In-situ hybridization using RNA probes prepared with GeneDetect™ ONE-STEP RNA probe synthesis templates.

(T7 Optiscript™ promoter). ^{35}S-labeled probe, frozen tissue sections. Detection by emulsion or film autoradiography.

(Last update: 15 July 2002, Ver 1.2)

GeneDetect.com Limited
Suite # 4, 209 Taylor Street, Auckland,
New Zealand
Phone, Fax: 64-9-353-1320
General: Info@GeneDetect.com
Sales: Sales@GeneDetect.com
Scientific: Scientific@GeneDetect.com

Introduction:

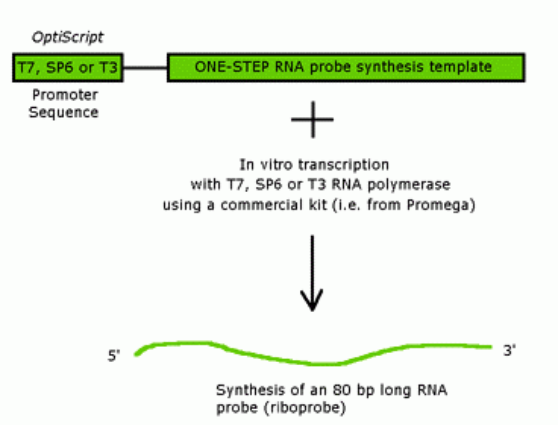
Non-radioactive probe labeling methods are becoming increasingly popular with in situ hybridization. Nevertheless the use of radioactive probes remains common due to the fact that they provide excellent sensitivity and allow detection of low abundance mRNAs in tissue sections. Single-stranded RNA probes also called complementary RNA (cRNA) or riboprobes (it must be noted that riboprobe is actually a registered trademark of Promega Corporation) are often used for in situ hybridization as they are 1. extremely sensitive (due to the fact that they can be labeled to high specific activity during probe synthesis), 2. RNA-RNA hybrids are more stable than DNA-RNA hybrids and 3. non-specific tissue signals can be removed after hybridization with RNase A since RNA duplexes (representing specific binding) are resistant to degradation by RNase A. One major disadvantage of using RNA probes is the amount of effort required to actually prepare these probes.

RNA probes are usually prepared by in vitro transcription. The RNA probe is transcribed from a linear DNA template using highly specific bacteriophage DNA-dependant RNA polymerases from the Salmonella bacteriophage SP6, and the E. coli. bacteriophages T3 and T7 (RNA polymerase T7, T3 or SP6). The investigator must therefore obtain sufficient quantities of a plasmid carrying the gene sequence of interest that can be used as the template for RNA probe synthesis. Furthermore, before a riboprobe can be transcribed the correct RNA polymerase promoter sequences must be available in the plasmid in the correct orientation with respect to the template sequence. If the cloned gene exists in a plasmid lacking these promoter regions the investigator is forced to subclone the gene into a more suitable vector. For example the transcription vectors pGEM (SP6 and T7 promoters, Promega) and pBluescript (T3 and T7 promoters, Stratagene) are commonly used. For in vitro transcription reactions the plasmid must also be in a linear form. The investigator must use restriction enzymes to linearize the plasmid. Even after the RNA probe has been transcribed successfully from the template, if the RNA probe is greater than 300-400bps in length it should be hydrolysed into shorter fragments since the optimal upper length for riboprobes for in situ hybridization is 150-200bps. Longer probes have poor tissue penetration. Lastly, and perhaps most limiting is the possibility that the investigator may not be able to easily source the cloned gene sequence required for RNA probe generation.

We have attempted to resolve these issues by making available our ONE-STEP RNA probe synthesis templates. Using our custom designed templates it is now possible to prepare an optimized RNA hybridization probe to any target gene cheaply and faster than ever before.

GeneDetect™ ONE-STEP RNA probe synthesis templates.

Your GeneDetect™ ONE-STEP RNA probe synthesis template is supplied as 50ug of a lyophilized 100mer PAGE purified DNA template. The template allows for in vitro transcription of an optimized 80bp RNA probe using any commercial riboprobe synthesis kit (we support the use of quality Promega reagents and kits). GeneDetect™ ONE-STEP RNA probe synthesis templates come standard with a GeneDetect OptiScript™ promoter sequence for T7 RNA polymerase. The standard T7 promoter has been redesigned to produce the GeneDetect OptiScript™ promoter which allows for optimal riboprobe generation by limiting premature abortive transcription of the probe. You are able to replace the T7 OptiScript™ promoter with another preferred promoter (either OptiScript™ T3 or SP6) when you initially make your purchase.



The "ONE-STEP" that is required is the re-constitution of the lyophilized template in water. The template can then be used directly with the in vitro transcription reaction to prepare the required 80bp RNA probe.

NB: DO NOT use DEPC-treated water to re-constitute the template since DEPC has been shown to inhibit RNA polymerases.

Other Materials/Kits required:

For best results with GeneDetect™ ONE-STEP RNA probe synthesis templates and in-situ hybridization we recommend using the following high quality reagents and kits.

1. 5'-(α-^{35}S) rUTP (10mCi/ml).

Amersham Pharmacia Biotech (APB) Cat # SJ1303. (at 250μCi/25μl or 1mCi/100ul)

NEN/Perkin Elmer Cat # NEG739H

APB, http://www.apbiotech.com
NEN/Perkin Elmer, http://lifesciences.perkinelmer.com/

2. In vitro transcription Kits. We suggest you use high quality kits from Promega.

Promega Riboprobe System – T7, Cat # 1440
Promega Riboprobe System – T3, Cat # 1430
Promega Riboprobe System – SP6, Cat # 1420

Promega, http://www.promega.com

3. 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

4. Novex Pre-cast 15% TBE-Urea gels (Novex Cat # EC68852 available from Invitrogen) and associated reagents.

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

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
x = times, as in wash 2 times
X = times (concentration), as in 2 X SSC

Protocol Summary (4 steps)

A. **Preparing your RNA probe (riboprobe)**
In vitro transcription (2 hours).
Purification/quantification (1 hour)

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

C. In-situ hybridization of ^{35}S-labeled riboprobe to tissue
Hybridization with probe (18-40 hours).
Post-hybridization washes (3-4 hours).

D. **Detection steps**

1. Film autoradiography (2-3 days)
2. Emulsion autoradiography (several weeks)

This protocol can easily be completed over 2-3 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. Preparing your RNA probe (riboprobe)

In vitro transcription (2 hours).

Please follow the manufacturers recommended instructions for the in vitro transcription kit you have purchased.

Alternatively follow this general protocol using Promega reagents.

ONE-STEP: Dilute the lyophilized template (50ug) into 150µl of autoclaved water (0.33ug/ul).

NB. DO NOT use DEPC treated water. DEPC may inhibit RNA polymerases.

1. Pipette 25µl ^{35}S-UTP (1000 Ci/mmol) into a 1.5ml microfuge tube. The final concentration of UTP should be ≥ 12µM to ensure transcription of full length riboprobe (at 1000 Ci/mmol, adding 250µCi into 10ul reaction = final concentration of ^{35}S-UTP = 25µM).

   Lyophilize the ^{35}S-UTP using a speed vac for about 1 hour until a white pellet is visible. (Do not use ^{35}S-UTP that has been previously thawed. Order either 250µCi or 1mCi vials and use up at first thawing and discard excess via radioactive disposal.)

2. In vitro transcription. To the tube with the dried ^{35}S-UTP add:

   2.0µl 5x Transcription buffer
   1.0µl DTT, 100mM
   1.0µl RNasin
   3.0µl **GeneDetect™ ONE-STEP RNA probe synthesis template** (i.e. 1ug total)
   2.0µl GTP+CTP+ATP Mix (stock solution containing 2.5mM of each nucleotide)

   Mix thoroughly and spin briefly to ensure solution is collected at the bottom of the tube. Promega kits include the RNase inhibitor RNasin.

   Add 1.0µl (20U) T7 RNA Polymerase (or T3/SP6 polymerase if T3/SP6 OptiScript™ promoter selected)

   Mix gently by pipetting, do not vortex. Incubate for 1 hour at **30°C**.

   Add a further 1.0µl (20U) T7 RNA Polymerase (or T3/SP6 polymerase if T3/SP6 OptiScript™ promoter selected)

   Mix gently by pipetting, do not vortex. Incubate for 1 hour at **30°C**.

   (Preferably order all of the above as ready-made stocks from Promega).

   Promega "RiboProbe Combination System" Packs contain all the required solutions except ^{35}S-UTP and the GeneDetect™ ONE-STEP RNA probe synthesis template.

3. To stop the reaction:

   Vortex

   Add 1.0µl RQ1 DNase to the transcription reaction

   Incubate 15 min, 37°C.

   To extract RNA after DNase step, add to the reaction:

   20µl 1x TE
   1.0µl tRNA (50mg/ml)

   Vortex

Purification/quantification (1 hour)

4. Equilibrate one G-50 Sephadex column to room temperature for each riboprobe (15-30 min. at room temperature).

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

   Measure the volume of riboprobe eluted from the G-50 column.

Calculating % incorporation.

You can calculate % incorporation of radioactive label and the specific activity of your radiolabeled riboprobe to ensure that the labeling reaction has been successful.

From a successful in vitro transcription reaction you might expect 25-90% incorporation of ^{35}S-UTP into probe.

Calculating % incorporation of label:

Total label: 25µl of ^{35}S-UTP added to the labeling reaction contains 250µCi (supplied at 250µCi in 25µl). Given that 1 µCi = $2.2x10^6$ cpm, 250µCi = $5.5x10^8$ 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) / $5.5x10^8$) X 100 = % incorp.

Confirming riboprobe integrity.

Run a denaturing TBE-Urea (7M), 15% polyacrylamide gel to assess the quality of the reaction product. The most important aspect is the amount of full-length transcript present. We recommend using Novex 15% TBE-Urea pre-cast gels (Novex Cat # EC68852 available from Invitrogen). Run 1ul of probe. Add probe to TBE-Urea sample buffer following the manufacturers directions (sample buffer contains Bromophenol Blue which runs at 10bps and Xylene Cyanol which runs at 40bps). Heat solution at 80ºC for 2 minutes and then load the sample. Run the gel until the bromophenol blue (the darker leading dye) is about to run off the gel. Dry the gel on a gel drier and then place against film overnight. One major band should be seen. A smear of smaller length transcripts may be seen. By performing the in vitro transcription reaction at 30°C, premature termination of full length transcript synthesis should be minimized.

NB. If required, the proportion of full-length transcript synthesized can be increased by increasing the concentration of the limiting nucleotide (i.e. UTP). Cold UTP (from the Promega kit) can be added at 1:1 (UTP: ^{35}S-UTP) or even 2:1 (UTP: ^{35}S-UTP) to increase the final concentration of limiting UTP in the in vitro transcription reaction to 75µM (0.75mM). The disadvantage of this however is that the specific activity of the generated probe will be reduced.

5. To the riboprobe add 1xTE to a final concentration of 300,000 cpm/µl.

6. Use immediately for in situ hybridization or store aliquots at -70° C up to one week in TE.

7. Just prior to starting in situ hybridization: Thaw the riboprobe and keep on ice.

Make up Hybridization Mix as described in section C.

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.

8. Fix 10 min. in 4% paraformaldehyde, 4°C.

9. Wash 5 min. in 0.5x SSC, RT.

10. Immerse slides in proteinase K solution , 1-5 µg/ml in RNase Buffer for 10 min., RT. The amount of proteinase K needs to be optimized

with each new preparation. Once optimized, aliquots can be frozen down and used for some time.

11. Wash for 10 min. in 0.5xSSC, RT.

PREHYBRIDIZATION: Dry around sections with Kimwipe, lay slides flat in an air tight box with a piece of filter paper which has been saturated with Box Buffer (4xSSC, 50% formamide) on the bottom.

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 Box buffer to keep the sections from drying out.

12. Cover each section with 100µl of hybridization buffer (HB) without probe (can use 50µl if the tissue is small). Incubate at 42°C for 1-3 hours.

C. In-situ hybridization of ^{35}S-labeled riboprobe to tissue

Hybridization with probe (18-40 hours).

13. HYBRIDIZATION MIX: for ^{35}S-labeled riboprobe.

    Assuming that you have used 100µl of prehybridization buffer combine the following: 2.0µl probe per slide (stock solution 300,000 cpm/µl in 1XTE) 1.0 µl tRNA per slide (50 mg/ml stock) Heat 3min, 95°C immediately add 17.0µl ice cold HB per slide, vortex, place on ice. (Adjust volumes if you have used less than 100ul for prehybridization).

14. HYBRIDIZATION: Add 20µl of above hybridization mix to each 100µl of prehybridization solution directly into the bubble covering the section. Incubate overnight at **45-55°C**. (Adjust volume if you have used less than 100µl for prehybridization).

Thus each section is incubated with 600,000 cpm of riboprobe. 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.

Post-hybridization washes (3-4 hours).

15. Wash 2 x 10 min. each in 2XSSC with β-Mercaptoethanol-EDTA, RT. (discard to radioactive WASTE)
16. Immerse in RNase A solution (20µg/ml in RNase buffer) 30 min, RT. (discard to radioactive WASTE)

17. Wash 2 x 10 min each in 2XSSC with β-Mercaptoethanol-EDTA, RT. (discard to radioactive WASTE)

18. Wash 2 hours in 4 liters of 0.1XSSC with β-Mercaptoethanol-EDTA, 55°C (Stringent wash)

19. Wash 2 x 10 min. in 0.5XSSC, RT.

20. Dehydrate 2 min. each in 50%, 70%, and 90% ethanol containing 0.3M NH_4Ac. (Dehydration buffers)

21. Dry in vacuum desiccator (3-4 hrs.) store with dessicant until autoradiography.

D. 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.

Place sections against photographic film for 2-3 days. Tape slides into the back of an autoradiography cassette (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 2-3 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.

Alternatively;

22. Dip slides in Kodak NTB2 nuclear emulsion diluted 1:1 with water at 42°C, dry for 2 hours in the dark, expose in the dark at 4°C with desiccant for 2-8 weeks.

23. Develop at 15°C:
    a) 3 min. Kodak D19 developer, diluted 1:1 with water
    b) 20 seconds in water stop rinse
    c) 3 min. Kodak Fixer, full strength
    d) Wash 3 times 5 min. each in water

Counterstain with Hematoxylin and Eosin

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.

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.

Probes against house keeping sequences.

Some genes are always expressed constitutively such as Actin or beta-tubulin. A low signal once again suggests tissue RNA degradation.

Positive control.

Perform in situ hybridization using the correct riboprobe 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 riboprobe. 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 5 min wash in 0.5XSSC following fixation with 4% PFA. These RNase-treated sections should be compared with sections also incubated with RNase solution (minus the RNase) for 1 hr at 37°C at the same step in the protocol. Following RNase treatment, sections should be washed 2 x 5 min in 0.5XSSC, RT 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.

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 template sequence that will

transcribe the correct and optimized riboprobe 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. RNase treatment of sections before labeled antisense probe hybridized. Is probe binding to mRNA?
2. Hybridize in parallel, labeled sense and labeled antisense riboprobes. Is the riboprobe binding to the tissue in a sequence-specific fashion?

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$_2$0 (distilled water). Must also be autoclaved.

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

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

100% Ethanol

Hybridization chambers (fill with Box buffer before overnight hybridization)

Oven @ 45-55°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)

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

DTT (various sources, 154mg/ml in dH$_2$O sterile = 1M solution, store as 1ml aliquots @ -20°C)

50XDenhardts (use as bought, if required dissolve with dH$_2$0, store 0.2ml aliquots @ -20°C)

RNase A (Sigma Cat # R5125)

Proteinase K (Sigma)

4% PFA (in 0.1M PB, phosphate buffer): 8g PFA dissolved in 100ml of H$_2$O. 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.

HB Hybridization Buffer

	Stock Concentration	**Volume of Stock**
10mM DTT		46.26mg
dH$_2$O		5.7ml
0.3M NaCl	5M	1.8ml
20mM TRIS, pH8.0	1M	600µl
5mM EDTA	250mM	600µl
1XDenhardt's	100X	300µl
10% Dextran Sulfate	50%	6.0ml
50% Formamide	100%	15.0ml
Total Volume		**30.0ml**

RNAse Buffer

	Stock Concentration	**Volume of Stock**
500mM NaCl	5M	100ml
10mM TRIS, pH8.0	1M	10ml
dH20		890ml
Total Volume		**1000ml**

RNAse Stock (10mg/ml)

10mg RNAse A (Sigma)
1.0ml RNAse Buffer
Heat treat as per Maniatis.

Working RNAse Solution-20mg/ml

300µl RNAse Stock in 150ml RNAse Buffer

2xSSC, β-ME, EDTA (β-ME = β-mercaptoethanol).

	Stock Concentration	**Volume of Stock**
2x SSC	20x	100ml
10mM β-ME	100%	875µl
1mM EDTA	250mM	4.0ml
dH20		860ml
Total Volume		**1000ml**

Box Buffer

	Stock Concentration	**Volume of Stock**
4x SSC	20x	50ml
50% Formamide	100%	125ml
dH20		75ml
Total Volume		**250ml**

Stringency Buffer

	Stock Concentration	**Volume of Stock**
0.1xSSC	20xSSC	20ml
10mM	beta-ME	3.5ml
1mM EDTA	250Mm	16.0ml
dH20		3960.5ml
Total Volume		
		4000ml

Dehydration Buffers:

	50%	**70%**	**90%**	**100%**
100% EtOH	100ml	140ml	180ml	200ml
3M NH4Ac	20ml	20ml	20ml	--
dH20	80ml	40ml	--	--
Total Volumes	**200ml**	**200ml**	**200ml**	**200ml**

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

Email us at Scientific@GeneDetect.com

Suite 4/209 Taylor Street Blockhouse Bay, Auckland, NEW ZEALAND ; Phone/Fax 64-9-353-1320 ; Email Sales@GeneDetect.com