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Strand specific detection of overlapping transcripts via purification involving denaturation of biotinylated cDNA

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ABSTRACT

Reverse Transcription Polymerase Chain Reaction (RT-PCR) is the most widely employed technique for gene expression analysis owing to its high sensitivity, easy reproducibility and fast output. It has been conceived that priming reverse transcription reactions with gene specific primers generates cDNA only from the specific RNA. However, several reports have revealed that cDNA gets synthesized even without addition of exogenous primers in RT reactions. Due to such self-priming activity, the signals from specific strands cannot be accurately detected and can confound the expression analysis, especially in context of overlapping bidirectional transcripts. The protocol described here was used for purification of biotin-tagged cDNA in conjunction with alkaline denaturation. This obviated the problem of background priming and enabled accurate strand specific detection of overlapping transcripts.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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1 2µg of DNase-I treated RNA was reverse transcribed at 55°C using Superscript-III First-Strand Synthesis System for RT-PCR (Invitrogen 18068-015) in 40µl RT reaction following manufacturer's instruction. The final concentration of each biotinylated GSP was 250nM.

2 50µl of Dynabeads™ MyOne™ Streptavidin C1 were transferred to a 1.5ml tube and collected over the magnetic stand.

3 Beads were washed twice with 400µl of 1x binding buffer consisting of 1M NaCl, 1mM EDTA, 0.1% Tween-20 and 10mM Tris-Cl (pH 7.5).

4 Beads were resuspended in 400 µl of 1x binding buffer with gentle tapping and 35µl of RT reaction was mixed. Importantly, use of pipette was avoided for dispersion of the beads.

5 Tube containing beads and RT mix was incubated at 15°C for 30 minutes with slow rotation for proper mixing.

6 Beads were collected by placing the tube over the magnetic stand for 2 minutes and supernatant containing unbound cDNA was aspirated out.

7 200µl of 0.15M NaOH was then added to the bead pellet and beads were dispersed with gentle tapping, followed by an incubation of 10 minutes at room temperature with intermittent mixing to denature the cDNA-cDNA duplexes. Supernatant was removed using the magnetic stand.

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8 Beads were washed once with 300µl of 0.1M NaOH to remove residual self-primed cDNA.

9 Beads were then washed twice with 400µl of 1x washing buffer consisting of 0.1M NaCl, 1mM EDTA, 0.1% Tween-20 and 10mM Tris-Cl (pH 7.5).

10 Finally, the beads were resuspended in 70µl of water and incubated at 70°C for 5 minutes, followed by cooling on ice to release biotinylated cDNA.

11 A short spin at 1500g was given at 4°C and enriched biotinylated cDNA was transferred to a fresh tube using the magnetic stand.

12 Enriched biotinylated cDNA (2 µl) was used for PCR. The amplification was carried out under conditions specifically optimized for each amplicon using gDNA and/or non-biotinylated cDNA as template.

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