Design and Synthesis of Immobilized Templates (Price Lab)

Immobilized templates are useful for studying initiation, elongation and termination of transcription. Under the appropriate conditions the three phases can be kinetically and/or physically separated. The document found here covers important considerations for the design and generation of immobilized templates.

This protocol details how a DNA template "tagged" with biotin is generated, purified, and then coupled to paramagnetic beads through a biotin/streptavidin linkage. Streptavidin is a 66 kDa protein with four identical subunits that each contain a high affinity binding site for biotin ($K_D = 10^{-15}$ M). The Dynabeads M-280/Streptavidin are uniform superparamagnetic, polystyrene beads with streptavidin covalently attached to the bead surface. Ideally, the template is synthesized using a PCR reaction with one of the two primers being biotinylated. Alternatively, biotin can be added to the end of a restriction fragment using a biotinylated nucleotide and the Klenow fragment in an end fill reaction. The tagged DNA must be separated from unincorporated biotinylated primer (or nucleotide) before coupling to the beads. Binding of DNA to beads is most efficient when performed at high ionic strength, presumably because of reduction of repulsive forces from immobilized DNA toward unbound DNA. The theoretical binding capacity of the beads for biotin is never achieved due to this repulsion and the amount of DNA bound is inversely proportional to the length of the DNA.

Design

Several parameters need to be considered in the design and synthesis of an immobilized template. The biotin should be located upstream of the promoter to allow polymerases that initiate to be able to efficiently run off the end of the template. The template should contain all known elements of the promoter required for efficient initiation in vitro. Practically, this means the biotin should be about 300 bp upstream of the start point of transcription. To facilitate examination of the elongation properties of the polymerase the length of the transcribed region should be between 500 and 1000 bp. However, the shorter the DNA fragment the more efficiently it binds to the beads.

Generation of Template by PCR

1. Use standard PCR reactions with plasmid DNA and two primers (one is biotinylated). The yield of DNA should be about 15 µg per 100 µl reaction. In general, start with ten 100 µl PCR reactions.

2. Check the PCR product by analyzing 2 µl of each reaction solution on an agarose gel.

3. Pool all (good) reaction solutions. Concentrate the DNA by ethanol precipitation. Dissolve the DNA into 100 µl TE buffer.

Purification of Template

1. Prepare a 3-5 ml G-200 gel-filtration column (height: about 15-20 cm, flow rate: 20 ml per hour, about one drop per 8 seconds).

2. Equilibrate with TE buffer + 0.5 M NaCl.

3. Load the DNA sample and elute with TE buffer + 0.5 M NaCl.

4. Collect 40 fractions (2 drops per fraction).

5. Draw the elution profile based on the absorbance at 260 nm. A biotinylated DNA peak will be expected between fraction 5 to 25. The other peak around fraction 30 is due to excess primers.

6. Pool the amplified DNA and quantify it. The total volume is about 0.5 ml containing 150 µg DNA.

Binding of Template to Beads

1. Take 1 ml streptavidin-conjugated Dynabeads M280 (Dynal). The beads can be concentrated in a microfuge tube using a magnet.

2. Wash the beads 3 times with TE buffer + 0.5 M NaCl by repeated concentration and resuspension.

3. Add the purified DNA to the concentrated beads.

4. Mix and incubate for 30 minutes. Quantify the DNA in solution after concentration of the beads every 10 minutes by UV absorbance or by agarose gel. The amount of the template that binds is related to the fraction of the primers that are biotinylated and the capacity of the beads for the specific template.

5. The unbound DNA can be analyzed on an agarose gel +/- streptavidin or streptavidin IgG conjugate to determine if it is biotinylated. The streptavidin will cause a mobility shift of the biotinylated DNA. The excess biotinylated DNA can be incubated with fresh beads to generate more immobilized template.

6. Wash the beads 3 times with TE buffer + 0.5 M NaCl followed by twice with TE buffer.

7. Resuspend into 0.5 ml TE buffer with final concentration 150 µg/ml. Use 2 µl for one transcription reaction.

8. Store the beads at 4 C.