

RNA probe labeling for DIG in situ.

Suggested timetables

Assemble all solutions and reagents before you start!

One-day timetable: Do everything on the same day. This will mean a very long day, and you may get less than ideal product yields.

Two-day timetable 1: Set up restriction digest overnight on Day 1; purify the DNA and do the rest of the protocol on Day 2.

Two-day timetable 2: Do restriction digest, purify the DNA, do the transcription reaction, and precipitate the RNA overnight on Day 1; resuspend the RNA on Day 2.

Three-day timetable : Set up restriction digest overnight on Day 1; purify the DNA, do the transcription reaction, and precipitate the RNA overnight on Day 2; resuspend the RNA on Day 3.

Solutions

10X dig U NTP mix – store at –20C

10 mM ATP

10 mM GTP

10 mM CTP

6 mM UTP

4 mM dig UTP (Boehringer is traditional, but other suppliers might be cheaper)

Make sure you use NTPs, not dNTPs!!!

10X transcription buffer – store at –20C

0.4 M Tris pH 7.5

0.06 M MgCl₂

0.1 M NaCl

0.02 M Spermidine-HCl

stop solution

0.2 M NaOAc

pH to 6.0 with acetic acid

2X Carbonate buffer (optional)

80 mM NaHCO₃

120 mM Na₂CO₃

You will also need:

Appropriate polymerases

50mM DTT

RNase inhibitor

20 mg/ml glycogen

4M LiCl

For solutions used in enzymatic reactions (transcription buffer, NTP mix, DTT, etc.), use commercial ultrapure RNase-free water, without traces of DEPC. Traces of DEPC may inhibit the enzymes.

DNA template preparation

Digest the plasmid with an enzyme that cuts the polylinker just upstream from the insert (for antisense probe) or just downstream from the insert (for sense probe). If possible, check the insert for internal restriction sites. It's OK if there is an internal site – you will get a probe corresponding to a partial gene sequence – but you do not want that partial sequence to be too short. Ideally, the plasmid should be digested for **4-5 hours or overnight** to ensure a complete digest. However, if you are short on time, 1.5-2 hours may do the trick.

For example, if the insert is directionally cloned (5' to 3') into the EcoR1 and Xho1 sites of pOT, digest with EcoR1 and transcribe with Sp6 for antisense probe, or digest with Xho1 and transcribe with T7 for sense probe.

After digestion, you will need to purify the DNA. Phenol-chlorophorm extraction is the traditional method of purification, but Quiagen columns should work, as well. Once the DNA is purified, precipitate it with ethanol for at least 10 minutes, spin down hard, dry completely, and resuspend in 5 μ l of RNase-free ultrapure water.

Digoxigenin-UTP labeling of RNA probes

10X transcription buffer	1 μ l
10X dig U NTP mix	1 μ l
50 mM DTT	1 μ l
RNase inhibitor (50 u/ μ l)	1 μ l
linearized DNA (1 μ g)*	5 μ l
T7, T3, or Sp6 RNA polymerase	1 μ l (10 to 20 u)

Notes:

Use commercial ultrapure RNase-free water, without traces of DEPC.

Make sure you use NTPs, not dNTPs!!!

*Some people say that for Sp6 polymerase you need to use 2-3 times more DNA template, and incubate the reaction at 40C instead of 37C.

--incubate at 37 C for **2 hours**

--add 40 μ l H₂O

Take a 5 μ l aliquot and run it on a gel to check probe quality and amount. Run the gel at <100V for roughly 15 minutes since RNA degrades quickly. If you want to be anal, treat gel box and comb with 0.2N NaOH for 30 minutes before use.

Ideally, you should get a nice band, not a smear – but smears also work sometimes. A very crude way to estimate how much probe you have made is to compare

the intensities of the RNA band to that of the DNA template band on your gel. If they are equal, you have synthesized roughly 2 μ g of RNA probe.

- add 50 μ l stop solution
- add 10 μ l 4 M LiCl
- add 5 μ l 20 mg/ml glycogen as carrier
- add 300 μ l ethanol
- mix and freeze (-20 C) **at least 15 min, preferably longer (1 hour - overnight).**
- spin top speed in microfuge 20 min in cold
- wash pellet in 70% ethanol
- dry completely
- dissolve in 150 μ l dH₂O and separate into several aliquots to avoid frequent thawing.
- store at -70 C
- use 1-3 μ l probe per 100 μ l hybridization buffer in reaction.

Hybridization cocktails containing the probe can be reused many times (>15 times), with results getting even better.

Optional fragmentation (carbonate hydrolysis) step:

You may want to chop your probe into pieces between 75 and 150 bp long. This may help if you are getting low signal/high background problem when the probe is not penetrating properly into the tissue. Normally, if you do a good Proteinase K digest, this step should not be required.

Resuspend pellet in 100 μ l H₂O. Add 100 μ l 2XCO₃⁻ buffer.

Incubate at 60°C for “**calculated**” time

Neutralize with 10 μ l 10% acetic acid.

Add 1/10 volume 3MNaAc (pH 5.2) and 2 volumes EtOH.

Precipitate at -20°C for **at least 15 minutes, preferably longer (1 hour - overnight).**

Rinse pellet with 70% EtOH.

Dry and dissolve as above.

Reaction time is calculated as follows:

$$\text{Time} = \frac{\text{Li} - \text{Lf}}{\text{K} \cdot \text{Li} \cdot \text{Lf}}$$

Li=initial length of probe (in kb)

Lf=final length of probe (0.15 kb)

K=0.11 kb⁻¹ min⁻¹

(The final fragment length of 150 bases is traditional).