

## DIG labeling of cDNA probes

Complex cDNA probes were generated from total RNA using AMV Reverse Transcriptase that incorporates DIG-11-dUTP into new synthesized cDNA. Add the following components to a nuclease free microcentrifuge tube:

RNase free sterile water to 13.5 ul

Oligo dT15 (0.5 ug/ul) 4 ul

Total RNA 4 ug

7.5 kb polIA RNA (1:1000) 1 ul

Heat the mixture to 65C for 15 min and quick chill on ice. Collect the contents of the tube by brief centrifugation and add:

5 X buffer 6 ul

Rnasin RNase Inhibitor 40 U 1 ul

dNTP (10 mM) dATP, dCTP, dGTP 1.5 ul

dTTP (4 mM) 1 ul

DIG-11-dUTP (1mM) Cat # 1573152 2.1 ul

AMV Rev. Transcriptase 50 U 5 ul

Mix the contents of the tube by gently vortexing. Incubate the reaction at 42C for 90 min.

## Incorporation DIG during PCR

Add the following components to a sterile microcentrifuge (0.2 ul) tube. Place the tube on in ice during pipetting. Use filter tips.

Reagents:

Water to 50 ul variable

PCR buffer 5 ul

Mg Chloride 50 mM 1.5 ul

PCR DIG labeling Mix Cat # 1585550 5 ul

Primer Mix 50 uM each 0.5 ul

Taq DNA Polymerase 5 U/ul 0.25 ul

Template DNA variable (10-50 ng of genomic DNA or 10 -100 pg of plasmid DNA)

Mix the reagents and centrifuge briefly to collect the sample at the bottom of the tube. Incubate the tube in termocycler. Perform 30 cycles of PCR amplification as follows:

Step 1 Denature 94C for 3 min

Step 2 Denature 94C for 45 sec

Step 3 Anneal 55C for 45 sec

Step 4 Extend 72C for 2 min (1 min for 1 kb template)

Step 5 Repeat Steps 2, 3, 4 30 times

Incubate for an additional 10 min at 72C and maintain the reaction at 4C.

After amplification analyze an aliquot of the reaction mixture (8 ul) by agarose gel electrophoresis.

## Estimating the yield of DIG labeled probes

The estimation of yield is performed by side comparison of the DIG labeled probe with a DIG labeled control DNA. Dilution series of both are prepared and spotted on a piece of membrane. Subsequently, the membrane is chemiluminescently detected and direct comparison of the intensities of sample probes and control allows the estimation of labeling yield.

Make a 4 fold dilutions of DIG labeled Control DNA (5 ng /ul) (Cat # 1 585 738) by mixing of 2 ul of DIG Control with 6 ul of water. Make 7 serial dilutions with final concentrations: 1,250 pg/ul, 312 pg/ul, 78 pg/ul, 19.5 pg/ul, 4.9 pg/ul, 1.2 pg/ul. Mix thoroughly between dilution steps.

Make serial 4 fold dilutions of the experimental probes, according to the same dilution scheme.

Spot 1 ul of the diluted controls on a piece of nylon membrane. In a second row, spot 1 ul of corresponding dilutions of experimental probe. Fix DNA to the membrane by cross-linking with UV light using UV Stratalinker 1800. Perform chemiluminescent detection using CDP Star and acquire the image using FluoroS Multiimager.

## DNA Dot Blotting

Dot blot is prepared by spotting 1 ul of denatured (heated at 100C for 5 min) PCR product diluted 3 fold (10 ul + 20 ul ) or 4 fold (10 ul + 30 ul). 96 dot blot is spotted by dipping Multi-Blot Replicator (V&P Scientific, Inc.) in diluted PCR products in a 96 well plate and transferring the aliquits into nylon Hybond N+ membrane (Amersham). Look for instruction into "The application instructions, care and procedures for Multi-Blot Replicators".

Fix the DNA to the membrane by UV crosslinking using UV Stratalinker 1800. The membrane can be used immediately for prehybridization, or can be stored dry at room temperature for future use.

## Prehybridization and Hybridization.

Place the blot in a hybridization tubes in 15 ml of prehybridization solution: 6 x SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) and 1% SDS. Prehybridize at 65C for 1 h with constant agitation in Micro Hybridization Incubator (SciGene Inc.). The complex cDNA probes and PCR probes are denatured by boiling for 5 min before adding to the prehybridization solution. Hybridization is performed overnight at 65C with gentle agitation.

To prevent background problem as a result of too high probe concentration, it is recommended to optimize the probe concentration by incubating small membrane pieces with a few DNA spots with different probe concentrations in the hybridization solution and subsequent detection. The highest probe concentration that gives acceptable background should be used for the hybridization experiment. Filtration of the probe also helps to lower spot like background. The probe can be filtered out through a 0.45 um filter. This should be performed after addition of the probe to prewarmed hybridization solution and filtration of this entire solution. cDNA and PCR probe concentrations are approximately 1 ng/ml hybridization solution and 1 pg/ml hybridization solution.

After hybridization, membranes are washed twice, 10 min per wash, in 20 ml of 2 x SSC and 1% SDS at room temperature, then twice for 30 min in 20 ml of 1 x SSC and 0.1% SDS at 68C.

Chemiluminescent detection is performed with anti-DIG antibody conjugated to alkaline phosphatase and CDP-Star. After posthybridization washes, membranes are washed briefly in Buffer 1 (100 mM maleic acid and 150 mM NaCl, pH 7.5) and incubated in 20 ml of Buffer 2 (1% blocking reagent in Buffer 1) for 1 h with gentle agitation in Hybridization Incubator. Anti-DIG-alkaline phosphatase is added to the fresh Buffer 2 to achieve a dilution of 1:10,000, followed by incubation for 1 h. Membranes are washed four or six times, 15 min per wash, in 20 ml of Buffer 1 with 0.3% of Tween 20 and then equilibrated in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl) for 3 min. Membranes are placed on plastic sheets and 0.5 ml (per 100 cm<sup>2</sup>) of 1:100 dilution of a 25 mM solution of CDP-Star is added on top of a membrane, scattering the drops over the surface of the membrane. Membranes are sealed in the plastic sheets and exposed to Kodak X-Ray film for 5 to 20 min to record chemiluminescence. Immediately after the film exposure, the same blot is placed into Fluoro-S MultiImager system. The image is acquire for a 30 min integration in the High sensitivity mode using the 50 mm f1.4 lenses without any filters.