For 20 mL add together:

4 mL 1 M $NaHPO_4$ (pH 7.0).
1 mL 0.1 M potassium ferrocyanide.
1 mL 0.1 M potassium ferricyanide.
120 µL Triton-X-100.
13.83 mL sterile distilled water.
Weigh out 30 µg X-Gluc and dissolve in 50 µL DMSO.
Add this to the above mixture, mix and add to plant tissue.
Incubate overnight at 37°C

This buffer can be made and stored at –20°C but repeated thawing and freezing is not recommended. It is easy to make it up from stock solutions when required. It can also be aliquoted if 20 mL is too large a volume for your tissue. If you are concerned about the substrate penetration into the plant tissue, you can vacuum infiltrate the tissue/buffer mix for about 2 minutes at RT before incubating at 37°C.

CHAPTER 3

Analysis of Transgenic Peanut Lines

3.1 Initial screen for transgenic tissues: PCR

POLYMERASE chain reaction (PCR) is a powerful technique to detect the presence of specific sequences in plant samples. It is both sensitive and rapid, and will allow the large-scale initial screening of putative transformed tissue for the presence of the transgene. We have used PCR to detect single and multiple copy transgenes in transformed regenerating peanut embryos and in the leaves of transgenic peanut plantlets growing in tissue culture or in the glasshouse. Oligonucleotide primers may target any area in the introduced gene, e.g. promoter, terminator, gene of interest, reporter gene, selectable marker gene or flanking regions. For PCR, DNA primers are used, short oligonucleotides, specific for the gene of interest. A PCR assay with these primers, even using relatively crude DNA as the template, will allow exponential amplification of the sequence between the primer binding sites. PCR should only be used as an initial, quick screen and does not replace Southern blot analysis to demonstrate integration into the peanut genome and approximate number of integrated copies. Care has to be taken when using PCR on tissue transformed with *Agrobacterium* to ensure that no traces of residual *Agrobacterium* persist. PCR cannot distinguish between the target sequence present in the bacterial plasmid or in the plant genome and will amplify both, potentially leading to false positive results.

There have been many methods published describing the analysis of transgenic plants using PCR. We have shown the method outlined below to be the simplest of methods to detect many different genes from a variety of tissue types of several different species (Higgins et al. unpublished). Importantly, it has also been successfully applied by different researchers demonstrating that it is a procedure adaptable to different laboratories.

This method is very simple. A small amount of tissue is soaked in template preparation solution (TPS) and heated to release the cellular contents, including the DNA. The tissue debris is then separated by centrifugation from the DNA-containing liquid, which is included in a PCR. This method has been used to amplify products up to 1500 bp in length. However, it is important to note the conditions under which you may observe a false negative result. You need to be aware of the following factors:

1. The ratio of the TPS volume to the amount of tissue can affect the amplification. When too little TPS is used relative to the amount of tissue, the level of PCR inhibitors (present in peanut tissues) can be high, even when the sample is diluted in water. This may be particularly noticeable, if you are trying to amplify a large fragment. We occasionally encounter false negatives diluting 2 mm^2 peanut tissue 1:10. The safest way is to measure the OD_{260} in a spectrophotometer and to adjust the DNA concentration in the TPS extract to 50–100 ng/μL sample for PCR (Sudarsono, pers. comm.).
2. The pH of the TPS can influence the success of the PCR. We found that by increasing the pH from 7.4 to 8.0 for peanut we obtained more predictable results.

If amplification is unsuccessful, we recommend reducing the amount of tissue being tested, increasing the volume of TPS or diluting the sample further than 1:10 prior to PCR.

Inclusion of primers for amplification of an internal multiple gene copy standard would also identify false negative reactions. The procedure we have outlined below has proven consistent for the PCR amplification of transgenes of up to 1500 bp in length from both peanut embryo and leaf tissue.

Leaf soak PCR

1. Remove a tissue sample of approximately 2 mm^2 from the plant(s) of interest or a whole somatic embryo from a culture plate.
   Place into a sterile Eppendorf tube.

The tissue can be either from a fresh plant or frozen tissue. The tissue can be either leaf or somatic embryos.

2. Add 50 µL of Template Preparation Solution (TPS) (100 mM Tris-HCl pH 8.0, 1 M KCl, 10 mM EDTA) to leaf tissue, 30 µL of TPS per somatic embryo.
   Grind the tissue briefly using a pestle suitable for microfuge tubes.
   Heat at 95°C for 10 minutes.
   Place on ice for 2 minutes.
   Centrifuge briefly to sediment any solid matter.

Glass or metal pestles designed for use with Eppendorf tubes can be purchased or prepared by your workshop. Plastic pestles can also be purchased together with specialist 1.5 mL tubes. While these pestles are designed to be disposable, we have used them several times by cleaning and autoclaving in-between uses.

3. Remove a 1 µl sample (diluted 1:10 in sterile distilled water) and add to the following mixture for a final volume of 25 µL:
   10 mM Tris-HCl pH 8.3. } 1 × PCR reaction buffer
   50 mM KCl. }
   1.5 mM $MgCl_2$. }
   50 µM dNTPs. }
   100 nM primer A.
   100 nM primer B.
   1.5 U *Taq* DNA polymerase.
   sterile distilled water to 25 µL
   If you are using a PCR machine that does not have a 'hot top', then add 30 µL of mineral oil to the tube.

In some cases, the TPS extract may inhibit the PCR, if undiluted. We believe that this is due to the presence of phenolic compounds within the crude plant extract as well as the high KCl concentration. It will probably be necessary to dilute the extract before adding to the reaction mixture. We have recommended a 1:10 dilution in sterile distilled water. If you still do not detect a PCR product, try diluting the extract further than 1:10.

Most companies that sell heat-stable DNA polymerase, also provide the buffer to go with it. We have used *Taq* from Roche Diagnostics (formerly Boehringer Mannheim). They provide a 10 × buffer that

has Mg^{2+} in it at the recommended concentration as well as other buffer components for PCR. If you use this buffer, you can add it instead of the Tris.HCl/KCl/ $MgCl_2$ by diluting it to a final concentration of 1×. Check the buffer you have to see if it provides all the components listed above at the correct concentrations. If not, either use what you have ensuring the Mg^{2+} concentration is correct or make it up yourself.

Depending on the type of PCR machine you have, you will use either a 200 or 500 µL tube to set up the PCR. The reaction outlined above utilises *Taq* DNA polymerase, however, there are a myriad of heat stable polymerases on the market now. We expect that other polymerase will also work fine under these reaction conditions, but check the manufacturer's information.

4. Carry out the PCR as follows:

Denaturation	1 cycle	94°C for 3 minutes.
Amplification	35 cycles	94°C for 45 secs.
		50–60°C for 45 secs.
		72°C for 90 secs.
Final extension	1 cycle	72°C for 10 minutes.
Soak		4–15°C until removal from machine.

We have carried out PCR under these conditions using PE Applied Biosystems GeneAmp PCR System 480 or 9700 **DNA** thermal cyclers. While it is possible that different PCR machines may contribute to the success of this PCR, we generally do not expect that to be an important factor. The most likely causes of failure will either be the presence of inhibitors in the TPS extract, or the sub-optimal design or annealing temperature of the primers. Depending on the primers that you use, the annealing temperature may need to be adjusted. As a rule of thumb, estimate the melting temperature (Tm) for your primers using the formula Tm~ 4 (G+C) + 2 (A+T), and set the annealing temperature to be Tm-5°C.

5. Analyse and record the PCR products by agarose gel electrophoresis according to the method outlined on **page 50**. Analyse 10 µL of the PCR product.

The percentage agarose you use to form the gel for the analysis of the PCR products depends on the size of the products you are expecting. For products of 0.5–4 kb in length, a 1% gel is appropriate. For products smaller than 0.5 kb, use a 1.5–2% agarose gel. Also use appropriate DNA molecular weight markers.

Total nucleic acid extraction

If you feel that the TPS extract is too crude and you would like to prepare a purer template, you can isolate pure genomic DNA as described on **page 47.** If you wish to prepare somewhat purer DNA than what is provided by the 'leaf-soak' method described above, but do not want to go to the trouble of preparing ultra-pure DNA then use the alternative protocol outlined below (Smith et al. 1992). We have used this method, as well as TPS extractions for the preparation of template DNA from peanut for use in PCR.

1. Remove approximately 100 mg of tissue from the plant to be analysed and freeze immediately in liquid nitrogen.
2. Transfer the tissue to a sterile Eppendorf tube.
   Grind the tissue to a powder with a pestle suitable for microfuge tubes.

3. Add 5 volumes (i.e. 500 µL) of 0.05 M sodium phosphate buffer, pH 8.0.
   Centrifuge for 5 minutes at 12000 × *g* at RT.
4. Working in a fume hood, transfer the supernatant to a fresh sterile Eppendorf tube.
   Extract with phenol/chloroform by adding an equal volume (500 µL) of phenol (saturated with 0.1 M Tris.HCl pH 8.0):chloroform:isoamyl alcohol (25:24:1).
   Centrifuge at 12000 × *g* for 5 minutes at RT.
   Working in a fume hood, transfer upper aqueous phase to a fresh, sterile Eppendorf tube.
5. Working in a fume hood (there will still be traces of the organic solvent), precipitate the DNA by adding 0.1 volume (50 µL) of 2.5 M sodium acetate pH 5.2 and 2 volumes (1000 µL) of absolute ethanol.
   Chill at –70°C for 15–20 minutes or –20°C for at least 2 hours.
   Centrifuge in a microfuge at 12 000 × *g* for 10 minutes at RT.
   Pour off the alcohol ensuring the pellet is not disturbed.
   Add 500 µL of 70% ethanol.
   Re-centrifuge at 12 000 × *g* for 5 minutes at RT.
   Pour off ethanol ensuring pellet is not disturbed.
   Centrifuge briefly again (~30 seconds) to bring the liquid to the bottom of the tube, pipette this liquid out without disturbing the pellet.
   Air dry 1–2 minutes.
   Resuspend in 100 µL of sterile distilled water.
   Use 1 µL in a PCR as described in the 'leaf soak PCR' section above.

3.2 Transgene integration: Southern blot

Once putative transgenic plants have been regenerated, it is important to confirm the presence of the transgene(s) in the plant(s). More importantly perhaps is confirming the integration of the transgene in the genomic DNA of the plant. It is also essential to ensure that the transgene is passed on through the germline to the progeny. Thus genomic DNA analysis of generations (i.e. T_1, T_2 etc.) beyond the primary transformant (the T_0 generation) is also important. For breeding purposes, it is essential to know how many copies of the transgene are present and if there are any rearranged or incomplete copies.

The presence of a transgene can be detected by either PCR (Chapter 3.1) or Southern hybridisation. PCR only determines if the transgene is present or not; it does not provide confirmation of the integration of the transgene. The presence *and* integration of the transgene can be confirmed by Southern hybridisation of restriction enzyme digested genomic DNA with a probe specific for either all or a portion of the transgene(s). Genomic DNA is extracted, then it is digested into smaller pieces with a specific restriction enzyme. The restriction fragments are then separated through an agarose gel by electrophoresis and then transferred to a nylon membrane. The DNA held on the membrane is then hybridised with a probe specific for the transgene that is labelled with either a radioisotope or with a non-radioactive label. This hybridisation is then detected by the appropriate method for the label used.

The presence of the transgene is shown by digesting the DNA with a restriction enzyme that will cut out the transgene sequence from the genomic DNA (Figure 15A). The actual integration of the transgene and an estimate of the transgene copy number are also confirmed

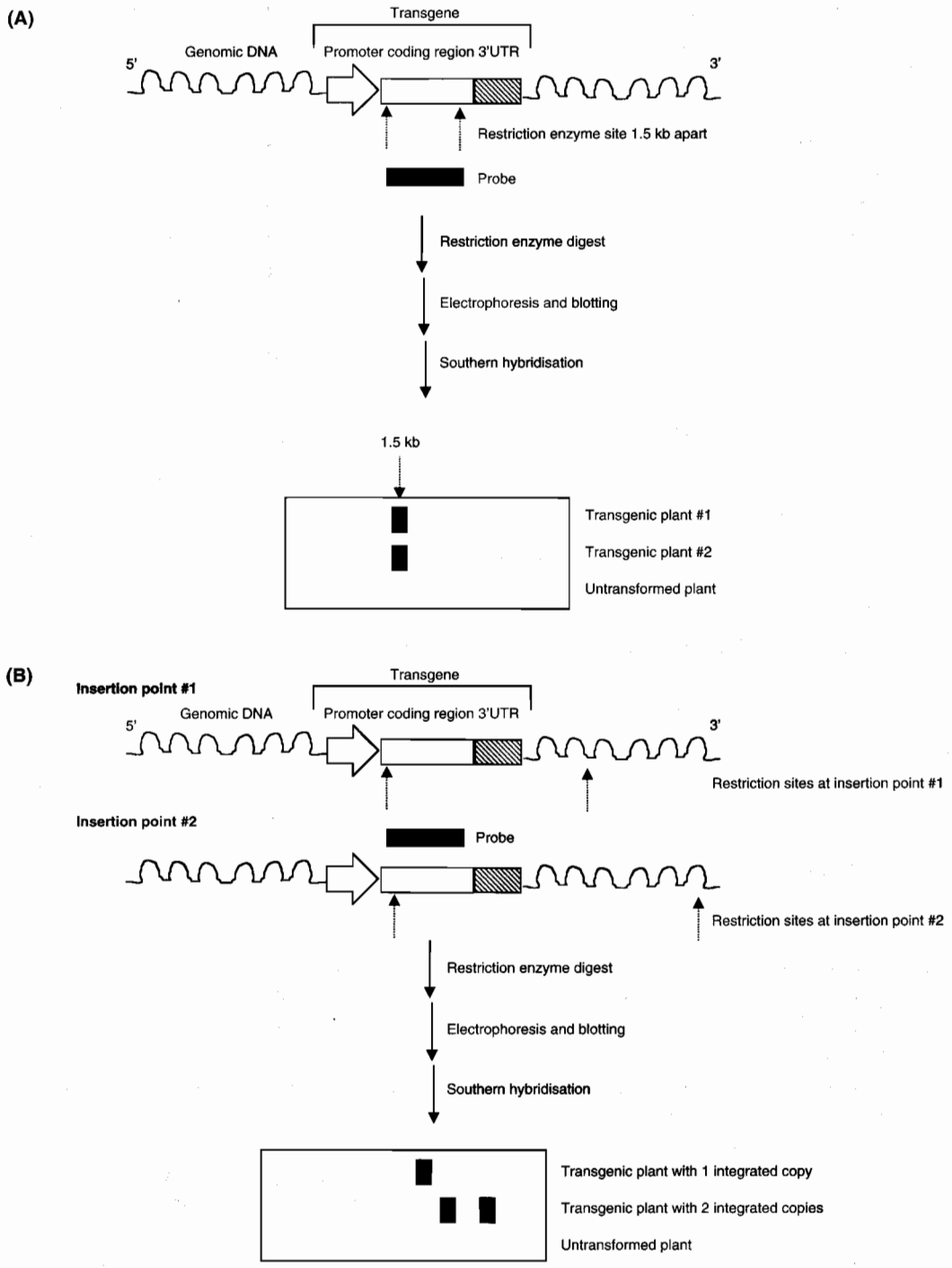

Figure 15. Southern blot schematic diagrams for the detection of a PStV CP transgene in the genome of transformed peanut plants. (A) confirmation of the presence of at least one PStV CP transgene; (B) estimation of transgene copy number.

by Southern hybridisation. If a restriction enzyme is used that cuts only once within the targeted transgene sequence, then the hybridisation of a probe specific for that gene will confirm integration and/or rearrangements of the transgene. The number of fragments observed gives an estimate of the copy number per plant genome (Figure 15B).

While there are many methods for carrying out these types of experiment, all of the methods have many features in common. A lot of mythology has arisen around these methods so that a researcher new to this area may be nervous about which ones to choose. From a technical point of view, these methods are in fact straight forward and with an understanding of the chemistry involved, any person skilled in molecular biology can carry out such experiments with ease. One word of caution we would like to offer is that if you are going to use radioactivity to label your probe, ensure that you are certified for the use of such chemicals and you understand the potential risks and dangers. It would also be useful to set aside a particular area within your laboratory specifically for the use of radioisotopes.

Southern Hybridisation Analysis

There are many procedures for the extraction of genomic DNA. Which one you choose depends on the type of analysis you wish to carry out. If you want to detect the gene by PCR, a relatively crude preparation is sufficient. If you wish to use restriction digestion and Southern hybridisation analysis you will need to prepare purer DNA, since many restriction enzymes can be inhibited by compounds present in crude plant extracts. Regardless of the analysis you wish to carry out, all methods rely on the disruption of the plant cells to release the contents by grinding. For analyses that require purer genomic nucleic acid preparations, this is followed by the denaturation and removal of proteins, carbohydrates and cell debris usually with phenol/chloroform. This is usually sufficient for PCR. For purer preparations, RNA is removed with RNase and then the genomic DNA is precipitated. The method described here for the preparation of pure genomic DNA has worked for several legume species as well as tobacco and cucurbits. If these do not work satisfactorily, we suggest you experiment with other procedures.

Extraction of pure genomic DNA

1. Remove up to 1 g of plant tissue and wrap in labelled aluminium foil. Place in liquid nitrogen immediately. The tissue can be extracted straight away or stored frozen at –80°C until required.

Any plant tissue can be used for the extraction of genomic DNA, however, leaf tissue generally gives the best yield of DNA. It is advisable to wear safety glasses while grinding tissue in liquid nitrogen and when using phenol/chloroform.

2. Place tissue into mortar and pour liquid nitrogen into mortar, over tissue.
3. Grind tissue in liquid nitrogen to a fine powder ensuring no large pieces of tissue remain.

   Add 3 mL of urea extraction buffer and transfer to a sterile polypropylene tube.

Use 3 mL of extraction buffer per gram of tissue. If you are extracting >1 g of tissue, scale up the amount of buffer accordingly. Increase the amount of phenol/chloroform appropriately (see below). Everything else can stay the same.

4. In a fume hood, add 3.5 mL of phenol/chloroform and shake for a minimum of 15 minutes at RT on either a rotating wheel or shaking table top. An emulsion between the phases must be formed.

If extracting 1 g of tissue, a 15 mL tube is appropriate, if extracting more, use a 50 mL tube. You can begin your extractions and place your samples on a rotating wheel or shaking table top while you are extracting the other samples. Make sure the last sample is extracted for at least 15 minutes. If using a shaking tabletop, also mix samples by hand regularly.

5. Separate the phases by centrifugation at top speed in a bench top centrifuge (~3000 r/min) for 10–15 minutes at 15–20°C.
6. In a fume hood, transfer the upper aqueous phase to a fresh polypropylene tube.
7. In a fume hood, add 500 µL of 4.4 M ammonium acetate, pH 5.2 and mix well.
8. Add an equal volume of isopropanol and mix gently.

At this stage, if there is a lot of genomic DNA, it will generally precipitate out as a mucous blob. If there is not much DNA you may not see a precipitate at this stage. Sometimes, the pellet is not very big and the mixing can break the precipitate up into small stringy pieces.

9. Spool DNA out and resuspend in 500 µL of sterile TE, pH 8.0.
   Transfer the DNA solution to a sterile 1.5 mL Eppendorf tube.

If a mucous blob type of precipitate has formed, it is much easier to dissolve the DNA if this precipitate is pulled or spooled out of the tube. The DNA can be removed from the tube using a hook made from a glass Pasteur pipette. Heat the end of a Pasteur pipette in the flame of a Bunsen burner to create a hooked end. Otherwise, try a sterile tip from an automatic pipette.

If the DNA does not form as an aggregated precipitate but has broken up into smaller stringy pieces, pellet the DNA by centrifugation for about 15 minutes at 12 000 × *g* at RT.
Pour off the supernatant leaving the pellet behind.
Air-dry and resuspend in 500 µL of TE, pH 8.0.
Transfer the DNA solution to a sterile 1.5 mL Eppendorf tube.

If no DNA precipitates at this stage, place at –20°C overnight or –70°C for 30 minutes. Centrifuge at 12 000 × *g* for 15 minutes.
Pour off isopropanol, ensuring pellet remains in the tube.
Air-dry and resuspend in 500 µL of TE, pH 8.0.
Transfer the DNA solution to a sterile 1.5 mL Eppendorf tube.

You must ensure that the DNA is fully resuspended. To help this along, you can heat the DNA at 65°C for about 10–15 minutes and leave overnight at 4°C. It is good to give the tube the occasional gentle flick during each of these treatments to encourage the DNA to dissolve. Do not vortex to avoid shearing the DNA.

10. When the DNA has dissolved, add 10 µL of 10 mg/ml RNase A.
    Leave at RT for 15 minutes.
    In a fume hood, do two rounds of phenol/chloroform extractions: add an equal volume of phenol/chloroform, mix the phases thoroughly, but gently and centrifuge at 12 000 × *g* for 10 min at RT. Transfer the upper aqueous phase to a fresh tube.
    Repeat, finally transferring the aqueous phase to a fresh tube.

A mixture of RNase A and RNase T1 will also work well. It is possible to omit the RNase step and subsequent phenol/chloroform extractions. RNA should not inhibit the restriction digestion of the genomic DNA; however, determination of the DNA concentration will not be accurate. If you use a spectrophotometer to measure the DNA concentration, the RNA will also be measured. If you use ethidium bromide staining of DNA in an agarose gel, the RNA will also chelate ethidium bromide and may make the visual estimation of DNA concentration difficult, especially if you compare to a standard that does not contain RNA.

You can measure the DNA as total nucleic acid concentration using a spectrophotometer (see below) and ignoring the presence of RNA. This has been adequate for detecting single copy gene sequences using radioactive probes but is not suitable for digoxigenin (DIG)-labelled probes. DIG-labelled probes are more sensitive to the amount of target DNA on a gel therefore if you are using this type of probe, you need to be more accurate when determining the concentration of the genomic DNA. Also, if you wish to quantify the amount of specific sequence within the genomic DNA, you will need to accurately determine the DNA concentration.

If you use an RNase step, you will notice at the phenol/chloroform extraction stage the interface will be quite substantial. Peanut is a starchy plant; thus it is likely that the interface is mostly starch. The interface may be particularly large from a glasshouse grown plant compared with a tissue culture grown plant. When removing the aqueous phase, avoid removing any of the interface. The presence of starch may interfere with subsequent restriction enzyme digestion. If it does, re-extract the DNA with chloroform, i.e. no phenol and re-precipitate.

11. Add 100 µL of 4.4 M ammonium acetate and mix gently.
    Add 700 µL of cold isopropanol and mix gently.
    Re-spool the DNA as described above.
12. Wash the precipitate in 70% ethanol. If you have a mucous blob, dip the pellet carefully into ethanol and drain on the side of the vessel. If you have had to centrifuge the precipitate, remove the ethanol from the pellet, add about 1 mL of 70% ethanol to the tube carefully without disturbing the pellet, gently wash the pellet by rolling the tube and pouring the ethanol off.
    Air dry for 1–2 minutes.
    Resuspend the DNA in 200 µL of TE, pH 8.0.
13. Determine the concentration from the A_{260} measurement.

Before measuring the A_{260}, ensure the DNA is fully resuspended as described above. The purity of the DNA can also be determined by calculating the A_{260}/A_{280} ratio. If it is around 2, then the DNA is pure; if it is less, then it is contaminated with protein. If you find in the next step that the DNA does not digest, it may be that the DNA needs re-extracting with phenol/chloroform to remove contaminating protein, or with ether to remove traces of phenol that may be present. Ether extractions are carried out exactly as for phenol/chloroform extractions except that the aqueous phase containing the DNA is the lower phase.

Digestion of genomic DNA with restriction enzymes

The choice of restriction enzyme will depend on the cleavage sites present within the transgene. To prove integration of the transgene, you need to choose a restriction enzyme that cuts once only within the transgene sequence.

1. Set up the following mixture:

Genomic DNA	10–20 μg.
10 × restriction buffer	5 μL.
0.1 M spermidine	2.5 μL.
Restriction enzyme	4U per μg DNA.
Sterile distilled water	to 50 μL total volume.

Most companies provide restriction enzymes with pre-made buffer either 10× or 5×. If you receive a 5× buffer, then adjust the volume accordingly. If you do not receive the buffer with the enzyme, you can make it up yourself as recommended for the particular enzyme by the company. Ensure the buffer you make up is sterile, since DNases that can break down the DNA may be present in an unsterile solution.

If the DNA is too dilute to make up a reaction of 50 μL, make up a mixture with 10–20 μg of DNA and scale everything else up. When the digest is complete, precipitate the DNA and resuspend in a smaller volume for loading on the gel.

The units of a restriction enzyme are defined as the amount of enzyme it takes to cut 1 μg of phage lambda DNA in 1 hour. You will need more enzyme per μg of genomic DNA than you do for lambda or plasmid DNA, hence 4 U per μg is recommended. Spermidine is included in the mixture to assist the enzyme to cut the high molecular weight DNA.

2. Leave overnight at the appropriate temperature for the particular restriction enzyme.
3. The next day, precipitate the DNA from the digest if you have had to scale up the reaction:
   Add 0.1 volume of 3 M sodium acetate, pH 5.2 and 2–2.5 volumes of cold (–20°C) ethanol.
   Leave at either –70°C for 15–20 minutes or –20°C for a minimum of 2 hours.
   Centrifuge the DNA at 12 000 × *g* for 20–30 minutes at RT.
   Pour off the supernatant, ensuring the pellet remains in the tube.
   Add 1 volume of cold 70% ethanol.
   Centrifuge at 12 000 × *g* for 15 minutes at RT.
   Pour off the supernatant carefully, ensuring the pellet remains in the tube.
   Briefly centrifuge to bring the remaining liquid to the bottom of the tube.
   Pipette off this liquid without disturbing the pellet.
   Air-dry for 1–2 minutes.
   Resuspend in ~20 μL of TE, pH 8.0.
   Leave on ice, if you are to load the DNA onto the gel soon, otherwise store at –20°C.

Agarose gel electrophoresis

Depending on the size of the wells that you use for your gel (see below), you may have to precipitate the DNA, i.e. the well may not be big enough to hold the entire volume of the digest. If this is not the case, you can load the digest directly. In some cases, you may prefer to do this, since by precipitating you risk losing some of the DNA. We have also had good experience in purifying the DNA digest using a Bresaclean kit (www.geneworks.com.au) to remove the enzyme and salts.

1. Prepare a gel tray before making up the gel mixture. This is done either by taping the ends of the tray or, if the gel system you use is designed for this, inserting the tray into the gel rig. See the instructions for your particular gel system. Insert the comb into the tray. Ensure the bench top you are using for pouring your gel is level.

The width of the wells is an important consideration when doing these experiments. You must ensure the well is big enough to hold the digest without spiling any of the DNA out. If the well is too wide, the hybridisation signal may be less intense than that with a narrower well, since the DNA will be spread over a larger area. If the well is too narrow, the hybridisation signal may appear as a blob rather than a discrete band. It is a question of balance.

2. To prepare 100 mL of a 1% gel, weigh out 1 g of agarose into a sterile flask or beaker Add 100 mL of 1 × electrophoresis buffer (i.e. TAE or TBE) (Sambrook et al. 1989)

Depending on the size of the restriction fragment(s) to be resolved, use either TBE or TAE as the electrophoresis buffer. Either buffer system works well for most circumstances but there are some differences, which you may like to consider. TAE resolves better in the upper end of the usual range of fragment sizes (i.e. 4–23 kb) while TBE resolves better at the lower end of the range (0.5–4 kb). It is a matter of convenience and desired result which one you choose. Also, if TAE gels are electrophoresed too long i.e. >20 hrs, a pH gradient will form between the ends of the gel tank and the DNA may run awry. If you need to electrophorese your gel for longer to resolve particular fragments, you can either use TAE and recirculate the buffer, or use TBE. The buffer you use to prepare the gel may contribute to background during hybridisation. If you do get unexplained background, try changing the buffer system.

3. Heat to boiling in either a microwave or over a Bunsen burner until the agarose has completely melted. Be sure you do not over boil the solution to the point where it spills out of the container or water evaporates.
   Cool to a temperature where you can hold the agarose solution reasonably comfortably in your hand but the agarose has not started to solidify.
   Add 1 µL of 10 mg/mL ethidium bromide to the agarose and mix by swirling, being careful not to create air bubbles.
   With one fluid movement, pour the agarose into the prepared gel tray.
   Leave at RT to set.

The ethidium bromide interchelates into the DNA molecules allowing visualisation of the DNA under UV transillumination. This compound can either be added into the gel mixture so that the DNA becomes stained as it migrates through the gel, or the gel can be soaked after electrophoresis in buffer containing 0.5 µg/mL ethidium bromide. There are reasons for and against both of these approaches, in the end, it is a matter of personal choice.

4. To a 50 µL digest add 10 µL of 6 × loading dye. If you have precipitated the digest and resuspended in 20 µL, add 4 µL.

There are many different recipes for loading dye solutions. The purpose of this solution is to make the digest heavier than water so the DNA will sink to the bottom of the well, and also to help you track how far the DNA has progressed through the gel during electrophoresis. The most commonly used dyes are bromophenol blue and xylene cyanol: bromophenol blue runs at ~500bp in a 1% gel The loading dyes contain either sucrose, ficoll or glycol (Sambrook et al. 1989).

5. Place the set gel into the gel rig, ensuring any tape and the comb have been removed.
   Pour 1 × electrophoresis buffer (TAE or TBE, but the same with which you made the gel) into the gel rig to cover the gel by ~1 cm.
   Load digested DNA into the wells, one lane per digest, ensuring the solution does not spill out.
   If planning to use a DIG-labelled probe, also load DIG-labelled DNA size markers (20–50 ng per lane). If using a radioactive probe, load unlabelled DNA markers (~0.25–0.5 µg per lane).

DIG-labelled size markers will tell you if your detection step has worked later in the procedure.

   You may also want to include as a positive control a lane of plasmid DNA carrying the gene(s) that was used to transform the plant. Of this DNA, load only ~10 pg.
   Another sample to include is digested genomic DNA from an untransformed plant, which serves as a negative control.

7. Assemble the gel rig and hook it up to a power supply, remembering that DNA is negatively charged at neutral pH and will migrate towards the positive electrode.
   Start the gel electrophoresis by setting the desired voltage.
   Continue until the bromophenol blue is ~1 cm from the bottom of the gel.

The appropriate speed at which to electrophorese a gel is described in Sambrook et al. (1989).

8. Take a photograph of the ethidium bromide-stained gel as a record.
   If you have included unlabelled markers, mark the position of each marker.

This can be done by either using a needle dipped in Indian ink and piercing each band once (wash the excess away) or cutting the edge of the gel at each position. The ink will transfer to the membrane as a faint black spot that allows you to see the positions of the markers. Cutting the gel may cause it to break at a later stage, so take care.

Polaroid photographs have been the method of choice to date for recording electrophoresis results. They are stable over time and provide very good contrast, so that even faint bands are often observable. More recently, digital cameras have emerged as alternatives by capturing video images onto thermal paper or preferably as computer files.

Southern blotting

Southern blotting was first described by Southern (1975). It refers to the transfer of DNA to a solid matrix, in this case a nylon membrane, where the DNA is fixed in position. The transfer can be done either by capillary blotting as described below or electroblotting in an electric field. Specific sequences within the DNA held on the membrane can be identified by hybridising the membrane with a labelled probe, either DNA or RNA. Prior to the transfer, the DNA being probed must be separated by electrophoresis as described above and then denatured in situ, so that the double-stranded DNA becomes single-stranded. If the DNA is not denatured, the probe cannot bind to the target sequence. Southern blot membranes or filters can be stored for some weeks prior to hybridisation.

1. (Optional) After electrophoresis of DNA through agarose and photography of the gel, transfer the gel to a tray containing 0.25 M HCl at RT.
   Place on a shaking table top and shake gently for 5 minutes. Ensure the gel is covered with liquid, as it may float.
   Pour off the HCl and briefly rinse the gel with distilled water.

This is a depurination step. Higher molecular weight DNA is more difficult to transfer to membranes than lower molecular weight. If the DNA is treated briefly with dilute HCl, this depurinates the DNA which helps in the transfer of this higher molecular weight DNA. If you allow the transfer to proceed for 20–24 hours, there will probably be no need to depurinate.

2. Transfer the gel to a tray containing denaturing solution (1.5 M NaOH/0.5 M NaCl).
   Place onto a shaking table top and shake gently for 20–30 minutes at RT. Ensure the gel is covered with liquid, as it may float.
   Replace the denaturing solution with fresh solution and shake gently for another 20–30 minutes.
   Wash the gel briefly in distilled water to remove traces of the denaturant.
3. Transfer the gel to a tray containing neutralising solution (1.5 M Tris-HCl, pH 7.0/0.5 M NaCl).
   Place onto a shaking tabletop and shake gently for 20–30 minutes at RT. Ensure the gel is covered with liquid, as it may float.
   Replace the neutralising solution with fresh solution and shake gently for another 20–30 minutes.
   The gel can remain shaking gently in neutralising solution for a while until you are ready to blot. When you are ready, wash the gel briefly in distilled water to remove traces of the neutralising solution.
4. Set up the blot as described below and following the diagram shown in the respective section of Sambrook et al. (1989).
   Fill a tray to about half way with 20 × SSC.
   Prepare a wick by cutting a sheet of Whatman 3 MM filter paper that is more than twice as long as the stage on which you will set up the blot. Wet this sheet with 20 × SSC.
   Place a stage in the tray of 20 × SSC and place wick over the top of the stage with the ends dipped into the 20 × SSC.
   Remove all air bubbles from the wick by rolling a glass pipette over the top.

For radioactively labelled probes, you can blot either onto neutral membranes (such as Hybond-N from Amersham) or positively charged membranes (such as Hybond N+ from Amersham). For DIG labelled probes, you must blot onto positively charged membranes. It is best to use the membrane supplied by Roche Diagnostics (formerly Boehringer Mannheim) since they have developed the DIG system and guarantee good results with their membrane.

5. Place the gel upside down on top of the wick.
   Remove all air bubbles.
   Trim off any parts of the gel that are not needed.

On loading a gel, the DNA sinks to the bottom of the well. Therefore, the DNA is actually closer to the underside of the gel. Hence you place it upside down for blotting since this means the DNA travels a shorter distance to get to the membrane.

6. Surround the gel with plastic wrap ensuring the wick is completely covered but the gel is not.

Placing plastic wrap around the gel prevents short-circuiting. If any of the paper towelling (see below) touches the wick, the buffer will travel to the towel directly without going through the gel and the DNA will not transfer efficiently.

7. Wearing gloves, cut a piece of nylon membrane to the size of the gel.
   With a pencil, label one corner on the side of the membrane that will be in contact with the gel.
   Fill a tray with 2 × SSC. Wet the membrane with this 2 × SSC by gently floating the membrane on the top until it has wet completely on the bottom and then gently submerge it.
   Place the membrane on top of the gel, with the labelled side closest to the gel.
   Remove all air bubbles by rolling a pipette over the top as before.
   Discard the membrane and use a fresh one if it moves during this process.
8. Cut 2 sheets of 3MM filter paper to slightly bigger than the gel and membrane.
   Wet these sheets with 2 × SSC.
   Place the 2 sheets on top of the membrane.
   Remove all air bubbles by rolling a pipette over the top as before.
9. Stack dry paper towelling over the top of the wet 3MM. The towelling should form an even, reasonably tidy stack to ensure good, even transfer.
   Place a flat sheet of glass or plastic over the stack and on top of that place a weight of about 1 kg such as a 1 L flask filled with water.
10. Leave the transfer proceed for 20–24 hours.
11. The next day, remove the towelling and 3MM paper from the membrane.
    Remove the membrane and immediately seal in plastic wrap to avoid it drying out.
    Make sure the wrap is reasonably smooth on the DNA side.
    Fix the DNA to the membrane by placing the membrane with the DNA side down (still in the plastic wrap) onto a UV transilluminator for 3 minutes. You can buy a box specifically for this purpose from BioRad (www.bio-rad.com).
    Remove the membrane from the warp and wash in 2 × SSC to remove any traces of gel.
    Immediately re-seal in a plastic bag to prevent it drying out.
    Store at 4°C until ready for use.
12. Disassemble the remainder of the blotting apparatus.
    The gel can be stained with ethidium bromide at this stage to check that DNA transfer has occurred.

If you do check the amount of DNA left on the gel, do not be surprised if you see high molecular weight DNA remaining in the gel. This is normal, the majority of the DNA will have transferred.

Southern hybridisation using DIG-labelled probes

Labelling probes with DIG

DNA probes are labelled using either PCR or random priming (also known as oligo-labelling). Both methods require a DNA template from which complementary sequence is synthesised by a DNA polymerase such as *Taq* (in the case of PCR) or Klenow fragment (in the case of random priming).

Roche diagnostics (formerly Boehringer Mannheim) developed the DIG labelling system as an alternative to radioactive labelling. They have published a technical manual for the labelling, use and detection of DIG labelled probes (see The DIG System User's Guide for Filter Hybridisation; http://biochem.roche.com). The labelling reaction incorporates digoxigenin-11-dUTP (DIG-11-dUTP). The molar ratio of the DIG-11-dUTP:dTTP determines in part the specific activity of the final product. Different products are available with 1:2 and 1:20 ratios. For probes to be used for Southern analysis, a higher level of DIG-dUTP incorporated per amount of template DNA (i.e. a higher specific activity) is generally required, especially for the detection of single copy genes within complex genomes. To make such probes, the lower ratio (i.e. 1:2) works better.

Hybridisation and washing

The hybridisation temperature used depends largely on the probe and the DNA you are probing. If using a probe with 100% homology to the target sequence, such as in the case of transgene detection, 50°C is an appropriate temperature.

1. Put on gloves and WASH TO REMOVE ANY TRACES OF TALCUM POWDER.

Talcum or other powder can cause spotting background when you come to detect the hybridisation signal.

Pre-warm DIG Easy Hyb solution to 50°C.

Pre-warm hybridisation oven (for bottles) or shaking water bath (for plastic bags) to 50°C.

2. Place membrane into a plastic bag or hybridisation bottle.

Hybridisation bottles are preferred since these are less messy than using plastic bags and good rotation of the membrane with the hybridisation solution can be assured. The use of bottles requires that you have an appropriate hybridisation oven that can rotate the bottles. If you are using plastic bags, ensure they are submerged into the water of the shaking water bath, otherwise the hybridisation will not be even. You can do this by using a small lead weight to hold them down at each corner.

You can have more than one membrane per bottle/plastic bag. Use nylon gauze between each membrane to ensure they do not stick to each other and the hybridisation solution can reach each one.

3. Place 10 mL of pre-warmed DIG Easy Hyb solution in with the membrane, close bottle ensuring no leakage of solution through the seal. Seal bag if using plastic bags, ensuring the solution does not leak out.

   Place into hybridisation oven or shaking water bath at 50°C.

   Prehybridise by rotating or shaking the membranes in the solution for at least 30–60 minutes at 50°C.

4. If using fresh, previously unused probe: in a sterile Eppendorf tube, add 10–20 µL of DIG-labelled probe directly to 1 mL of DIG Easy Hyb solution.

   Denature for 10 minutes by placing tube in a boiling water bath. Ensure the lid of the tube does not pop open during boiling as you may lose or dilute the probe.

   Quench on ice immediately.

   Discard DIG Easy Hyb solution, used for prehybridisation and add 5–10 mL fresh pre-warmed DIG Easy Hyb.

   Add denatured, quenched probe to this solution in the bottle/plastic bag by pipetting.

Do not pour the solution in as you may pour it onto the membrane. If this occurs, you are likely to get a big blob of background where it hits the membrane.

DIG-labelled probes can be re-used several times. When using a DIG-Labelled probe for the first time, its activity needs to be determined. That is, you need to determine how well the probe has been labelled with the DIG label. Refer to the DIG user's manual for details.

   If using a previously used probe: replenish the used DIG-labelled probe by adding 25% unused probe.

   Boil for 10 minutes by placing the tube in a boiling water bath. Ensure the lid of the tube does not pop open during boiling as you may lose or dilute the probe.

   Quench on ice.

   Remove the prehybridisation solution from the bottle/plastic bag.

   Pour in the boiled hybridisation solution.

5. Hybridise overnight at 50°C by rotating bottle/shaking plastic bag.

   Following hybridisation, pour off the probe into a fresh tube and store at –20°C until needed again.

If using bottles, you can carry out the following washing steps with the membrane still in the bottle. If using plastic bags, remove the membrane from the bag and wash in trays. The trays will need to be as small as possible and with lids so that the water of the shaking water bath does not accidentally get into the trays and damage the membranes.

6. Wash membranes at RT.

These washes are carried out to remove excess probe from the membrane and to remove any non-specific hybridisation of the probe to sequences other than the target sequence. The first wash is at low stringency (i.e. high salt, low temperature) to remove the excess probe. The second wash is at high stringency (i.e. low salt, high temperature) to eliminate any non-specific binding. Depending on the homology of your probe for the target sequence and the length of the probe, you can adjust these washing conditions. If you are trying to hybridise a probe with low homology to the target sequence, you may wish to stop at the low stringency wash. Remember that with DIG-labelled probes, unlike with radioactively labelled probes, you cannot detect after the first round of washing and then do more washes to increase the stringency. Once you have detected the hybridisation, the only thing you can do is to strip the probe off and rehybridise, washing at a different stringency the next time.

Bottles: Set a hybridisation oven to 68°C for later high stringency washes.
Pour in ~100 mL of 2 × SSC/0.1% SDS.
Rotate the bottles quickly at RT for 5 minutes.
Pour off the solution and add fresh solution.
Repeat wash twice at RT for 5 minutes each.

Plastic bags/trays: Set a water bath to 68°C for later high stringency washes.
With membranes in a washing tray, pour in 2 × SSC/0.1% SDS to ensure membrane is well covered.
Cover tray with lid and place on shaking tabletop for 5 minutes at RT.
Pour off the solution and add fresh solution.
Repeat wash twice at RT for 5 minutes each.

7. Replace 2 × SSC/0.1%SDS with 0.2 × SSC/0.1% SDS.
   Carry out 2 × 20 minutes washes as described above, this time at 68°C.
8. Cool oven/water bath to RT.
   Rinse membrane in 0.2 × SSC/0.1% SDS at RT to cool blot and bottle.

The blot needs to be cooled at this stage since if it is hot, the blocking solution to be used in a later step will curdle and rendered ineffective.

Detection of the hybridisation signal

If using bottles, you can carry out the detection steps with the membrane still in the bottle. If using plastic bags, continue to use trays.

1. Bring the 10 × blocking solution to room temperature (this is usually stored at –20°C)
   Equilibrate the wet membrane with 10 mL of 1 × DIG wash buffer.
   Wash 1–2 minutes at RT.
2. Prepare 2 × blocking solution by diluting the 10 × stock 5-fold in 1 × maleic acid buffer.
   Discard the wash buffer and replace with 20 mL of 2 × blocking solution brought to RT.
   Agitate for 60 minutes at RT.
3. Centrifuge anti-DIG alkaline phosphatase-conjugated antibody in a microfuge for 5 minutes at RT.

This ensures any precipitate or particulate matter is at the bottom of the tube and not accidentally pipetted onto the membrane where it can contribute to background noise.

Pipette 1 µL of the antibody conjugate directly into the blocking solution already in the bottle/tray (this gives a 1:20 000 working dilution).
Rotate/shake at RT for 30 minutes.

Do not add directly onto the membrane as the concentrated antibody will cause uneven detection and high background in that spot. There are different chemiluminescent substrates available for the alkaline phosphate conjugated antibody. CSPD does not provide as sensitive a detection (ca. 25 × less) as CDP-Star. For CSPD, dilute the antibody 1:10 000; for CDP-star dilute the antibody 1:20 000.

4. Discard the antibody solution from the bottle/tray.
   Wash the membrane in 100 mL of 1 × DIG wash buffer, twice for 15 minutes each at RT.
   Remove substrate from storage and bring to RT, ensure there is no precipitate in the solution.
5. Wash membrane for 2–5 minutes in 10 mL 1 × DIG detection buffer to equilibrate the membrane to the new buffer.
6. Mix 100 µL of CSPD or CDP-star with 10 mL of 1 × detection buffer, i.e., dilute 1:100.
   ENSURE POWDER IS REMOVED FROM GLOVES BY WASHING
7. Stretch some plastic wrap over the bench, taping each of the corners down to obtain a smooth surface.
   Remove membrane from bottle/tray and place, DNA side up onto the plastic wrap.
   Cover each membrane with 5 mL of diluted substrate prepared in step 6.
   Leave 5 minutes on the bench at RT, ensuring the filter is evenly wet and does not lift during this period.
8. Drip membranes dry and blot back dry onto 3MM paper briefly.
   Place damp membranes between 2 sheets of overhead transparency ensuring no air bubbles are present. Do not rub membranes as this may smudge the signals.
   For CSPD substrate, place at 37°C for 10 minutes. This is not necessary for CDP-star substrate.
   Expose to x-ray film in a film cassette without intensifying screens at RT for 1–2 hours initially.
   Develop film and re-expose to film for shorter or longer periods, if necessary.

Use write-on transparencies you use with an overhead projector. Ensure that the transparencies you use have the same texture on each side. Do not use those with one rough side (photocopier film), as they stick fast together and mask the signal and you will not detect anything.

Southern hybridisation using radioactively-labelled probes

Labelling probes with radioactivity

As for DIG-labelled probes, radiolabelled DNA probes are made using either PCR or random priming (also known as oligo-labelling). The most commonly used label is [α^{32}P]-dCTP although [α^{33}P]-dCTP is also used.

Most molecular biology companies such as Pharmacia, Roche Diagnostics etc. supply kits for the labelling of DNA probes. We recommend the use of these since the preparation of the buffers etc required for oligolabelling can be tedious. The use of kits has simplified and sped up considerably the process of preparing radiolabelled probes.

Hybridisation and washing

As for DIG-labelled probes, membranes can be hybridised in either bottles or plastic bags. If at all possible, use hybridisation bottles, as they are much easier to handle than plastic bags. The introductory notes for using DIG-labelled probes regarding hybridisation temperature also apply to the use of radioactively labelled probes; however, the hybridisation method described here uses an aqueous hybridisation solution unlike the DIG Easy Hyb. Hence, the temperature of hybridisation will be higher to achieve the desired stringency.

1. Pre-warm hybridisation oven (for bottles) or shaking water bath (for plastic bags) to 65°C.
2. Place membrane into a plastic bag or hybridisation bottle.
3. Place 10 mL of hybridisation solution in with the membrane, close bottle ensuring no leakage of solution through the seal. Seal bag if using plastic bags, ensuring the solution does not leak out.
   Place into hybridisation oven or shaking water bath at 65°C.
   Prehybridise by rotating or shaking the membranes in the prehybridisation solution for a minimum of 60 minutes (up to overnight is fine, 4 hours is probably better than 1 hour) at 65°C.
4. Have freshly labelled probe in an Eppendorf tube.
   Add 100 µL of 10 mg/mL sheared herring or salmon sperm DNA.
   Denature for 10 minutes by placing tube in a boiling water bath. Ensure the lid of the tube does not pop open during boiling.
   Quench on ice.
   Either add directly to the prehybridisation solution in the bottle/plastic bag by pipetting or replace the prehybridisation solution with a fresh 10 mL to which the denatured herring sperm DNA has been added.

Herring sperm or salmon sperm DNA is added to reduce the background. This DNA binds non-specifically to the membrane, preventing the radioactively-labelled probe from doing so.

Do not pour the solution in as you may pour it onto the membrane. If this occurs, you are likely to get a big blob of background where it hits the membrane.

5. Hybridise overnight at 65°C by rotating bottle/shaking plastic bag.
   Following hybridisation, discard the radioactive hybridisation solution appropriately.

Radioactively-labelled probes can be reused, depending on the isotope used and the specific activity of the probe. If it is a very 'hot' probe made with fresh isotope, then it can probably be reused within the next 2 weeks.

6. Washing is carried out as described for DIG-labelled probes with the following modifications:
   Wash 2 × 20–30 minutes at 65°C with 2 × SSC/0.5% SDS.
   Wash 2 × 20–30 minutes at 65°C with 0.2 × SSC /0.5% SDS.

Detection of the hybridisation signal

After washing, remove membrane form bottle or tray and seal in plastic (removing air bubbles) before it dries out.

Using a Geiger counter, monitor the level and location of radioactivity. Compare with a corner of the membrane, which should be 'cold'.

Depending on the level of radioactivity, expose to x-ray film in cassettes using intensifying screens up to overnight at –80°C

Develop film and re-expose to film, if necessary.

3.3 Transgene expression: mRNA

Analysis of transgenic plants at the RNA level allows the researcher to determine the expression of the gene transferred into the plant species of interest. Genes are transcribed into messenger RNA (mRNA) which is then, in turn, translated into protein. Thus, a measure of the mRNA level from a specific gene gives a measure of the transcriptional activity of that gene i.e., when the mRNA is present, the gene must be transcriptionally active.

The presence of a specific mRNA molecule can be detected by many different methods. Here we briefly describe the use of northern hybridisation using DIG-labelled and radioactively-labelled probes as well as reverse transcription -PCR (RT-PCR) for measuring the cellular steady state levels of mRNAs. The rate of transcription of a specific mRNA in the cells' nuclei can also be measured by an assay called 'nuclear run on'. We do not describe this here but if you wish to find out more information, please refer to Current Protocols in Molecular Biology (Ausubel et al. 1997).

Northern hybridisation is similar to Southern hybridisation. Either total RNA or poly(A)$^+$ RNA (mRNA is usually polyadenylated to direct it into the cytoplasm) is extracted and separated according to size via denaturing agarose gel electrophoresis. The RNA is then transferred to a nylon membrane by blotting and hybridised with a mRNA-specific probe which has been labelled with either a radioisotope or with a non-radioactive label such as digoxigenin (DIG). This hybridisation is then detected by the appropriate method for the label used.

Detection of specific mRNA molecules from a total RNA population relies on the mRNA being reasonably abundant. If the mRNA is not abundant, the sensitivity of this procedure may not be sufficient. Thus, it may be necessary to purify the mRNA by isolating polyA$^+$ RNA.

The presence of a transgene transcript can also be detected by RT-PCR. This is a similar procedure as described for genomic DNA analysis except that the RNA is first used as a template for synthesising cDNA, which is then used as the template for the PCR. Please refer to Dietzgen (2000) for a detailed description of RT-PCR.

Preparing an RNase-Free Environment

RNA is very susceptible to degradation by ribonucleases (RNases). RNases are very stable enzymes that generally require no cofactors to function. Thus, even very small quantities of RNase contamination can cause serious degradation of the RNA. RNases can come into contact with RNA when cells are lysed during extraction procedures as well as from the outside from the researcher's hands, solutions and equipment. Thus, it is important to try to maintain an RNase-free environment when preparing and working with RNA. This is almost impossible; however, there are things that can be done to reduce the risk of contamination. The most important aspect of working with RNA is being aware of the where the risks of contamination are and trying to avoid them. There is a good description of RNase contamination and how to avoid it in Sambrook et al. (1989). It is not difficult to work with RNA if you have planned and prepared well and if you are aware of potential contamination as you work.

As much as possible, use sterile unused disposable plasticware. Such plasticware is free of RNases and can be used without pre-treatment. All other plasticware and glassware must be treated prior to use to remove contaminating RNases. It is especially important to remember that as you work in the laboratory, you will come into contact with many surfaces that are potentially contaminated. Hands are also a major source of RNases, thus wear gloves and be aware of what you have touched, changing your gloves regularly. Sterilise your work area.

Use fresh, sterile solutions that are dedicated to RNA work; using the same solutions for RNA and DNA work increases the risk of RNase contamination. You can also reduce the risk of contamination by using only sterile pipette tips, graduated pipettes or sterile measuring cylinders etc for dispensing solutions for RNA work. While this is not necessary, if you are having problems with RNase contamination, you may wish to reserve chemicals, glass and plasticware and equipment for RNA work.

The minimal pre-treatment is to sterilise by autoclaving, although this does not remove all RNases. Methods to prepare RNase-free glassware (baking 240°C) and plasticware and solutions (using the chemical 'diethylpyrocarbonate', DEPC) are described in Sambrook et al. (1989). Be careful when handling DEPC as it is a suspected carcinogen. DEPC cannot be used for solutions containing Tris because it reacts with amines. For solutions containing Tris, make up solutions with DEPC-treated water in an RNase-free bottle and add to RNase-free solutions. If possible, reserve a bottle of Tris crystals for RNA work.

Northern Hybridisation Analysis

RNA can be purified from the rest of a cell's components using one of several procedures. As for extraction of genomic DNA, the methods for RNA extraction involve disrupting the plant cells to release their contents followed by the denaturation and removal of proteins, carbohydrates and cell debris, usually with phenol/chloroform. During cell lysis, RNases must be denatured to render them inactive. In the method described below, phenol and detergent are used to achieve this. An alternative is to use guanidinium isothiocyanate. The higher molecular weight nucleic acids (chromosomal DNA) are precipitated leaving the lower molecular weight nucleic acids (including RNA) behind. The RNA is then precipitated, or alternatively it is pelleted through a caesium chloride cushion. The RNA purification methods described below have been used for the purification of total RNA from peanut.

Northern hybridisation was developed after Southern hybridisation. Its name refers to the transfer of RNA, rather than DNA, to a solid support, such as a membrane, and hybridisation of that RNA with a DNA or RNA probe. The transfer is done as it is done for Southerns. The major difference is that rather than denaturing the nucleic acids after gel electrophoresis, RNA is denatured prior to loading the gel and during gel electrophoresis. Northern analysis gives a measure of the steady state level of a specific mRNA and its size. It does not give a measure of the transcription rate of the mRNA nor the structure of this molecule.

Total RNA extraction

1. Remove ~1 g of plant tissue and wrap in labelled aluminium foil (record weight). Freeze in liquid nitrogen immediately. The tissue can be extracted straight away or stored frozen at –80°C until required.

2. Place tissue in mortar and add liquid nitrogen.
3. Working in a fume hood, grind tissue in liquid nitrogen to a fine powder ensuring no large bits of tissue remain.
   Add 2 mL NTES per 1–2 grams of tissue and 1.5 volumes (1.5 mL/g) of phenol/chloroform/isoamyl alcohol.
   Grind tissue until it has thawed and become totally liquid.
   Pour into a polypropylene tube such as a Falcon tube (15 or 50 mL) that can be capped well and will be less than half full with the extract.
   Vortex vigorously for 5 minutes.
4. Separate the phases by centrifugation for 15 minutes at 4°C at top speed in a benchtop centrifuge. A swing out rotor is best; 12 000 × *g* is the ideal speed. If the centrifuge cannot spin at this speed, increase the time.
   In a fume hood, remove upper aqueous phase to a sterile Oakridge tube (ensure no phenol is transferred).
5. Add 0.1 volume 2 M sodium acetate pH 5.8 and 2 volumes of absolute ethanol.
   Leave overnight at –20°C.
6. Spin at 9000 r/min in a Beckman JA20 rotor or equivalent for 20–30 minutes at 4°C.
   Pour off the ethanol carefully, ensuring the pellet remains in the tube.
   Drain by standing tube upside down on tissue, ensuring the pellet remains in the tube.
   Wash the pellet with 70% cold (–20°C) ethanol.
   Centrifuge for 5 minutes at 12 000 × *g* at 4°C.
   Pour off 70% ethanol carefully, ensuring the pellet remains in the tube.
   Drain by standing tube upside down on tissue, ensuring the pellet remains in the tube.
   Air-dry briefly.
7. Dissolve pellet in 1 mL of sterile distilled water per 1–2 g of plant tissue.
   Add 1 volume of 4 M LiCl (or 0.25 volume of 10 M LiCl).
   Stand for 3 hours on ice, or overnight at 4°C.
   Centrifuge at 12 000 × *g* for 10 minutes at 4°C.
8. Pour off supernatant carefully, ensuring the pellet remains in the tube.
   Dissolve pellet in sterile distilled water (0.5 mL/g tissue).
   Add 0.1 volume of 2 M sodium acetate pH 5.8 plus 2 volumes of absolute ethanol.
   Leave at –20°C for more than 2 hours.
   Centrifuge at 12 000 × *g* for 10 minutes at 4°C.
   Wash pellet with 70% ethanol.
   Centrifuge for 5 minutes at 12 000 × *g* at 4°C.
   Pour off 70% ethanol carefully, ensuring the pellet remains in the tube.
   Air-dry briefly.
9. Dissolve pellet in sterile distilled water (200 µL/g tissue).
   Determine the concentration from the A_{260} measurement. An OD of 1 is equivalent to 40 µg/mL of single stranded RNA.
   Once the concentration of the RNA is known, calculate the total amount of RNA present within the tube, i.e. the yield.

Before measuring the A_{260}, ensure the RNA is fully resuspended. The purity of the RNA can be determined by calculating the $A_{260/A280}$ ratio. If it is 1.8–2, then the RNA is pure, if it is less then it has protein and/or phenol contamination and the concentration measurement will not be accurate.

10. Store RNA precipitated under ethanol, i.e. add 0.1 volume of 2 M sodium acetate pH 5.8 and 2 volumes of absolute ethanol. Store at either –20°C or –70°C.
11. When you wish to remove some RNA, place onto ice when removing from freezer.
    Vortex thoroughly.
    Remove the volume equivalent to the amount of RNA you wish to analyse and transfer to a fresh, sterile Eppendorf tube.
    Place the stock RNA back in the freezer.
    Centrifuge the RNA you have removed at 12 000 × *g* at 4°C for 20–30 minutes.
    Wash pellet with 70% ethanol.
    Centrifuge for 5 minutes at 12 000 × *g* at 4°C.
    Pour off 70% ethanol carefully, ensuring the pellet remains in the tube.
    Air-dry briefly.
    Resuspend in the appropriate volume of sterile distilled water to give the RNA concentration you require.

Poly(A)$^+$ RNA extraction

mRNA represents only about 1% of the total RNA within a cell. If you find that you cannot detect a specific mRNA from within a total RNA population, it maybe that this mRNA is not very abundant and the sensitivity of the assay is not high enough. The sensitivity can be increased using the mRNA population only. With rare exceptions, mRNA molecules have a string of adenine residues at their 3' end known as a polyA tail. Affinity chromatography based on the hybridisation of adenine and thymidine is used to isolate mRNA, thus, the polyA tail is used as kind of hook for separating mRNA from the other RNA forms. A total RNA population is combined with oligo(dT) molecules bound to a solid matrix such as cellulose. The mRNA molecules then become physically separated from the other RNA forms as their polyA tails hybridise to the oligo(dT). Obviously, for the purification of poly(A)$^+$ RNA, it is critical that the starting RNA be intact and undegraded.

A method describing the isolation of mRNA can be found in Sambrook et al (1989). However, many molecular biology companies sell products specific for the purification of mRNA. These products have simplified and sped up this procedure greatly.

Agarose gel electrophoresis of RNA

Some researchers recommend having gel electrophoresis equipment set aside for RNA work only to reduce the risk of contamination with RNases. If you do not have the resources for this, it is possible to use equipment for common purposes for RNA work. Ensure the gel tank, tray and comb are cleaned with detergent and rinsed thoroughly in DEPC-treated water. Wipe with ethanol and air dry. Some recommend then soaking the equipment in 3% H_2O_2 for 10 minutes at RT and rinsing thoroughly with DEPC-treated water (Sambrook et al. 1989).

Even though RNA is a single stranded molecule, it can fold into secondary structures via intramolecular base pairing forming double stranded regions. To probe RNA sequences effectively, this secondary structure must be denatured. This is done prior to loading RNA onto an agarose gel. To prevent the RNA from refolding as it migrates through the agarose, a denaturing gel containing formaldehyde can be used.

1. Prepare deionised formamide and formaldehyde.
   In a fume hood, add 1 g of Mixed bed resin to 100 mL of formamide/formaldehyde.
   Stir gently for 30 minutes and filter through Whatman #1 paper into sterile containers such as 50 mL Falcon tubes.

Formamide and formaldehyde are hazardous chemicals and as such should be treated with caution by wearing a lab coat, gloves and working with them in a fume hood. They need to be deionised since ions present within these solvents can negatively affect the way the RNA migrates through the agarose gel. Greater than 100 mL can be deionised and stored at –20°C for future use.

2. Up to 5 µg of total RNA will be loaded onto the gel, thus, remove the volume equivalent to 5 µg from your RNA stock and prepare as described above, resuspending finally in 3.5 µL of sterile distilled water.

Generally, 5 µg of total RNA has been sufficient for several legume species, including peanut, for the detection of specific mRNAs within a total RNA population. If you wish, you can load >5 µg by scaling up the preparation of the sample as described below. If you do this, ensure the gel well can hold the increased volume.

3. Prepare the gel tray before making up the gel mixture. See Chapter 3.2 for notes on this.
   Prepare a 65°C water bath.
4. Using a sterile bottle/flask to make up a 100 mL gel, melt 1.4 g of high quality agarose in 10 mL of 10 × MOPS buffer and 85 mL of sterile, RNase-free distilled water.
   Melt in a microwave oven or over a Bunsen burner without over boiling.
   Check the volume by using a sterile plastic measuring cylinder.
   Make up to 95 mL with sterile, RNase-free distilled water.
   Pour back into bottle/flask.
   In a fume hood, add 5 mL of deionised formaldehyde and mix well.
   In one action, pour the agarose mixture into the prepared gel tray that is waiting in the fume hood.
   Remove air bubbles form the gel with sterile pipette tip or Pasteur pipette.
   Leave to set at RT.

Caution: Ensure you are wearing safety glasses, gloves and a lab coat when handling formaldehyde-containing solutions. Do not put your face over the gel after it is poured to avoid inhaling formaldehyde fumes.

5. While the gel is setting, prepare 1 × MOPS buffer from 10 × stock. Depending on the gel rig you are using, you may need up to ~1L of this.
   Also prepare the sample denaturing solution. For each sample, mix together in a sterile Eppendorf tube:
   10 µL deionised formamide.
   3.5 µL deionised formaldehyde.
   2 µL 10 × MOPS buffer.
   1 µL 10 mg/mL ethidium bromide.
   Mix thoroughly and leave on ice until required.

6. Place the set gel into the gel rig, ensuring any tape and the comb have been removed. Pour 1 × MOPS buffer into the gel rig so that it is level with the top of the gel but does not submerge the gel.
7. Mix together 3.5 µL (= ~5 µg of total RNA) and 16.5 µL of the sample denaturing solution. Place in the 65°C water bath for 5 minutes.
   Quench on ice.
   Add 5 µL of RNA loading dye and load immediately onto gel.
   Other samples to load would be positive and negative controls as well as RNA size markers.
   Assemble the gel rig and hook it up to a power pack, remembering that RNA being negatively charged at neutral pH will run towards the positive electrode.
   Start the gel running by setting the desired voltage.
   Slowly electrophorese the RNA about 1 cm into the gel.
   Turn off the current and pour in more 1 × MOPS buffer to submerge the gel ~1 cm.
   Turn the current back on and run until the bromophenol blue has reached ~1 cm from the bottom of the gel.
8. If you are using a horizontal gel rig, remove the gel and check how far the RNA has run and its integrity.
   If the RNA has run far enough, take a photograph of the gel as a record.
   If you have run unlabelled markers, mark the position of each marker and/or the rRNA bands which should be clearly visible. This can be done using Indian ink or cutting the gel as described in chapter 3.2. Once the rRNA has been transferred to the membrane, they should be clearly visible. You may wish to wait and mark their position in pencil on the membrane after blotting.
   You should also include a negative control such as RNA prepared from an untransformed plant, and a positive control such as RNA from a plant you know is expressing the RNA or a synthetic RNA prepared from the cloned gene.

Northern blotting

1. Transfer the gel from the gel rig to a tray.
   Wash gently in sterile, RNase-free distilled water for 15 minutes.
   Replace the water with 20 × SSC and wash for 20 minutes at RT.
   Repeat the 20 × SSC wash.
2. Set up the blotting rig as described for Southern blotting (see page 53).
   The next day, disassemble the blotting apparatus as described.
   Remove the membrane and immediately note the positions of the rRNA bands (which should be visible) with a pencil on the side of the membrane. Then cover in plastic wrap to avoid it drying out.
   Fix the RNA to the membrane as described for Southern membranes.
   Remove the membrane from the wrap and wash in 2 × SSC to remove any traces of agarose.
   Immediately seal in a plastic bag to prevent it drying out.
   Store at 4°C until ready for use.
3. Look at the gel after transfer on a UV transilluminator to confirm that most of the RNA was transferred.

Northern Hybridisation

Labelling probes

DNA probes are labelled either with DIG or radioactive isotopes as described for Southern hybridisation. RNA probes (riboprobes) are often used for probing northern blots as these probes provide greater sensitivity than DNA probes and allow strand specific detection of mRNA. To make an RNA probe, you must have your sequence of interest cloned into a plasmid that carries a promoter for a DNA-dependent RNA polymerase such as T7, T3 or SP6. Also, the sequence must be cloned in such a way so that transcription from the promoter through the sequence of interest will give rise to antisense (complementary) synthetic RNA that can then hybridise to the sense mRNA molecule within the RNA found on the membrane. If you transcribe a sense RNA probe, it will not hybridise to the mRNA but rather to any complementary antisense RNAs that may be present. For more information about the desired features of DNA templates for preparing riboprobes, please refer to the technical information provided by the various companies that supply the relevant kits.

While DIG-labelled probes have been used for the detection of mRNAs on northerns, the sensitivity may not be as high as you need. Thus, we recommend the use of radioactively-labelled probes for northern hybridisation analysis. In doing so, we also urge you to ensure you are adequately prepared for the use of radioisotopes.

Hybridisation and washing

Hybridisation is carried out essentially as described for Southern hybridisation. The difference is the hybridisation mixture and/or temperature of hybridisation. Generally, hybridisation solutions containing formamide are used in northern hybridisation analysis. We recommend the use of a formamide containing hybridisation solution and hybridisation at 42°C as a starting point. Always include RNA prepared from an untransformed plant as a negative control on your northerns, this will tell you if you have specific hybridisation to the transgene or not.

Carry out hybridisation and washing according to the instructions outlined for Southern hybridisation analysis, substituting 42°C for the prehybridisation and hybridisation temperatures and substituting the following mixture for the Southern hybridisation solution:

50% formamide.
5 × SSC.
2 × Denhardt's solution.
0.1% SDS.
100 µg sheared herring sperm DNA/mL.

Detection of the hybridisation signal

Detection of the hybridisation signal is as described for Southern hybridisation (Chapter 3.2).

3.4 Transgene expression: protein

Analysis of the transgene product provides information about the activity of the transgene within a transformed plant. If the transgene has been engineered to give rise to a protein, a measure of the specific transgene product gives a measure of gene activity as well as the translatability of the transgene mRNA. Synthesis of the transgene product provides further proof of the transcriptional activity of the transgene.

The presence of a specific protein can be detected by many different methods, most of which rely on the use of an antibody specific for the particular protein of interest. We have used plate-trapped antigen enzyme-linked immunosorbent assay (ELISA) as well as western or immunoblotting to measure the steady state levels of transgenic protein.

Plate-trapped antigen ELISA (PTA-ELISA) involves coating polystyrene wells with crude plant extracts and washing away any excess material before binding of the protein-specific antibody. This antibody is then detected by a secondary antibody, which has been covalently linked to an enzyme. The substrate for this enzyme is added and hydrolysed to yield a coloured soluble product which is detected spectrophotometrically. The method is semi-quantitative, i.e. the intensity of colour is an indication of the amount of specific protein present within the crude preparation.

Immunoblotting or western blotting is similar to southern and northern blotting in that target molecules (here: proteins) from a crude mixture are separated according to size by denaturing gel electrophoresis and then transferred to a membrane. The specific protein is detected using a specific antibody, like in ELISA. Again, a secondary antibody is applied which is conjugated to an enzyme. The enzyme's substrate is added and the resulting insoluble product either emits light energy or is coloured.

Plate-Trapped Antigen (PTA) — ELISA

1. Remove 3 samples of ~5 mm^2 per leaf (cork borer No.4) and place into a labelled sterile Eppendorf tube.
   Place immediately on ice. Use immediately or store at –20°C until required.
2. Add 400 µL coating buffer and grind in the tube using a small pestle. Place samples on ice while grinding the remaining samples.
   Centrifuge for 2 minutes in a microfuge and return to ice.
   Transfer 100 µL of each sample to a well of an ELISA plate in duplicate.
   Place wet paper towels into a plastic box, lying them flat.
   Place ELISA plate into box on top of wet paper towels and place lid on box.
   Incubate for ~ 16 hrs at 4°C overnight.

In this assay, we use flat bottom polystyrene ELISA plates with a 96-well format. Please note that the plastic used to manufacture 96-well plates and the shape of the wells can vary. The wet paper towels are used to prevent the liquid from evaporating.

3. Place some PBS-Tween into a wash bottle.
   Empty contents of plate by inverting the plate and shaking into a sink.
   Wash plate with PBS-Tween by squirting solution vigorously into each well. All the green colour should disappear after this first wash. Ensure wells are left filled with PBS-Tween. Leave 3 minutes at RT. Empty contents by flicking plate. Repeat PBS-Tween washes/ incubations twice.
   Finally empty wells by flicking the plate and with a vigorous action, slam the plate down 5 times onto some cushioned paper towels without breaking the plate.

It is important that as much liquid is removed from each well as possible between each wash. This reduces the amount of carry over between treatments.

4. Remove antibody (usually prepared from a rabbit) from freezer and dilute appropriately in sample buffer. For a good antiserum, we use a 1:1000–1:10 000 dilution.
   Add 100 µL of diluted antibody to each well.
   Incubate for 2 hours at RT in the boxes with wet paper towels.
5. Wash with PBS-Tween as described above.
6. Dilute commercial secondary antibody (goat anti-rabbit IgG-alkaline phosphatase) in conjugate buffer as recommended by the manufacturer.
   Add 100 µL to each well.
   Incubate in the boxes for 3 hours at RT.

This secondary antibody is generally purchased from a company such as Sigma. We have used a goat anti-rabbit IgG since the primary antibody was raised in a rabbit. It does not matter what the source of the secondary antibody is as long as it is specific for the species in which you raised the primary antibody. The dilution at which to use this antibody will be recommended by the manufacturer. It is generally in the order of 1:10 000–1:30 000.

7. Wash with PBS-Tween as described above.
8. Prepare a fresh 1 mg/mL solution of p-nitrophenyl phosphate in substrate buffer.
   Add 100 µL to each well.
   Incubate for 1 hour at RT.

This incubation time can be from 15 minutes to 2 hours, depending on how fast colour develops — you need to watch and decide empirically. Whatever length of time you choose, you should use the same length of time for all your experiments so you can compare your data. Depending on what is available in your laboratory, other enzymes such as peroxidase or penicillinase and their respective substrates and buffers can be used.

9. Stop reaction by adding 50 µL of 1 M NaOH per well.
10. Measure the $A_{410/405nm}$ values using an ELISA plate reader.

For this particular substrate, you will notice the colour turning yellow where you have binding of the antibody to the protein of interest. Hence, the absorbance is read at 410 or 405 nm. When you carry out a PTA-ELISA experiment, ensure you have included positive and negative controls. A negative control would be a leaf extract from an untransformed plant and buffer only. This will tell you the non-specific background binding of the antibody to plant proteins or the plastic. A positive control would contain the specific protein that the antibody detects, i.e. in the case of an expressed viral coat protein, an extract from a virus-infected plant could be used.

Western Blotting

SDS-polyacrylamide gel electrophoresis

This procedure for electrophoresis of proteins is based on Laemmli (1970).

We recommend using a Mini Protean II System available from BioRad for the electrophoresis of samples and the subsequent electroblotting of these to nitrocellulose membrane.

1. If using a Mini-Protean II System, assemble the gel plates as described by the manufacturer. If using home made plates, put together the gel plates by taping and clamping so as to avoid leakage.
2. Prepare a lower (separating) gel as follows:
   In a 50 mL beaker, mix together distilled water, lower Tris buffer and acrylamide/bisacrylamide (29:1) mixture according to the proportions outlined below for the percentage gel you wish to prepare.
   When you are ready to pour the gel, add the freshly prepared 10% ammonium persulphate stock and TEMED, swirl to mix while avoiding the formation of air bubbles.

	7.5%	8%	10%	12%	15%
Distilled water	6.63 mL	6.4 mL	5.53 mL	4.63 mL	3.3 mL
Lower Tris	3.33 mL	3.33 mL	3.33 mL	3.33 mL	3.33 mL
Acryl/bis	3.33 mL	3.56 mL	4.43 mL	5.33 mL	6.67 mL
10% amm. persulfate	40 µL	40 µL	40 µL	40 µL	40 µL
TEMED	7 µL	7 µL	7 µL	7 µL	7 µL

The recipes given above are for the Mini-Protean II System. This size gel is generally suitable for the analysis of transgenic plants; however, if you wish to obtain greater resolution, use a larger gel system and scale up the above recipes.

Smaller proteins will migrate through the gel more quickly than larger ones during electrophoresis. For analysis of proteins of 25–50 kDa, use a 10% gel; for smaller proteins up to 10 kDa, 12% is recommended.

3. Add the mixture by pouring it carefully between the plates, using a wide-bore pipette. Try to avoid trapping air bubbles, move the plates about to encourage any air bubbles to the top of the gel mixture. Ensure the separating gel mix does not go above ~ 1.5–2 cm (a finger wide) from the top.
   Overlay the mixture by carefully pipetting some water-saturated butanol over the top with a Pasteur pipette.
   Allow the mixture to polymerise for 1 hour at RT.

The butanol is to provide an anaerobic environment for the gel to set and an even surface on which to pour the stacking gel. To saturate butanol with water, add ~10% (v/v) distilled water, shake vigorously and wait until the phases have separated. The butanol will be on the top.

4. Prepare a 5% upper (stacking) gel as follows: In a 20 mL beaker mix 1.93 mL distilled water, 0.85 mL Upper Tris buffer, and 0.58 mL acrylamide/bisacrylamide (29:1) solution.
5. Pour off the butanol from the polymerised separating gel and, using a wash bottle, carefully rinse the surface of the gel with distilled water and remove any excess liquid with an absorbent tissue.
   When you are ready to pour the stacking gel, add 20 µL 10% ammonium persulphate and TEMED, swirl to mix avoiding the formation of air bubbles and pour immediately (the gel should set within 10–15 minutes).

Carefully and quickly pour the stacking gel mixture to the top of the separating gel until it reaches ~2 mm from the top of the plate. Try to avoid trapping air bubbles.
Carefully insert the comb into the stacking gel mixture, trying not to trap air bubbles at the bottom of the comb's teeth.
Allow to polymerise for 30 minutes.

6. Once the gel has polymerised, assemble gel rig according to the manufacturer's instructions. If you are using a home made rig, assemble so that no buffer will leak from the upper reservoir into the lower reservoir. You may need to use Vaseline to seal the plates securely to the rig.
   Carefully remove the comb from the gel avoiding damaging the wells.
   Using a wash bottle, carefully rinse each well with electrophoresis buffer.
   Press plates into holder and fill the upper and lower reservoirs with electrophoresis buffer.
   Using a bent syringe, blow out the bubbles from between the bottom of the plates.
7. Using a mortar and pestle grind plant sample in a suitable low salt buffer such as10 mM phosphate, PBS or TE buffer, or in distilled water at a ratio of 1:20 (w/v). You may want to add a pinch of acid-washed sand to help in the homogenisation of the plant material. Centrifuge briefly to remove coarse plant material and place sample on ice.
   Mix together in a fume hood one part clarified plant extract with one part 2 × SDS-PAGE sample buffer in an Eppendorf tube.
   Close and secure lid before boiling for 10 minutes and place on ice immediately to cool to RT.
8. Add 1–15 µL samples to each well. The smaller the volume, the better the stacking and subsequent separation of proteins. Try to load similar volumes across the gel to avoid distortion of bands.
   Attach the electrodes and hook gel rig up to power pack. Apply 100V constant voltage to run the sample through the stacking (upper) gel; once all of the sample has entered the separating (lower) gel, increase to 180V for the remainder of the electrophoresis until the bromophenol blue almost reaches the bottom of the gel.

Include prestained protein molecular weight markers as one of the samples. This will allow you to follow 1. separation of proteins during electrophoresis and subsequently 2. transfer from the gel to the membrane.

9. When the electrophoresis has finished, turn off the power and remove the electrodes.
   Pour off the electrophoresis buffer.
   Remove the gel plate assembly from the rig and place onto paper towels.
   Carefully separate the plates with a spatula leaving the gel on one glass plate.
10. If the proteins in the gel are to be stained, at this point carefully lift the gel off the plate and transfer it into a container with Coomassie Blue stain and leave for 2 hours to overnight. Destain the gel by washing in several changes of destaining solution.
    If the gel is to be electroblotted, at this point set up the blotting apparatus according to the instructions on page 72.

Western immunoblotting

Wear gloves throughout this procedure. Do not let your bare skin come into contact with the nitrocellulose membrane as your skin will leave oil traces that will contribute to the background.

1. Cut a nitrocellulose (0.45 μm pore size) sheet to the size of the gel. For the Mini Protean II System, this is 8 × 10 cm.
   Cut two pieces of Whatmann 3MM filter paper 8 × 10 cm.
   Have 2–3 L of transfer buffer prepared and stored at 4°C.

Nylon membrane is generally not suitable for western blot detection and nitrocellulose has to be used.

2. Remove the gel from the glass plate and cut away the stacking gel and marker dye front. Mark the top left hand corner by cutting away a small piece of gel.
   Transfer the gel to a plastic box containing transfer buffer and gently agitate for 10 minutes (10–15% gel) to 30 minutes (5–10% gel).
3. Have a second plastic box containing transfer buffer.
   Carefully lower the nitrocellulose filter into the buffer, avoiding trapping air bubbles. Once it has wet through on the underside, gently submerge the filter in the buffer.
   Also soak the filter paper and transfer sponges in the transfer buffer.
4. Prepare the sandwich for electroblotting by keeping everything submerged; this helps to avoid trapping air bubbles. Assemble on the cathode (the grey plastic support for the Mini Protean II System) in the following order: sponge-filter paper-gel-nitrocellulose-filter paper-sponge-clear plastic. Do this by placing a wet transfer sponge onto the cathode. Next, place a sheet of wet 3MM onto the sponge, removing air bubbles carefully. Place gel onto filter paper, removing air bubbles. Place the wet membrane onto the gel, placing it carefully into position over the gel. Rub the membrane with gloved fingers to remove air bubbles and to establish electrostatic interaction with gel. Place the other sheet of wet 3MM onto the filter, carefully removing air bubbles. Place other wet transfer sponge onto 3MM, removing air bubbles.
   Close the sandwich (still submerged in transfer buffer), taking care not to slide the sandwich.
5. Place the electrophoresis tank onto a magnetic stirrer and add stirring bar.
   Fill the transblot chamber with cold transfer buffer and an ice filled cooler, which will not leak into the buffer.
   Slowly place assembly into the transblot chamber, the grey side facing the black electrode.
   Hook up to power pack, ensuring the current direction is correct.
   Electroblot at 100V (constant voltage) for 70 minutes.
   Replace ice-filled cooler after 30 minutes.
6. Disassemble the apparatus and place membranes face-up on a piece of filter paper.
   Air-dry and store at RT or proceed directly to detection steps.
   Evaluate the success of the transfer by lack of bubble impressions on the membrane and by complete transfer of stained marker proteins. The gel can be stained with Commassie Blue as described above to assess extent of transfer.

Immuno detection

1. Have ready a plastic box containing rinse buffer with 0.1% Triton X-100.
   Gently wet the membrane by placing it on top of the rinse buffer and once wet through underneath, gently submerging it.
   Shake gently for 10 minutes at RT.
2. Place membrane in 50 mL Blocking Solution (rinse buffer + 0.1% Triton X-100 + 5% (w/v) low fat (skim) milk powder)
   Incubate with gentle shaking for at least 1 hour at RT or overnight at 4°C.

The blocking solution should have a smooth consistency. Do not use it, if the milk powder will not fully dissolve or form clumps. If this happens, use a fresher batch of milk powder.

3. In as small a container as possible with membrane lying flat, place membrane face-up and add 10 mL of fresh rinse buffer containing 0.1% Triton X-100 + 1% skim milk powder + antiserum specific to the protein to be detected.
   Incubate with gentle agitation for 90 minutes at RT.

In most cases the antiserum used would have been generated in a rabbit. The dilution to be used will need to be determined empirically and depends on the specific antibody titre. A 1:10 000 dilution is a good place to start.

4. Pour off antibody solution.
   Wash vigorously in 4 changes of rinse buffer + 0.1% Triton X-100, 20 minutes each. Do not place more than one membrane per container, since membranes may stick to each other and prevent proper washes.
5. Add 10 mL of rinse buffer + 0.1% Triton X-100 + 1% skim milk powder + secondary goat anti-rabbit IgG-alkaline phosphatase conjugate. Incubate with gentle agitation for 90 minutes at RT or overnight at 4°C

For the dilution to use, follow the manufacturer's recommendation for immunoblot applications. Sigma Co. recommends 1:30 000 for its conjugate. If the source of your primary antibody is not from rabbit blood, you will need to use the appropriate antibody conjugate, which is specific for the species used.

6. Pour off antibody conjugate solution. Wash vigorously in 4 changes of rinse buffer + 0.1% Triton X-100, 20 minutes each. Do not place more than one membrane per container.
7. Equilibrate membrane by washing for 5 minutes in 10 mL of 1 × DIG detection buffer.
8. Dilute 10 µL of 10 mg/mL CPD Star luminescent substrate in 1 mL of 1 × DIG detection buffer.
   Stretch a large piece of plastic wrap onto the bench and tape down corners so that it is flat.
   Place membrane face-up on plastic wrap.
   Cover membrane with diluted CPD Star substrate solution. You will need about 1 mL per 25 cm^2 of membrane.
   Leave for 5 minutes at RT.

There are other sensitive chemiluminescent substrates for alkaline phosphatase or horseradish peroxidase. Your choice depends on what is available in your laboratory. Colourimetric substrates are generally not as sensitive.

9. Drain excess liquid from membrane and blot face-up briefly onto 3MM paper. Do not let the membrane dry out.
   Place damp membrane between sheets of overhead transparency and expose to x-ray film.
   Refer to chapter 3.2 for notes regarding exposure to film.

3.5 Recipes

Nucleic Acid Extraction

4.4 M ammonium acetate

In a sterile bottle, dissolve 169.6 g of ammonium acetate in 400 mL of sterile distilled water.
Make up to 500 mL final volume.
Store at RT.

4 M LiCl

Dissolve 17 g of lithium chloride in 100 mL of distilled water.
Add 0.1 mL diethyl pyrocarbonate (DEPC) and incubate for at least 12 hours at 37°C
Autoclave to sterilise.
Store at RT.

NTES

0.1 M NaCl.
10 mM Tris-HCl pH 8.0.
1 mM EDTA.
1.0% SDS.

Solutions containing SDS cannot be autoclaved as the detergent will foam. Therefore, make up the NTES mixture without the SDS, autclave and add the SDS later. Ensure the SDS stock you use is RNase-free by making it up with DEPC-treated sterile distilled water. Alternatively, make the NTES up from sterile, DEPC-treated stock solutions and mix together in a sterile bottle.

Phenol/chloroform

The information provided here is from Sambrook et al. (1989). Phenol/chloroform is a 1:1 mixture of these two solutions. Phenol can come either as a clear, colourless liquid, which can be used without redistilling or in a crystalline form which does require redistilling. Sometimes, the liquefied form may be pink or yellow. If this occurs, do not use and return to the supplier for replacement. We recommend buying the liquefied form of phenol as it is simpler to work with. If you can only purchase the crystalline form, it must be redistilled at 160°C to remove oxidation products. If these are not removed, these products can cause cross-linking of RNA and DNA or cause the break down of the phosphodiester bonds of these molecules.

Phenol should be equilibrated to pH >7.8 otherwise the DNA will not partition into the aqueous phase. A method for the equilibration of phenol can be found in Sambrook et al. (1989).

Please note that phenol should be handled with extreme care in a fume hood and wearing protective clothing such as gloves, lab coat and safety glasses. It is a highly corrosive substance that can cause severe burns. If skin burns occur, wash with soap and water. Do not use ethanol to wash the area.

The phenol/chloroform mixture usually also contains isoamyl alcohol. The phenol denatures proteins, the chloroform debaures proteins and starches and facilitates the separation of the phases and the isoamyl alcohol reduces foaming during the extraction. Only the phenol requires treatment prior to making up this mixture.

To make 500 mL of a 25:24:1 mixture of phenol:chloroform:isoamyl alcohol mix together the following:
250 mL equilibrated phenol.
240 mL chlorform.
10 mL isoamyl alcohol.
Mix thoroughly.
Overlay with either 100 mM Tris-HCl pH 8.0 or TE pH 8.0.
Store in a dark bottle at 4°C for up to 1 month.

The shelf life can be extended with the addition of 8-hydroxyquinoline, which slows the oxidation process. Add a few crystals of this compound and dissolve by shaking. The organic phase will turn yellow, add enough crystals to turn the mixture canary yellow in colour.

10 mg/mL RNaseA

RNase A can be purchased as a ready-made solution from most companies which sell molecular biology reagents. Ensure the RNase A is free of DNase. You can also buy it as a powder and make a stock solution as described below; however, it may be contaminated with DNase which must be removed by boiling.

Weigh out 100 mg RNase A into a sterile container.
Add 10 mL of sterile distilled water and dissolve the powder completely.
Place tube containing the solution into a boiling water bath for 10–15 minutes.
Aliquot into sterile Eppendorf tubes.
Store at –20°C.

While boiling the solution, ensure that the lid does not pop open, as any evaporation will change the concentration of the solution.

10% SDS

Wearing a lab coat, gloves, safety glasses and a face mask, weigh out 50 g of SDS powder into a sterile bottle.
Add ~900 mL sterile distilled water carefully so as not to disturb the powder.
Once dissolved, make up to 1 L with sterile distilled water.
Store at RT.

Do not autoclave as this solution. At colder temperatures, the SDS may precipitate out of solution. If this occurs, heat until it has dissolved before you use it. For RNA work, ensure the solution is made with DEPC-treated sterile water. Use dedicated solutions for RNA work only.

TE (10 mM Tris-HCl/1 mM EDTA, pH 8.0)

10 mL 1 M Tris-HCl pH 8.0.
2 mL 0.5 M EDTA pH 8.0.
988 mL distilled water.
Sterilise by autoclaving.
Store at RT.

For RNA work, make this solution using DEPC-treated water. Do not treat with DEPC afterwards.

TPS

Using sterile stocks solutions, prepare 100 mL as follows in a sterile bottle:
10 mL 1 M Tris-HCl pH 8.0 (100 mM final concentration).
33.35 mL 3 M KCl (1 M final concentration).
2 mL 0.5 M EDTA pH 8.0 (10 mM final concentration).
54.67 mL sterile distilled water.
Store at RT.

Urea extraction buffer

168 g urea.
25 mL 5 M NaCl.
20 mL 1 M Tris-HCl pH 8.0.
16 mL 0.5 M EDTA pH 8.0.
20 mL 20% sarcosine.
Make to 400 mL with sterile distilled water (~190 mL).
Store at RT.

Do not autoclave this solution as it contains urea. Instead, use autoclaved stocks and bottles.

0.05 M sodium phosphate buffer pH 8.0

Combine 46.6 mL of 1 M Na_2HPO_4 pH 8.0 and 3.4 mL NaH_2PO_4 pH 8.0.
Make up to 1 L with distilled water and autoclave.
Store at RT.

DNA Restriction Digestion and Electrophoresis

10 mg/mL ethidium bromide

Ethidium bromide is a suspected mutagen. Where possible, avoid handling this compound in powder form to avoid inhaling it. When handling the liquid, always wear gloves. If possible, purchase pre-made ethidium bromide stock solution from a company selling molecular biology chemicals. If you must prepare the stock solution from powder, follow these instructions:

Ensure you are wearing a lab coat, safety glasses, gloves and a face mask.
In a fume hood, weigh out 100 mg ethidium bromide powder into a sterile bottle.
Carefully add 10 mL of sterile destilled water so as not to disperse the powder into the air.
Add a magnetic stirring bar to the bottle, seal the bottle and cover with aluminium foil.
Place on a magentic stirrer overnight.
Store at 4°C.

The magnetic stirring bar should be dedicated for use only with ethidium bromide.

6 × DNA loading dye

40% sucrose (w/v) in sterile distilled water.
0.25% bromophenol blue.
Aliquot into 1 mL aliquots and store at 4°C.
(This and alternative dye solutions are described in Sambrook et al. (1989)).

0.1 M spermidine

See the recipe section in chapter 2.3.

50 × TAE

242 g Tris base.
57.1 mL glacial acetic acid.
100 mL 0.5 M EDTA pH 8.0.
Make up to 1L with distilled water and autoclave.

10 × TBE

108g Tris base.
55 g boric acid.
40 mL 0.5 M EDTA pH 8.0.
Make up to 1L with distilled water and autoclave.

Alternatively, a 5 × stock can be made. For either concentration, a precipitate forms over time when concentrated TBE is stored. Try to store stocks in glass bottle at RT and discard batches the form the precipitate. While we have recommended a working strength of 1x, for agarose gels a 0.5 × solution will also provide enough buffering and current for electrophoresis.

10 × MOPS buffer

0.2 M MOPS.
0.05 M sodium acetate.
0.01 M EDTA pH 8.0.
Adjust pH to 7.0 with glacial acetic acid.
Sterilise by autoclaving.
Store in the dark at RT (either in a darkened cupboard or cover the bottle in aluminium foil).

This solution will turn yellow on autoclaving.

RNA loading dye

8% ficoll.
0.02% bromophenol blue.
0.04% xylene cyanol.
Aliquot into 100 µL or smaller aliquots.
Store aliquots at RT.

Discard each aliquot after use.

Protein Extraction

30% Acrylamide/0.8% bisacrylamide (w/v)

Puchase ready-made solution from a company.

4 × Upper Tris buffer

6.06 g Tris-base (0.5 M Tris-HCl).
Adjust pH to 6.8 with concentrated HCl.
4 mL 10% SDS.
Make to 100 mL with distilled water.
Store at RT.

4 × Lower Tris buffer

18.17 g Tris-base (1.5 M Tris-HCl).
Adjust pH to 8.8.
4 mL 10% SDS.
Make to 100 mL with distilled water.

Tris-glycine electrophoresis buffer

3 g Tris-base.
14.3 g glycine.
2 g SDS.
Make to 1L with distilled water.
Store at RT.

SDS-PAGE sample buffer

10 mL glycerol.
5 mL 2-mercaptoethanol.
30 mL 10% SDS.
12.5 mL 4 × Upper Tris buffer.
Make up to 100 mL with distilled water.

Coomassie Blue staining solution

Dissolve 0.1g Coomassie Blue R in 1 mL methanol.
Add this to 200 mL 25% isopropanol/10% acetic acid.

Destaining solution

10% isopropanol/10% acetic acid.

Southern/Northern Blotting and Hybridisation

0.25 M HCl

Add 21.55 mL of concentrated HCl into 800 mL of distilled water.
Make up to 1 L with distilled water.
Store at RT.

Denaturing solution (1.5 M NaOH/0.5 M NaCl)

Dissolve 60 g NaOH and 29.22 g NaCl in ~800 mL of distilled water.
Make up to 1 L with distilled water.
Store at RT

Neutralising solution (1.5 M Tris-HCl pH 7.0/0.5 M NaCl)

Dissolve 181.71 g Tris base and 29.22 g NaCl in ~800 mL of distilled water.
Add concentrated HCl until the pH reaches 7.0.
Make up to 1 L with distilled water.
Store at RT.

20 × SSC

Dissolve 175.3g g of NaCl and 88.2 g of tri-sodium citrate in ~800 mL of distilled water.
Make up to 1 L with distilled water.
Sterilise by autoclaving.
Store at RT.

For RNA work, treat this solution with DEPC prior to autoclaving and use only for RNA work.

10 mg/mL herring/salmon sperm DNA

Weigh out 200 mg of herring or salmon sperm DNA.
Add ~12 mL of distilled water.
Dissolve the DNA by stirring and heating.
Make up to 20 mL with distilled water.
Denature the DNA by sucking up and squeezing out through a 21 gauge needle.
Further denature by autoclaving.
Aliquot into 0.5 mL aliquots in sterile Eppendorf tubes.
Store at –20°C.

This solution is quite viscous before autoclaving. It is difficult to dissolve the DNA without heating. Take care not to exceed the total volume of 20 mL since the DNA itself takes up a large volume.

Hybridisation solution for radioactively labelled probes

0.9 M NaCl.
1% NaCl.
10% dextran sulphate.
100 μg/mL herring or salmon sperm DNA.

To make up 100 mL of this solution, using sterile solutions where appropriate, combine together.
18 mL 5 M NaCl.
10 mL 10% SDS.
10 g dextran sulphate.
Add ~20 mL sterile distilled water.
Stir and heat to dissolve the dextran sulphate.
Slowly add the remaining volume of water (52 mL) to make up to 100 mL, ensuring the dextran sulphate is dissolved completely.
Store at RT.

This solution is fairly viscous. The dextran sulphate is difficult to dissolve and takes up a large volume, thus you need to be careful that you don't add all of the water in one go as the final volume will be greater than 100 mL once the dextran sulphate has dissolved. On storage, some of the dextran sulphate comes out of solution so that just prior to use, heat for 1–2 minutes in a microwave or over a Bunsen burner until it is completely dissolved.

Make up this mixture without the herring sperm DNA and store at RT. Add the herring or salmon sperm DNA just prior to hybridisation as described in the procedure outline.

DIG Easy Hyb Solution

This particular solution must be purchased from Roche Diagnostics (formerly Boehringer Mannheim). The recipe for this solution is a trade secret and therefore cannot be reproduced in the laboratory.

Northern hybridisation can be carried out using DIG labelled probes, although the sensitivity is not as great as with radioactively labelled probes. If you wish to use a DIG-labelled probe for northern, use a fresh bottle of DIG Easy Hyb and keep it dedicated for RNA work.

2 × SSC/0.1% SDS

Mix together 100 mL 20 × SSC and 10 mL 10% SDS.
Add 890 mL sterile distilled water.
Store at RT.

Refer to the notes for 10% SDS.

0.2 × SSC /0.1% SDS

Mix together 10 mL 20 × SSC and 10 mL 10% SDS.
Add 980 mL sterile distilled water.
Store at RT.

Refer to the notes for 10% SDS.

10% Blocking solution

Blocking reagent must be purchased from Roche Diagnostics. As for DIG Easy Hyb, the recipe for this reagent is a trade secret and therefore can't be reproduced in the laboratory.

Aliquot blocking reagent upon opening into ~50 mL aliquots, using sterile 50 mL tubes or bottles.
Prior to aliquoting, store at RT. After opening and aliquoting, store at –20°C.

If using DIG-labelled probes for RNA work, keep an aliquot dedicated for this.

For immediate use as 2 × solution mix:
20 mL Blocking Reagent.
80 mL 1 × maleic acid buffer.

10 × Maleic acid buffer

This buffer can be purchased from Roche Diagnositcs. However, the recipe for this is available and can thus be made up in the laboratory as follows:

To 800 mL distilled water add:
116 g maleic acid.
87.6 g NaCl.
70 g NaOH beads.
Adjust pH to 7.5 with 8 N NaOH.
Fill up to 1 L with distilled water.

To make a 1 × working solution, dilute the stock 10×. For RNA work, ensure the solution is RNase-free.

10 × DIG washing buffer

This buffer can be purchased from Roche Diagnositcs. However, the recipe for this is available and can thus be made up in the laboratory as follows:

Add 30 mL Tween 20 to 1 L 10 × maleic acid buffer.

To make a 1 × working solution, dilute the stock 10×. For RNA work, ensure the solution is RNase-free.

10 × DIG detection buffer

This buffer can be purchased from Roche Diagnositcs. However, the recipe for this is available and can thus be made up in the laboratory as follows:

121.14 g Tris.
58.44 g NaCl.
Fill up to 1 L with distilled water.
Adjust pH to 9.5.

To make a 1 × working solution, dilute the stock 10×. For RNA work, ensure the solution is RNase-free.

ELISA buffers

All the buffers described below are as described by Clark and Adams (1977).

10 × Phosphate buffered saline (PBS) pH 7.4

80 g/L NaCl.
2 g/L KH_2PO_4.
11.5 g/L Na_2HPO_4.
2 g/L KCl.
Adjust pH to 7.4.
Sterilise by autoclaving.
Store at **RT**.

To make a 1 × working solution, dilute the stock 10×.

PBS-Tween

To 1 L of 1 × PBS pH 7.4, add 0.5 mL of Tween-20.
Store in wash bottles at 4°C.

Sample buffer

In a sterile bottle, add 2 g of soluble polyvinyl pyrrolidone (PVP) to 100 mL of fresh PBS-Tween.
Adjust pH to 6.0 and store at 4°C.

Conjugate buffer

In a sterile bottle, add 2 g PVP and 0.2 g bovine serum albumin (BSA) to 100 mL of fresh PBS-Tween.
Store at 4°C.

Coating buffer

In a sterile bottle, dissolve 0.159 g of Na_2CO_3 and 0.293 g $NaHCO_3$ in 70 mL of sterile distilled water.
Adjust pH to 9.6 with glacial acetic acid.
Make up to 100 mL with sterile distilled water and store at 4°C.

Substrate buffer

In a sterile bottle, mix 9.7 mL diethanolamine with 80 mL sterile distilled water.
Adjust pH to 9.8 with HCl.
Make up to 100 mL with sterile distilled water and store at 4°C.

Protein Transfer buffer

14.4 g/L glycine (192 mM).
3.03 g/L Tris base (25 mM).
200 mL/L ethanol (20%).
Add distilled water to 1 L; do not adjust pH.
Store at 4°C.

10 × Rinse buffer

12.1 g/L Tris-HCl pH 7.4 (10 mM).
87.6 g/L NaCl (150 mM).
3.7 g/L EDTA (1 mM).
Add distilled water to 1 L.
Store at 4°C.

REFERENCES

Atreya, C.D., Rao, J.P. and Subrahmanyam, N.C. 1984. In vitro regeneration of peanut (*Arachis hypogaea* L.) plantlets from embryo axes and cotyledon segments. Plant Science Letters, 34: 379–83.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. 1997. Identification of newly transcribed RNA. In: Current Protocols in Molecular Biology. John Wiley and Sons Inc, USA, 4–10.

Baker, C.M. and Wetzstein, H.Y. 1992. Somatic embryogenesis and plant regeneration from leaflets of peanut, *Arachis hypogaea.* Plant Cell Reports, 11: 71–75.

Baker, C.M. and Wetzstein, H.Y. 1998. Leaflet development, induction time, and medium influence somatic embryogenesis in peanut (*Arachis hypogaea* L.). Plant Cell Reports, 17: 925–29.

Baker, C.M., Durham, R.E., Burns, J.A., Parrott, W.A. and Wetzstein, H.Y. 1995. High frequency somatic embryogenesis in peanut (*Arachis hypogaea* L.) using mature, dry seed. Plant Cell Reports, 15: 38–42.

Brar, G.S., Cohen, B.A., Vick, C.A. and Johnson, G.W. 1994. Recovery of transgenic peanut (*Arachis hypogaea* L.) plants from elite cultivars utilising ACCELL technology. The Plant Journal, 5: 745–53.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. 1994. Green fluorescent protein as a marker for gene expression. Science, 263: 802–805.

Cheng, M., His, D.C.H. and Phillips, G.C. 1994. Recovery of primary transformants of Valencia-type peanut using *Agrobacterium tumefaciens*. Peanut Science, 21: 84–88.

Cheng, M., Jarret, R.L., Li, Z. and Demski, J.W. 1997. Expression and inheritance of foreign genes in transgenic peanut plants generated by *Agrobacterium*-mediated transformation. Plant Cell Reports, 16: 541–544.

Cheng, M., Jarret, R.L., Li, Z., Xing, A. and Demski, J.W. 1996. Production of fertile transgenic peanut (*Arachis hypogaea* L.) plants using *Agrobacterium tumefaciens.* Plant Cell Reports, 15: 653–657.

Chilton, M.D. 1983. Crown gall gene as a vector for plants. Bio/technology, 1: 163–164.

Clark, M.F. and Adams, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology, 34: 475–483.

Clemente, T.E., Robertson, D., Islieb, T.G. and Beute-Marvin, K. 1992. Evaluation of peanut (*Arachis hypogaea* L.) leaflets form mature zygotic embryos as recipient tissue for biolistic gene transfer. Transgenic Research, 1: 275–284.

De Freitas, V.G., Lacrote, C., Sachette, M.G., Krul, W.R., De Oliveira, D.E., Neves, L.J. and Mansur, E. 1997. Identification of competent cells for Agrobacterium-transformation and in vitro regeneration in peanut leaf and cotyledon explants. Revista Brasileira de Fisiologia Vegetal, 9: 157–167.

Dewi, I.S., Harjosudarmo, J., Birch, R.G. and Dietzgen, R.G. 1997. The effect of 2,4-D, NAA, and picloram on somatic embryogenesis and plant regeneration form immature seed. Jurnal Bioteknolgi Pertanian, 2: 23–30.

Dietzgen, R.G. (ed.) 1999. Elimination of aflatoxin contamination in peanut. Australian Centre for International Agricultural Research, Canberra, ACT. Proceedings No. 89.

Dietzgen R.G. 2000. Applications of PCR in plant virology. In: Khan, J.A. and Dijkstra, J. (ed.). Trends in Plant Virology. Harworth Press, New York.

Eapen, S. and George, L. 1993. Somatic embryogenesis in peanut: Influence of growth regulators and sugars. Plant Cell, Tissue and Organ Culture, 35: 151–156.

Eapen, S. and George, L. 1994. *Agrobacterium tumefaciens* mediated gene transfer in peanut (*Arachis hypogaea* L.) Plant Cell Reports, 13: 582–586.

Egnin, M., Mora, A. and Prakash, C. 1998. Factors enhancing *Agrobacterium tumefaciens*-mediated gene transfer in peanut (*Arachis hypogaea* L.). In Vitro Cellular and Developmental Plant Biology, 34: 310–318.

Fang, X., Xu, Z., Zhang, Z. Yan, L., Chen, K., Jia, S., Zhang, C., Livingstone, M.D., Birch, R.G. and Dietzgen, R.G. 1996. Plant regeneration and Agrobacterium-mediated gene transfer in leaflets of peanut (*Arachis hypogaea* L.). Oil Crops of China, 4: 52–56.

Fang, X., Xu, Z., Zhang, Z., Yan, L., Chen, K., Luo, L., Chen, J., Birch, R.G. and Dietzgen, R.G. 1999. Expression and inheritance of GUS gene and NPTII gene in transgenic peanut plants generated by *Agrobacterium*-mediated transformation. Peanut Science and Technology, 35: 241–245.

Finer, J.J., Vain, P., Jones, M.W. and McMullen, M.D. 1992. Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Reports, 11: 323–328.

Franklin, C.I., Shorrosh, K.M., Trieu, A.N., Cassidy, B.G. and Nelson, R.S. 1993. Stable transformation of peanut callus via *Agrobacterium* — mediated DNA transfer. Transgenic Research, 2: 321–324.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Molecular Biology Reporter, 5: 387–405.

Hazra, S., Sathaye, S. and Mascarenhas, A.F. 1989. Direct somatic embryogenesis in peanut (*Arachis hypogaea* L.). Bio/technology, 7: 949–951.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. The Plant Journal, 6: 271–282.

Hood, E.E., Helmer, G.L., Frayley, R.T. and Chilton, M.D. 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. Journal of Bacteriology, 168: 1291–1301.

Khan, M.R.I., Tabe, L.M., Heath, L.C., Spencer, D. and Higgins, T.J.V. 1994. *Agrobacterium* — mediated transformation of subterranean clover (*Trifolium subterraneum* L.). Plant Physiology, 105: 81–88.

Kochert, G.1996. Molecular markers and genome mapping. In: Darussamin, A. (ed.) Current Status of Agricultural Biotechnology in Indoensia, 89–108. AARD, Jakarta.

Kokalis-Burelle, N., Porter, D.M., Rodriguez-Kabana, R., Smith, D.H. and Subrahmanyam, P. (ed.) 1997. Compendium of Peanut Diseases, Secondnd Edition. St. Paul, USA: APS Press.

Lacorte, C., Mansur, E., Timmerman, B. and Cordeiro, A.R. 1991. Gene transfer into peanut (*Arachis hypogaea* L.) by *Agrobacterium tumefaciens*. Plant Cell Reports, 10: 354–357.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 227: 680–685.

Li, Z., Jarret, R.L., Pittman, R.N., Dunbar, K.B. and Demski, J.W. 1993. Efficient plant regeneration from protoplasts of *Arachis paraguariensis* Chod. Et Hassl. using a nurse culture method. Plant Cell, Tissue and Organ Culture, 34: 83–90.

Livingstone, D.M. and Birch, R.G. 1995. Plant regeneration and microprojectile-mediated gene transfer in embryonic leaflets of peanut (*Arachis hypogaea* L.). Australian Journal of Plant Physiology, 22: 585–591.

Livingstone, D.M. and Birch, R.G. 1999. Efficient transformation and regeneration of diverse cultivars of peanut (*Arachis hypogaea* L.) by particle bombardment into embryogenic callus produced form mature seeds. Molecular Breeding, 5: 43–51.

Mansur, E.A., Lacorte, C., De Freitas, V.G., De Oliveira, D.E., Timmerman, B. and Vordeiro, A.R. 1993. Regulation of transformation efficiency of peanut (*Arachis hypogaea* L.) explants by *Agrobacteirum tumefaciens*. Plant Science, 89: 93–99.

McFarlane, M. and Wilson, J.B. 1996. A model for the mechanism for precise integration of a microinjected transgene. Transgenic Research, 5: 171–177.

McKently, A.H., Moore, G.A., Doostdar, H., Niedz, R.P. 1995. *Agrobacterium*-mediated transformation of peanut (*Arachis hypogaea* L.) embryo axes and the development of transgenic plants. Plant Cell Reports, 14: 699–703.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiologia Plantarum, 15: 473–497.

Newton, T.R. 1997. *Agrobacterium*-mediated transformation of peanut (*Arachis hypogaea* L.) Honours Thesis, The University of Queensland.

Ozias-Akins, P. 1989. Plant regeneration from immature embryos of peanut. Plant Cell Reports, 8: 217–218.

Ozias-Akins, P., Fan, H. and Wang, A. 1996. Expression of GUS under the control of a soybean promoter modulated by carbohydrates. Proceedings of the American Peanut Research and Education Society Inc., Page 27.

Ozias-Akins, P., Schnall, J.A., Anderson, W.F., Singsit, C., Clemente, T.E., Adang, M.J. and Weissinger, A.K. 1993. Regeneration of transgenic peanut plants from stably transformed embryogenic callus. Plant Science, 93: 185–194.

Sambrook, J., Fritsch, E.F., Maniatis, T. 1989. Molecular Cloning, a Laboratory Manual, Second Edition. Cold Spring Harbour Laboratory Press, New York

Sarker, R.H., Chowdury, A.P. and Hoque, M.I. 1997. Preliminary studies on *Agrobacterium*-mediated genetic transformation of peanut *(Arachis hypogaea* L.) Bangaldesh Journal of Botany, 26: 155–162.

Smith, N. 1998. More T-DNA than meets the eye. Trends in Plant Science, 3: 85.

Smith, R.L. and Beliaev, D.V. 1995 Isolation of peanut seed coat- and pod-specific genes using differential hybridization and differential display methods. Proceedings of the American Peanut Research and Education Society Inc., 27.

Smith, G.R., van de Velde, R. and Dale, J.L. 1992. PCR amplification of a specific double-stranded RNA region of Fiji disease virus from diseased sugarcane. Journal of Virological Methods, 39: 237–246.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology, 98: 503–517.

Urban, L.A. and Weissinger, A.K. 1996. Enhanced regeneration and transformation of Valencia A peanut with the osmotin gene. Proceedings of The American Peanut Research and Education Society Inc., Page 27.

Yang, H., Singsit, C., Wang, A., Gonsalves, D. and Ozias-Akins, P. 1998. Transgenic peanut plants containing a nucleocapsid protein gene of tomato spotted wilt virus show divergent levels of gene expression. Plant Cell Reports, 17: 693–699.