DIG RNA Probe Synthesis and Purification

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[Abstract] *In situ* hybridization is an effective method to examine the expression level and location of a gene of interest in tissues or cells. To do this, RNA can be labeled with digoxigenin-UTP (DIG) by *in vitro* transcription with SP6 and T7 RNA polymerase. The method provided in this protocol is a detailed description of synthesizing an antisense DIG-labeled RNA probe for *in situ* hybridization using the DIG RNA labeling kit from Roche.

Materials and Reagents

1. CI (Chloroform: Isoamylalcohol = 24:1)
2. NaOAc
3. EtOH
4. LiCl
5. DEPC H_2O
6. RNasin Plus Protease Inhibitor (Promega Corporation, catalog number: N2611)
7. 70% ethanol
8. PCI (Phenol: Chloroform: Isoamylalcohol = 25:24:1, volume, Sigma-Aldrich, catalog number: P3802)
9. Glycogen (QIAGEN, catalog number: 158930)
10. DIG RNA labeling kit (Roche Diagnostics, catalog number: 11175025910)
11. Quick Spin Columns (Roche Diagnostics, catalog number: 1274015)

Equipment

1. Standard tabletop centrifuges
2. RNase-free Eppendorf tube
3. NanoDrop
4. Falcon snap cap tube
5. Water bath

Procedure

A. Linearizing the cDNA

1. Cut 5 µg of cDNA in a 100 µl reaction. I use ~2-3 µl of enzyme.
2. Add 1 volume (100 µl) PCI, vortex and centrifuge 2 min.
3. Transfer upper phase in a new tube and add 1 volume (100 µl) CI, vortex and centrifuge 1 min.
4. Transfer upper phase to new tube. Ethanol precipitate this phase.
5. Add 200 µl EtOH, 10 µl 3 M NaOAc, 0.5 µl 1 mg/ml glycogen and precipitate at -20 °C overnight.
6. Spin down and wash with 70% ethanol.
7. Spin again and resuspend in 11 µl nuclease-free water. Take 1 µl of linearized DNA to spec the concentration.

B. DIG probe synthesis (using the Roche labeling kit)

* = provided with kit

1. Add the following to an RNase-free Eppendorf tube (20 µl reaction):
   1 µg linearized DNA template
   2 µl NTP labeling mix*
   2 µl transcription buffer*
   2 µl of RNA polymerase (T7, T3, SP6)*
   1 µl RNase inhibitor*
   RNase-free water to 20 µl total volume.
2. Mix by flicking tube gently and briefly centrifuge.
3. Incubate reaction at 37 °C for 2 h.
4. Add 2 µl DNase I* to the reaction.
5. Incubate for 15-20 min at 37 °C.

C. Purifying the DIG probe

Method 1: Precipitation of RNA probe

1. Add:
   3.7 µl DEPC H_2O
   1.33 µl 7.5 M LiCl
   75 µl EtOH
2. Precipitate at -20 °C O/N.
3. Centrifuge 4 °C, 13,000 rpm for 30 min.
4. Wash pellet in 500 µl 70% DEPC-EtOH (-20 °C).

5. Centrifuge 5 min, 13000 rpm, RT.
6. Air dry 5-10 min.
7. Add:
   50 µl DEPC H_2O
   20 units RNasin (0.35 µl)
   Resuspend RNA probe
   Take 2 µl to check on agrose gel and quantify RNA using NanoDrop.
8. Store RNA probe at -20 °C.

Method 2: Quick Spin Columns

1. Remove the quick spin columns from the storage bag and gently invert several times to resuspend the medium.
2. Remove the top cap from the column and then the bottom cap. Some of the buffer will run out of the column. Discard it.
3. Place the column in one of the collection tubes (provided in kit) and put both the column and collection tube into a falcon snap cap tube (clear tube, like the ones we use for maxi preps).
4. Spin the sample in the table-top centrifuge at 1,100 *x g* for 2 min.
5. Discard the buffer and if the tip of the column is submerged in the buffer when you take it out of the first spin, then spin it again to make sure that all of the buffer is cleared from the column.
6. Place the column in a new collection tube (provided with kit).
7. Add your DIG probe to the center of the column without touching the beads.
8. Spin for an additional 2 min at 1,100 *x g*. The eluate from this spin is your DIG probe sample. Transfer it to a clean Eppendorf tube (about 20 µl) and bring up to 100 µl with nuclease-free water. This will give you a concentration of about 100 ng/µl of probe.

Notes

I usually synthesize 3-4 separate dig probe reactions of the same probe and add all of them to the same G50 column. This way you have made quite a bit of probe using only 1 column.

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