Riboprobe synthesis from a plasmid template

Mendelsohn Group, GUDMAP Consortium

Plasmid Preparation
Clone/vector

cDNA is replicated in *E. coli* strains transformed with a recombinant plasmid and the cells are lysed to isolate the plasmid containing the cDNA sequence of interest. Each recombinant plasmid has a specific selectable marker, such as antibiotic resistance. All cultures that are described below are grown in Luria Broth (LB) media to which is added a specific antibiotic to select for those bacteria containing the recombinant plasmid.

Most plasmids were purchased through Open Biosystems and are I.M.A.G.E clones.

Plating out a glycerol stock

- Streak a glycerol stock of a strain containing the recombinant plasmid onto a dropout LB agar plate using a sterile pipette tip.
- Ensure the plates contain the appropriate concentration of antibiotic to select for the bacteria containing the recombinant plasmid.

Antibiotic	Final concentration
Ampicillin	50µg/ml
Kanamycin	10µg/ml
Chloramphenicol	25µg/ml

- Grow overnight (approximately 16 hours) at 37°C.

Mini culture

- Isolate a single white colony using a sterile pipette tip and transfer it in to 5ml of LB media containing the appropriate concentration of antibiotic.
- Incubate in an orbital shaker at 37°C for approximately 6 hours.

Midi Culture

- Following incubation the culture should be slightly cloudy and drifts of *E. coli* should be seen when shaken.
- Add 500µl of this culture to seed a 50ml LB culture with the appropriate concentration of antibiotic. Incubate in an orbital shaker overnight at 37°C.

Midi plasmid preparation

- Pour the bacterial culture into a 50ml centrifuge tube.
- Pellet the bacteria by centrifugation for 15 minutes. Centrifuge: Eppendorf Centrifuge 5810R at 3,500rpm.
- Discard the supernatant.
- The plasmid is isolated using the QIAGEN Plasmid Midi Kit (Qiagen #12143) according to the manufacturer's instructions.
- The plasmid DNA is eluted with 100-200µl of TE buffer (pH8).

- The plasmid concentration is determined using a NanoDrop 2000c**.**
    - The machine is first calibrated with 2µl of H_2O.
    - 2µl of the purified plasmid is run on the machine and the DNA concentration recorded.
- A small aliquot of plasmid DNA is run on a 1% gel for qualitative analysis.
- All plasmid cDNA is stored at -20°C.

Linearization of the plasmid template

- The plasmid is linearized with the appropriate restriction enzyme determined from the vector map.
- For linearization, a 500µl mix is made as follows:

Reagent	**Amount**
Plasmid	20µg
Buffer	50µl (1/10th final volume)
Restriction enzyme	2µl (20-40U/µl)
H_2O	make up to a 500µl volume

- Restriction enzymes are purchased from New England BioLabs, and the appropriate buffer for each enzyme is listed on the product and the manufacturer's website.
- Vortex and briefly centrifuge to collect the digestion mix to the bottom of the microfuge tube.
- Incubate overnight at the optimal temperature for the restriction enzyme being used, usually 37°C.

Precipitation of DNA

- Vortex and briefly centrifuge.
- Add the following to the linearized plasmid:

1µl glycogen
1/10th volume of 3M NaOAc
2X volume of cold 100% ethanol

- Invert tube several times to mix.
- Incubate at -80°C for at least 15 minutes. The incubation can be left overnight.
- Centrifuge at 13k rpm for 15 minutes at 4°C. (Centrifuge: Eppendorf Centrifuge 5417C)
- Carefully discard the supernatant and wash pellet with 400µl cold 70% ethanol.
- Centrifuge at 13k rpm for 10 minutes at 4°C.
- Carefully discard the supernatant and dry the pellet at room temperature or at 37°C. Do not over-dry the pellet.
- Dissolve the DNA pellet in H_2O at a concentration of about 1mg/ml.
- Incubate at 37°C for at least 15 minutes to allow the DNA to dissolve.

Quantification of DNA
Run ~100-200ng linearized cDNA alongside a 1Kb Plus DNA Ladder & Low DNA Mass Ladder (Invitrogen #10787-018 & #10068-013) to verify the concentration.

In-vitro transcription of Digoxigenin-labeled riboprobes

Ensure RNase-free conditions are maintained and that reagents are kept on ice. In-vitro transcription & digoxigenin labeling are carried out using the Roche DIG RNA Labeling Mix (Roche #11 277 073 910). The manufacturer's instructions are followed and are described briefly below.

- Mix together the following reagents to make a total volume of 20µl:

Reagent	Volume
1ug linearized plasmid DNA	xµl
DIG RNA labeling mix, 10X	2µl
Transcription buffer, 10X	2µl
Sterile RNase free double distilled water	make up to a final volume of 18µl
RNA polymerase, T7, Sp6, or T3 (20U/µl)	2µl

- Vortex and centrifuge briefly.
- Incubate for 2 hours at 37°C.
- Take a 2µl aliquot of the reaction mix to run on 1% agarose gel to compare the quality of this RNA to the RNA following clean up procedures. Store at -20°C.
- Optional: Add 2µl of DNase I, RNase-free (10,000U stock / Roche #10 776 785 001) and incubate at 37°C for 15 minutes.
- Add 2µl of 0.2M EDTA (pH 8.0) to stop the reaction.
- Use Illustra ProbeQuant G50 micro columns (GE Healthcare Life Sciences #28-9034-08) to clean the probe by following the manufacturer's instructions.
- Take a 2µl sample to run on a 1% agarose gel to verify the clean band.
- Add 50µl of hybridization buffer as described in the *in-situ* hybridization protocol.
- Store probes at -20°C.

Quantification of Riboprobe
A 1% agarose gel can be run with the two samples collected against a 1Kb Plus DNA Ladder & Low DNA Mass Ladder (Invitrogen #10787-018 & #10068-013) to approximate the probe concentration after cleaning.

Alternatively, the 2µl RNA taken after cleaning (before addition of hybridization buffer) can be run on a NanoDrop 2000c.

Media & Solutions

Luria Broth (LB)/ 1L

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g

Dissolve components in 1L of distilled water.
For LB agar - Add agar to a final concentration of 1.5%.

Sterilize by autoclaving at 15 psi, from 121-124°C for 15 minutes.

TE Buffer pH 8.0
10mM Tris pH 8.0
1mM EDTA