

Digoxigenin-labeled riboprobe synthesis from plasmid template

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Plasmid Preparation

All plasmids are I.M.A.G.E clones which can be obtained from Open Biosystems (<http://www.lifesciences.sourcebioscience.com>).

Sterile conditions are maintained at all times when working with bacteria and the glycerol stocks.

Day 1: Plating out from a glycerol stock

1. Take the culturing plates out of the fridge and let them warm up at room temperature for 15-30 minutes.
2. Ensure that the plates contain the correct antibiotic to select for the bacteria containing the recombinant plasmid. Kanamycin (Kan) and chloramphenicol (Cam) can be added directly to non-ampicillin plates. Add 20µl of Kan (50mg/ml) or Cam (25mg/ml) on to the plate and spread.
3. Spread 4µl of 25mg/ml Xgal for the blue/white colony screen.
4. Streak plate with the glycerol stock using a sterile pipette tip.
5. Incubate upside down overnight at 37°C.

Day 2: Mini Culture

1. Set up a small culture in the late afternoon.
2. Select a white colony using a sterile pipette tip and transfer in to 2ml Terrific Broth (TB) with the appropriate concentration of antibiotic added.
3. Incubate overnight at 37°C in an orbital shaker set at 225rpm.

Day 3: Midi Culture

1. Store the 2ml culture at 4°C until later in the afternoon.
2. Add 2mls of this culture to 50ml TB containing the appropriate concentration of antibiotic.
3. Incubate overnight at 37°C in an orbital shaker set at 225rpm.

Day 4: Midi plasmid preparation

1. Pour the bacterial culture in to a 50ml centrifuge tube.
2. Pellet the bacteria by centrifugation. Centrifuge: Avanti J26-XP, Beckman Coulter Centrifuge at $\geq 20,000 \times g$.
3. Drain the supernatant. The pellet can be frozen and stored at -20°C.
4. Extract the plasmid DNA using a QIAGEN Plasmid Midi Kit according to the manufacturer's instructions.

5. Resuspend the plasmid DNA in 100µl of TE buffer (pH 8.0).
6. Determine the yield, DNA concentration & purity of the plasmid DNA using spectrophotometry at 260 nm. For further qualitative and quantitative analysis run a small aliquot of plasmid DNA on a 1% agarose gel.

Day 5: Plasmid template linearization

1. Set up the following restriction digest to linearize the plasmid template.

Plasmid	20µg
Restriction enzyme	2µl
Restriction buffer	10µl
Make up to 100 µl with H ₂ O	
2. Incubate overnight at the appropriate temperature for the restriction enzyme digest.

Day 6: Restriction digest clean up with phenol:chloroform

1. Add 1 volume of phenol:chloroform (50:50 mix) to the digest.
2. Vortex and centrifuge at 13K rpm for 1min in a bench centrifuge.
3. Transfer the aqueous layer to a clean tube (discard organic layer).
4. Add 1 volume chloroform, vortex and centrifuge at 13K rpm for 1min.
5. Transfer the aqueous layer to a clean tube.

Precipitate DNA

1. Vortex.
2. Add 2.5 volumes ethanol, mix by inversion and place on dry ice for 10-20 minutes.
3. Centrifuge in the cold room at 13K rpm for 15 minutes.
4. Aspirate supernatant taking care not to lose the pellet
5. Wash pellet by adding 70% ethanol and vortex.
6. Centrifuge at 13K rpm for 10 minutes.
7. Carefully aspirate the ethanol.
8. Pulse spin to bring down remaining ethanol and aspirate again to remove the alcohol.
9. Leave the tube open on the bench for 15 minutes to allow the ethanol to evaporate from the pellet.
10. Resuspend pellet in 20µl DEPC-treated H₂O.
11. To check the quality and quantity of the linearized DNA load 1µl of DNA with 4 µl loading buffer and run on a 1% agarose gel. If band is a single clean band label the probe on the same day.

***In vitro* transcription of digoxigenin-labeled riboprobes**

Ensure RNase-free conditions are maintained and that the reagents are kept on ice.

1. Mix together the following:

DEPC H ₂ O	Xµl*
5X T3/T7/Sp6 buffer	4µl
DTT	2µl (Promega #P1171)

DigUTP Nucleotide mix	2µl (Roche #11277073910)
DNA (1µg)	Xµl*
Placental Rnase inhibitor	0.5µl (Promega #N2511)
T3, T7 or Sp6 enzyme	2µl (Promega, T3 #P4024, T7 #P4074 or Sp6 #P4084)

*Adjust DNA and H₂O to give a 20µl total volume

2. Incubate overnight at 37°C.
3. Add 80µl RNase free water
4. To extract the RNA use mini Quick Spin RNA Columns (Roche #11814427001).
5. Determine the yield, concentration & purity of the RNA using spectrophotometry at 260 & 280nm.

Media & Solutions

Terrific Broth (TB) with salts, no amp (1l)

12 g tryptone (Fisher Scientific #BP1421500)
 24 g yeast extract (Fisher Scientific #BP1422500)
 4 ml glycerol (Fisher Scientific #BP229-4)
 Dissolve to a final volume of 900 ml with ddH₂O
 Autoclave at 121°C for 45 minutes.
 Cool to touch on stir plate.
 Add 100 ml 10X TB salts.
 Store 50 ml aliquots at -20°C.

10X TB salts (250ml)

5.78 g KH₂PO₄
 31.35 g K₂HPO₄
 Dissolve to a final volume of 250 ml with ddH₂O
 Autoclave at 121°C for 45 minutes.

Antibiotic Stock Solutions

Ampicillin	60mg/ml
Kanamycin	50 mg/ml
Chloramphenicol	25 mg/ml

Add antibiotic to the media at a concentration of 1µl/ml.

Luria Broth (LB)+ Ampicillin plates

Add 1l ddH₂O to 2l flask with stir bar.
 Add 40 g LB Agar (Acros #61189-5000)
 Stir until dissolved.
 Autoclave at 121°C for 45 minutes.
 Cool to touch on stir plate.
 Add ampicillin to a final concentration of 100 mg/l.
 Immediately pour plates and allow to cool (keep a flame going).
 Once cool, cover and mark sizes with black (LB) and red (amp) lines.

Store in plastic sleeve with lids facing down at 4°C.

TE Buffer pH 8.0

1mM Tris pH 8.0

1mM EDTA

For 500ml

1M Tris pH 8.0 (5ml)

0.5M EDTA pH 8.0 (1ml)

dH₂O 496ml.

Loading Buffer

Gel loading dye, Blue 6X (New England BioLabs #B7021S)