, Vector Labs) and evaluate staining using a fluorescent microscope.

FITC, Green: Excitation $_{max}$[nm]: 494, Emission $_{max}$[nm]: 523 (pH 8.0)

Rhodamine, Red: Excitation $_{max}$[nm]: 555, Emission $_{max}$[nm]: 580 (pH 8.0)

2. Indirect detection of GreenStar* DIG-labeled oligonucleotides using an anti-DIG antibody conjugated to alkaline phosphatase for moderately expressed mRNAs.

GreenStar* DIG-labeled oligonucleotides can be detected after hybridization by an anti-DIG antibody conjugated to the enzyme alkaline phosphatase (AP) which catalyzes a chemical reaction for indirect probe detection.

An antibody (anti-Digoxigenin-AP, Fab fragments, made in sheep) for the detection of DIG-labeled compounds is available from Roche Molecular Biochemicals.

Hint: Small antibody aggregates in the Anti-DIG-AP may lead to higher background staining. It is therefore suggested that the vial be centrifuged for 5 min at 13,000 rpm before its first use. Thereafter, it is sufficient to centrifuge for 1 min directly before dilution.

Transfer slides to Tris-buffered saline (TBS, 100mM Tris HCl, 150mM NaCl, pH 7.5)

Wash sections 3 x 5 mins in TBS.

Optional. Cover sections for 30 mins with blocking solution (TBS + 0.1% Triton X-100 + 1% normal sheep serum from Sigma or 1% proprietary blocking agent from Roche).

Hint: The use of fetal calf serum as a blocking agent is not advised due to its endogenous alkaline phosphatase activity.

Pour off blocking solution and incubate sections for 4 hrs minimum at RT with anti-DIG antibody diluted 1:100 to 1:1000 (1:200 initial recommended dilution) in TBS + 0.1% Triton X-100 + 1% normal sheep serum from Sigma or 1% proprietary blocking agent from Roche).

Wash sections 3 x 5 mins in TBS.

Dissolve one NBT/BCIP ready to use tablet from Roche in 10mls of dH_20 to make 10 mls of staining solution or alternatively prepare yourself (below).

10mls of NBT/BCIP staining solution:

0.4 mg/ml NBT (Nitro blue tetrazolium chloride)

0.19 mg/ml BCIP (5-Bromo-4-chloro-3-indolyl-phosphate, toluidine salt)

100mM Tris buffer, pH 9.5
50mM $MgSO_4$

Optional: Add 10µl of stock 1M levamisole solution per 10 mls staining solution (1mM levamisole). Although unlikely, some endogenous phosphatase activity may remain at this stage of the protocol and cause increased non-specific binding. The option is to add levamisole (Sigma). For convenience prepare a 1M stock solution in dH_2O which will be stable at 4°C for several weeks. If endogenous phosphatase activity is particularly high (found in certain tissue types) increase the amount of levamisole added to the detection solution.

Incubate sections in a Coplin jar or similar with the staining solution. A blue precipitate will form. The development time will depend on numerous factors but is usually between several minutes for high abundance to several hours or even overnight for low level mRNAs.

Stop the reaction by rinsing the slides several times in tap water.

Finally rinse in dH_2O and mount slides with any water-soluble mounting medium (do not use xylene-based mounting medium) or optionally counter stain the sections.

3. Tyramide Signal Amplification (TSA) for low abundance mRNAs.

Tyramide Signal Amplification (TSA) is a patented technology that can be used to amplify DIG signals in standard in-situ hybridization protocols, resulting in significant increases in sensitivity without loss of resolution or increase in background.

TSA is easily integrated into any protocol following an initial addition of horseradish peroxidase (HRP) to the probe. This is accomplished using anti-DIG-HRP conjugated antibodies. TSA is extremely sensitive and is highly recommended for detecting mRNAs expressed at low levels.

We have used TSA successfully with in situ hybridization on frozen tissue sections. Follow the manufacturers recommended procedures to amplify the DIG signal.

1. DakoCytomation, GenPoint™ Catalyzed Signal Amplification System, Cat#K0620 with GenPoint™ Ancillary package, Cat#K0621. With this kit you will also need to purchase the Anti-DIG-HRP conjugated antibody, Cat#P5104 from DakoCytomation.

Controls:

Of course the most important part of any experimental procedure is the inclusion of controls. However often with insitu hybridization experiments controls not used properly, if at all. In carrying out an insitu 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 2 x 5 mins washes with PBS 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 2 x 5 min in PBS 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, Sigma P9403, dissolve to 10 mg/ml in dH_2O, store 0.5ml aliquots @ -20°C)

ssDNA (Salmon sperm/Salmon testes DNA, 10 mg/ml, Sigma D7656, use as bought, store 0.5ml aliquots @ -20°C)

tRNA (use as bought, Sigma R5636, 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)

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.

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