Probe generation by in vitro transcription (RIBOPROBES)

Probes can be generated by in vitro transcription reaction in the presence of labeled nucleotides. The target DNA fragment to be detected is cloned into the polylinker region of a transcription vector containing promoters for either SP6, T3 or T7 RNA polymerase. To synthesize an anti-sense "run-off" transcript, a specific restriction endonuclease should be used to linearize the DNA template and to create a 5' overhang on the anti-sense strand before transcription. The procedure described here incorporates one biotinylated nucleotide for approximately for every 20-25 nucleotides in the newly-synthesized transcript.

Equipment and reagents

- 10% SDS
- Proteinase K (10 mg/ml) (Roche Molecular Biochemicals)
- Phenol/chloroform/isoamyl alcohol (25:24:1) (Gibco)
- SP6, or T3, or T7 polymerase, 20 units/μl (Roche Molecular Biochemicals)
- Ethanol
- Biotin-16-UTP 10 mM (Roche Molecular Biochemicals)
- NTPs 10 mM (Roche Molecular Biochemicals)

Method

1. Perform restriction digest of 20-25 μg of template DNA with specific restriction endonuclease which cut the template ~200-300 nt downstream of the promoter region to create an 5' overhang to linearize the DNA template used for "run-off" transcription in 1.5 ml test tube
2. After the restriction digest add 10 μl 10% SDS and 2 μl proteinase K (10 mg/ml) and add enough water to make a total reaction volume of 200 μl
3. Incubate the tube for 30 min at 50ºC
4. Add 8.5 μl 0.5 M EDTA (pH 8.0), 1.5 μl water and 210 μl phenol/chloroform/isoamyl alcohol (25:24:1).Vortex for 1 min
5. Centrifuge the tube at 13,000 x g for 5 min at room temperature

6. Collect upper aqueous layer into new 1.5 ml test tube. Add 200 μl phenol/chloroform/isoamyl alcohol (25:24:1). Vortex for 45 seconds
7. Centrifuge the tube at 13,000 x g for 5 min at room temperature
8. Collect 180 μl upper aqueous layer. Add 20 μl 3M sodium acetate (pH 5.2) and 500 μl pre-chilled (-20ºC) 100% ethanol. Let the precipitate form for at least 20 min at –20°C
9. Centrifuge the tube at 13,000 x g for 30 min at 4ºC
10. Discard the supernatant. Add 200 μl pre-chilled (-20ºC) 70% ethanol
11. Centrifuge the tube at 13,000 x g for 30 min at 4ºC
12. Discard the supernatant
13. Dry the pellet under vacuum. Dissolve the pellet into 10 μl nuclease-free water. Adjust the final concentration to ~ 1 μg/ μl. If desired, store at –20°C
14. To set up in vitro transcription reaction add the following to a 1.5 ml microcentrifuge tube on ice, 2 μl 10x transcription buffer (supplied with SP6, T3 or T7 RNA polymerase), 2 μl 10 mM ATP, 2 μl 10 mM CTP, 2 μl 10 mM GTP,1.3 μl 10 mM UPT, 0.7 μl 10 mM biotin-16-UTP, 1 μg purified linearized DNA template, 0.5 μl RNA pol (SP6, T3 or T7) (20U/ μl). Enough nuclease-free water to make a total reaction volume of 20 μl.
15. Mix the components and centrifuge briefly.
16. Incubate the tube for 2 h at 37ºCa
17. To the reaction tube, add 2 μl 0.2 M EDTA (pH 8.0) to stop the reaction, 2 μl 4M LiCl and 60 μl pre-chilled (-20ºC) 100% ethanol. Let the precipitate form for at least 20 min at –20ºC
18. Centrifuge the tube at 13,000 x g for 15 min at 4ºC
19. Discard the supernatant
20. Wash the pellet with 50 μl pre-chilled 70% (v/v) ethanol
21. Centrifuge the tube at 13,000 x g for 10 min
22. Discard the supernatant
23. Dry the pellet under vacuum. Dissolve the RNA pellet for 30 min at 37ºC in 50 μl nuclease-free water plus 1 μl RNase inhibitor (20U/ μl)

24. To estimate the yield of the transcript. Run an aliquot of the transcript on an agarose gel beside an RNA standard of known concentration. Stain with ethidium bromide. Compare the relative intensity of staining between the labeled transcripts and the known standard[b].

[a]If you want to remove the template DNA, add 2 units DNase I, RNase-free to the test tube and incubate for 15 min at 37ºC and then go to the step 4

[b]If you are not going to use the labeled probe immediately, store the probe solution at –70ºC