SOP: Synthesis of DIG-labeled RNA probes for ISH

By

Richard Yu

Research fellow

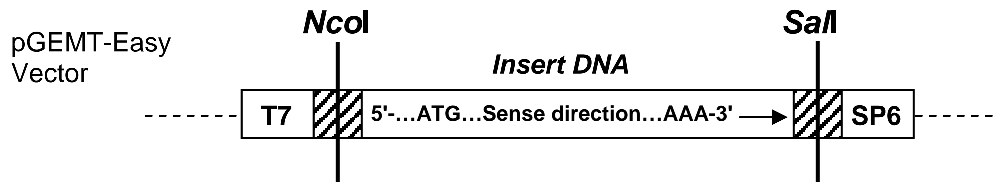
Centre for Marine Environmental Research and Innovative Technology (MERIT)
Department of Biology and Chemistry
City University of Hong Kong
(Email: bhrichyu@cityu.edu.hk)

PART A: Preparation of template DNA

Method 1: Digestion of plasmid DNA

1. Digest 10 µg plasmid DNA using appropriate restriction enzymes (Note: Don't use enzymes that leave a 3' overhang). For making antisense probes, use a unique restriction site 5' to the insert which contain a sense 5'→3' sequence. For making sense (control) probes, use a unique restriction site 3' to the insert.

Example



Sense probe: Linearize plasmid with *Sal*I and use T7 RNA pol.
Antisense probe: Linearize plasmid with *Nco*I and use SP6 RNA pol.

2. Set up the following reaction in a 1.5-ml microtube:

10× Buffer	10 µl
Plasmid DNA	10 µg
Restriction enzyme	5 µl (10–15 U/µl)
Sterile water	make to 100 µl
	37°C for 3 h

3. Run 2 µl digestion product on gel to confirm the completion of digestion.

4. Purify linearized DNA using the Wizard SV Gel and PCR Clean-up System (Promega). Elute DNA with 50 µl RNase-free water.

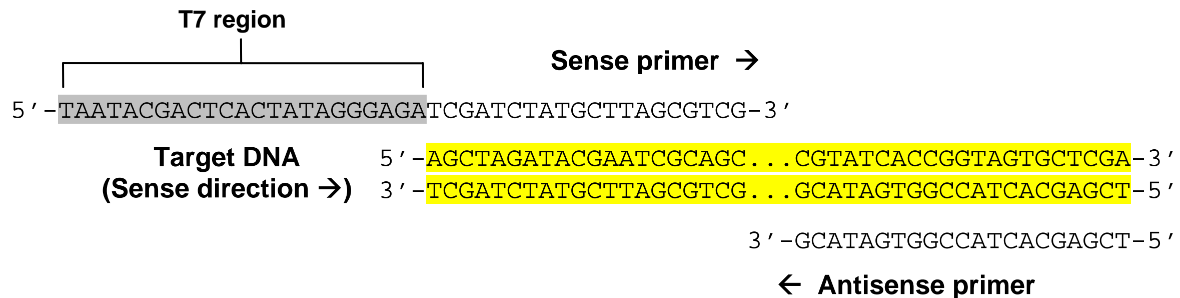
5. Measure the concentration of the eluted DNA at 260 nm.

Method 2: PCR approach

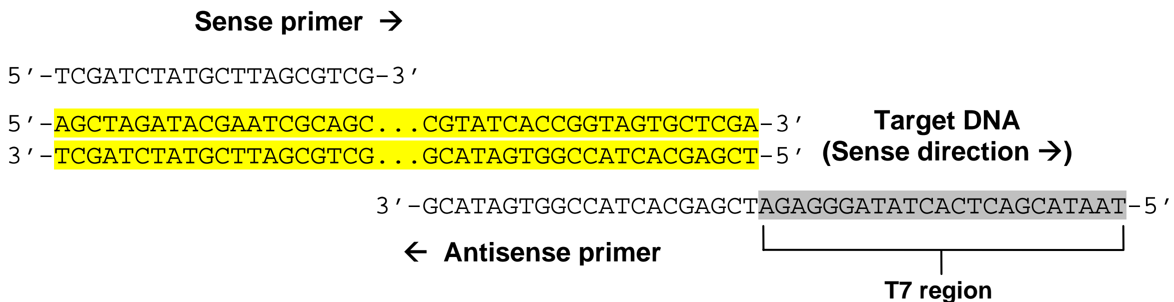
(Source: Ambion technical bullet #154: http://www.ambion.com/techlib/tb/tb_154.html)

This method utilizes PCR to add phage promoters to specific DNA sequences, generating DNA templates for *in vitro* transcription without the need to subclone into a phage transcription vector. The simplest approach is to append the phage promoter sequence to one of the PCR primers such that the promoter is incorporated into the PCR product. An example of this strategy is shown as below. One of the PCR primers has the 23-base T7 promoter sequence appended at the 5' end. Amplification of the target DNA yields PCR product that contains the T7 promoter upstream of the sequence of interest.

Making sense probe:



Making antisense probe:



Example:

(1) PCR recipe (use iTaq DNA Polymerase, BioRad):

	Vol. per rnx (µl)
10× iTaq buffer	5
50 mM $MgCl_2$	1.5
10 mM dNTP	1
10 µM Forward primer	1
10 µM Reverse primer	1
cDNA (i.e. RT product) or plasmid DNA (ca. 8 pg/µl)	3
Sterile water	37.25
iTaq DNA Polymerase	0.25
Total:	50

Prepare two such 50-µl reactions and combine them at the end.

(2) Thermal profile:

1 cycle of	3 min, 95°C
35 cycle of	30 sec, 95°C 30 sec, 55–60°C 30 sec–1 min, 72°C (sufficient for probes ranging from 200–1000 bp)
1 cycle of	10 min, 72°C
1 cycle of	4°C, forever

(3) Run 5 µl PCR product on gel to assess the amplification specificity.

(4) Purify PCR product using a Wizard SV Gel and PCR Clean-up System (Promega). Elute DNA with 50 µl RNase-free water.

(5) Measure the concentration of the eluted DNA at 260 nm.

PART B: Probe synthesis by *in vitro* transcription

1.

Linearized plasmid DNA		1 µg
or Purified PCR product		200 ng
10× DIG RNA labeling mix		2 µl
10× Transcription buffer		2 µl
RNaseOut (40 U/µl)		1 µl
SP6 or T7 RNA pol. (20 U/µl; Roche)		2 µl
Sterile water	make to	20 µl

Incubate for 2 h at 37°C.

2. Optional: Add 2 µl RQ1 RNase-free DNase (Promega) to remove template DNA. Incubate for 30 min at 37°C.

3. Add 2 µl RQ1 DNase Stop Solution to terminate the reaction. Incubate at 65°C for 15 min to inactivate the reaction.

> **Comment**: Activity of T7 RNA polymerase is often 2–3 times higher than that of SP6 RNA polymerase. If you need high probe yield, T7 RNA polymerase becomes the only choice. In this case, you can screen and select plasmid clones with both insert orientations or use T7 promoter site-appended primers to generate DNA templates representing sense and antisense probes.

PART C: Purification of unincorporated DIG labeling mix

Choice 1: **MEGAclear™ Purification Kit** (Ambion, Cat# 1908) — Filter-based column purification for large scale transcription reactions (http://www.ambion.com/techlib/prot/bp_1908.pdf)

Protocol (for two to five 20-µl *in vitro* transcription reactions):

1. Bring the RNA sample to 100 µl with Elution Solution. Mix by pipetting.
2. Add 350 µl of Binding Solution Concentrate to the sample. Mix gently by pipetting.
3. Add 250 µl of 100% ethanol to the sample. Mix gently by pipetting.
4. Apply the sample to the Filter Cartridge attached to a Collection/Elution Tube.
5. Spin at 14,000 rpm for 30 sec.
6. Discard the flow-through and replace the Collection/Elution tube.
7. Wash the column twice with 500 µl Wash Solution (100% EtOH pre-added) by centrifugation at 14,000 rpm for 30 sec each.

8. Remove traces of Wash Solution by an additional centrifugation at 14,000 rpm for 1 min.
9. Place the Filter Cartridge into a new Collection/Elution Tube.
10. Apply 50 µl of Elution Solution/RNase-free distilled water to the center of the Filter Cartridge. Close the cap of the tube and incubate in a heat block set to 65–70°C for 5–10 min.
11. Recover eluted RNA by centrifugation at 14,000 rpm for 1 min at RT.

Choice 2: **Quick Spin Column for radiolabeled RNA purification** (Roche, Cat# 11274015001) — pre-packed G-50 Sephadex spin column (https://www.roche-applied-science.com/pack-insert/1274015a.pdf)

Protocol (for one to four 20-µl *in vitro* transcription reactions)

1. Remove a column from the storage container and gently invert it several times to resuspend the medium.
2. Remove the top cap from the column, then remove the bottom tip. This sequence is necessary to avoid creating a vacuum and uneven flow of the buffer. Allow the buffer to drain by gravity and discard it.
3. Place the column in a collection tube and centrifuge at 1100 ×*g* for 2 minutes. Discard the collection tube and the eluted buffer.
4. Keeping the column in an upright position, very carefully apply the RNA sample (**up to 100 µl**) to the center of the column bed. Avoid applying the sample to the sides of the column; if this occurs, nucleotides flow around the medium and are not retained. Overloading the column (volume 100 µl) also results in nucleotides flowing through, contaminating the RNA sample.
5. Being careful to keep the column in an upright position, place the column in a second collection tube. Maintaining the column in an upright position is very important, specially after centrifugation. Tipping the column causes back-flow of the RNA sample, resulting in reduced recovery.
6. Centrifuge for 4 minutes at 1100 ×*g*.
7. Save the eluate from the second collection tube. This contains your purified RNA sample.

PART D: MOPS-formaldehyde gel electrophoresis

1. Prepare 50 ml 1.2% MOPS-formaldehyde agarose gel as follows:

- Melt 0.6 g agarose in 42.5 ml Milli-Q water in a microwave oven.
- Allow to cool to approximately 55–60°C under running tap water.
- Add 5 ml of 10×MOPS buffer, 2.5 ml of 36.5% formaldehyde and 5 µl of 5 mg/ml ethidium bromide. Mix the contents well.

- Allow the gel to solidify (15–20 min) within a fume hood.

2. Mix 2 µl RNA sample with 2 µl 2× Loading Dye Solution (Fermentas, Cat #: SM0423). Heat the mixture for 10 min at 75°C. Chill on ice for 2 min.

3. Apply the samples and 5 µl RNA ladder, high range, ready-to-use, (Fermentas, Cat #: SM0423) on gel and run it at 100 V for 1 h. For optimal results, add ethidium bromide to the electrophoresis buffer (1× MOPS), producing a final concentration of 0.5 µg/ml (e.g. 35 µl of 5 mg/ml ethidium bromide to 350 ml of 1×MOPS buffer).

4. Examine the gel under UV using the Gel-Doc 2000 system (BioRad).

5. Mix 2 µl RNA sample with 68 µl RNase-free distilled water. Measure OD at 260 nM with a Gene Quant RNA/DNA Calculator (Pharmacia Biotech) or equivalent (e.g. Eppendorf BioPhotometer). Convert OD to RNA concentration (ng/µl) using the following formula:

$$\text{RNA concentration (ng/µl)} = \text{OD} \times 35 \times 40 \text{ ng/µl}$$

6. Divide the RNA probe into multiple aliquots (e.g. 2 µl per tube). Store the RNA at −80°C.