DIG DNA PROBEs: PCR Labeling Protocol

Step 1: Creation of DIG Probe

-DIG Labeled Probe PCR Reaction Components:

-5 ul PCR Buffer 10x (Vial 3)
-5 ul PCR DIG Labeling Mix (Vial 2)
-Upstream Primer, Variable
-Downstream Primer, Variable
-Template, Variable, optimum template concentration is 10-100 pg for plasmid DNA and 1-50 ng for genomic DNA. For plasmids this is usually 1 ul of a 1:100 dilution of a standard mini-prep.
- 0.75 ul Enzyme Mix (Vial 1)
-Sterile Water, Variable

Total Volume = 50 ul*

***This reaction can be cut in half to save reagents.**

A typical reaction mixture that I used to create DIG labeled probes from cDNA clones:

-5 ul PCR Buffer Vial 3
-5 ul DIG Labeling Mix Vial 2
-1 ul (10 uM) Upstream Primer
-1 ul (10 uM) Downstream Primer
-1.25 ul Template **(1/100 dilution Mini-Prep DNA)**
-0.75 ul Enzyme Mix Vial 1
-36 ul Sterile Water

Total Volume = 50 ul

ALSO NEED TO DO A **CONTROL RXN, WHICH USES **VIAL 4** (REGULAR DNTPs WITHOUT DIG TAGGING) **IN PLACE OF VIAL 2**. USE 3 ul OF VIAL 4

*Run the PCR Reaction according to the manufacturer's instructions, adjusting for the Tm of your primers.

Run 3-5ul of DIG PCR and also the Control PCR on an agarose gel to make sure product is there – the DIG PCR product should be larger in size due to the tagging.

Step 2: Hybridization

-Pre-heat appropriate volume of DIG Easy Hyb solution to hybridization temperature. You may calculate the optimal hybrid temp for your reaction (See DIG Easy Hyb manual for formula). Typically, the hyb temp is between 50 and 60 C.

-For small membranes pre-heat 5 ml of DIG Easy Hyb solution, for larger membranes use 8-10 ml. **Generally preheat 20ml for Southerns, in 50ml Falcon tube non-agitating in the Hybrdiization oven at desired temp.**

-Pre-hybridize membranes with only 10ml (of the 20ml that was pre-heated) of DIG Easy Hyb solution for 20 minutes at appropriate hybridization temperature. Make sure the DNA side (top) of membrane is facing INWARD in the tube.

-While membranes are pre-hybridizing, denature **10ul only** of the 50ul rxn of DIG-labeled DNA by boiling (100 C) in the PCR machine for 5 minutes then rapidly cool on ice.

-Pour off the initial 10ml of DIG Easy Hyb solution that was used to pre-hybridize. Discard.

-Add appropriate amount of Denatured Probe. Optimum concentration is 25 ng/ml of hybridization solution. I have been adding approximately 0.5 ul of DIG probe to 5 ml Hyb solution for the virtual northerns. The probes are very sensitive, you don't need very much! If you add too much probe your blots will have VERY high background. For Southerns you need more probe, so I add 1 ul of DIG probe per ml of hybridization solution ….. AVOID BUBBLES.

-Incubate with agitation in hybrid oven for desired time. For single gene detection, overnight is best.

Step 3: Post-Hybridization Washes

Can pour off the overnight hybridization with DNA probe solution and save at -20C for re-use. Probes are typically good for 2-4 uses.

1. Wash membranes 2x for 5 minutes in 2x SSC, 0.1% SDS (Wash 1) at room temp. **Shaking on RT shaker.** Pour off first wash and then add second wash.

-To make 50 ml of Wash 1:

-5 ml of 20% SSC
-250 ul of 20% SDS
-44.75 ml water

-For small membranes, 10 ml per wash is sufficient. It really depends on the container you choose to use. Just make sure membrane is immersed and under agitation. **Generally make 50ml of solution to use 25ml twice.**

2. Wash membranes 2x 15 minutes **at 68 C shaking in hybridization oven** in Wash 2 = 0.1% SDS, 0.1% SSC under constant agitation. Pour off first wash and then add second wash. **Generally make 50ml of solution to use 25ml twice.**

-To make 50 ml Wash 2:

-250 ul 20% SSC
-250 ul 20% SDS
-49.5 ml water

Step 4: Detection procedure

3. Wash membranes briefly (1-5 minutes) in 25 ml of 1X Washing Buffer provided in DIG Wash kit. The buffer comes at 10x concentration. To make 100 ml of buffer add 10 ml concentrate to 90 ml water. (Save the remaining 1X wash buffer for step 6.)

4a. Make 1X Blocking Solution. **IMPORTANT: YOU MUST DILUTE BLOCKING SOLUTION IN 1X Maleic ACID BUFFER**. Both the blocking solution and maleic acid buffer come in 10x concentration. Dilute maleic acid buffer to 1x in water. Then dilute blocking solution to 1x in 1x maleic acid buffer.

4b. Incubate membranes for 30 minutes in blocking solution. Make sure membrane is covered in solution. Amount depends on container. For virtual northerns I have been using 20 ml.

5. Incubate membrane in Anti-body solution for 30 minutes. To make anti-body solution, add 1 ul anti-body to 20 ml 1x blocking solution. Amount needed depends on container used. Make sure membrane is immersed.

6. Wash membrane 2x 15 minutes in 1x Washing buffer. Make sure membrane is immersed in buffer.

7. Equilibrate membranes 2-5 minutes in Detection Buffer. Detection buffer is 10x concentration, dilute to 1x using water. **20ml only needed.** Use 18-19ml in this step, and save 1 to 2 ml for CDP-Star Solution in step 9.

8. Place membrane into clear ziplock bag and tape into autorad cassette, leaving the top of bag open.

9. Make CDP-Star detection solution. For large membranes make 2 ml, for small membranes 1 ml is sufficient. Add 10 ul CDP-Star solution to 1 ml of 1x Detection buffer. **CDP-STAR is light-sensitive** so make this buffer AFTER the ziplock bag with membrane in it are taped to cassette.

10. Add CDP-Star solution to membrane. Make sure there are no wrinkles in the bag/membrane. Close bag and close cassette. Incubate room temp for 5 minutes.

11. After 5 minutes, develop autorad on film for 30-60 seconds ONLY! For larger blots, southerns etc. 5 minutes is typically necessary. For small blots, northerns, 45 seconds is sufficient. The longer you expose, the more background you will get.

Catalog Numbers from Roche:

1. **PCR DIG Probe Synthesis Kit - Cat. No. 1 636 090**
2. **DIG Easy Hyb – Cat.No. 1 603 558**
3. **Anti-Digoxigenin-AP Fab Fragments, Cat No. 1 093 274**
4. **CDP-STAR Cat No. 1 685 627 (1ml volume) Cat no. 1 759 051 (2 X 1 ml)**
5. **DIG Wash and Block Buffer Set Cat No. 1 585 762**
6. **Nylon Membranes Cat No. 1 209 272**
7. **Lumi-Film Chemiluminescent Detection Film Cat No. 1666657 (8x10 inches)**

I have found that you get better signals with the Roche membranes and film which are design specifically for chemiluminescent detection. I have used standard membranes like H bond from Amersham and standard X-Ray film and they work fine, but you just get cleaner better looking blots with the Roche products in my opinion.

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