RIBOPROBE GEMINI SYSTEM TRANSCRIPTION OF CLONED DNA

The protocols listed below are a modification of Melton, D.A., et al. (1984) Nucl. Acids Res. ***12,****7035-7056.*

REAGENTS

1. 5x transcription buffer
   200 mM Tris-HCl, pH 7.5 measured at 37°C
   30 mM $MgCl_2$
   10 mM spermidine
   50 mM NaCl

2. rNTP stocks
   10mM ATP
   10mM CTP
   10mM UTP
   10mM GTP

(Note: The ribonucleotide mixtures should be neutralized to pH 7. The ribonucleotides provided as components of **Riboprobe Gemini** System II have already been neutralized).

3. 100mM DTT (0.5mM EDTA can be added to increase the stability of the DTT.)

4. RNasin® ribonuclease inhibitor (recommended because of possible glassware and reagent contamination).

5. Labeled rNTP (Optional, only when radiolabeled RNA is desired.)

The 5x transcription buffer should be autoclaved or filter sterilized. The DTT and rNTP stocks should be prepared in DEPC-treated water. DEPC (diethylpyrocarbonate) is available from Sigma Chemical Company, St. Louis, Mo.

DEPC-treated water (100 ml dH_2O)

1. Add 1.0 ml of 10% DEPC in absolute ethanol.
2. Let sit at room temperature for 5 minutes.
3. Heat to 90°C for 5 minutes.
4. Cool to room temperature.

TRANSCRIPTION (Suggested protocols)

Below are listed suggested protocols for use with the **Riboprobe Gemini**™ system. These protocols are applicable for transcription reactions using either the **Riboprobe** SP6 RNA polymerase or the **Riboprobe** T7 RNA polymerase. Transcription protocol #1 has been optimized for the synthesis of RNA probes. Transcription protocol #2 has been optimized for the synthesis of large quantities of unlabeled RNA. They are the standard conditions used at Promega Biotec in testing quality control of our enzyme preparations and are intended for use as

guidelines in setting up transcription reactions. They can be modified to suit your particular needs.

TRANSCRIPTION PROTOCOL #1

High Specific Activity RNA Probe Synthesis (20 µl)

1. Add DEPC-treated water to a final volume of 20 µl.
2. 4.0 µl 5x transcription buffer
3. 2.0 µl 100mM DTT
4. 0.8 µl RNasin^{R} ribonuclease inhibitor
   (25 units/µl stock) ([final] = 1 unit/µl)
   (Optional; see reagent section #4)
5. 4.0 µl 2.5mM each of ATP, GTP, and UTP (Mix together equal amounts of 10mM ATP, GTP, UTP, AND H_2O.)
6. 2.4 µl 100µM CTP ([final] = 12 µM)
7. 1.0 µl 0.2-1.0 mg/ml linearized plasmid template DNA
   (0.2-1.0µg) in water or Tris-EDTA buffer
8. 5.0 µl 10mCi/ml a-^{32}P-CTP (50µCi)
   (other labaled rNTPs can be used (10mCi/ml; >400Ci/mmmole)).
9. 0.5-1.0 µl either **Riboprobe** SP6 RNA polymerase or **Riboprobe** T7 RNA polymerase (5-10 units)

Incubate for 1 hour at 37°C-40°C.

Notes:

1. The above reaction can be run in the absence of unlabeled CTP. For a 20 microliter reaction, 100µCi of 400 Ci/mmole a-^{32}P-CTP is approximately 12µM. The yield of full-length transcripts is reduced as the concentration of limiting nucleotide falls below 12µM.

2. The components should be added in the order shown and the mixture should not be kept on ice, but rather at room temperature during addition of each successive component. It has been found that DNA can sometimes precipitate (because of the spermidine) if the components are incubated at 4°C.

TRANSCRIPTION PROTOCOL #2

Synthesis of Large Amounts of Unlabeled RNA (100 µl)

1. Add DEPC-treated water to a final volume of 100 µl
2. 20.0 µl 5x transcription buffer
3. 10.0 µl 100mM DTT
4. 4.0 µl **RNasin**^{R} ribonuclease inhibitor
   (25 units/µl stock) ([final] = 1 unit/ul)
   (Optional: see reagent section #4)

5. 20.0 µl 2.5mM each of ATP, CTP, UTP, and GTP (Made by mixing together equal amounts of the rNTP stocks.)
6. 2.0 µg linearized plasmid DNA in water or Tris-EDTA buffer
7. 10-20 units either **Riboprobe** SP6 RNA polymerase or **Riboprobe** 77 RNA polymerase.

Incubate for 1-2 hours at 37°C-40°C.

Notes:

1. Using the above conditions for the synthesis of large amounts of unlabeld RNA, yields of 5-10 micrograms RNA/microgram plasmid DNA are attainable.

2. The components should be added in order shown and the mixture should not be kept on ice, but rather at room temperature during addition of each successive component. It has been found that the DNA can sometimes precipitate (because of the spermidine) if the components are incubated at 4□C.

REMOVAL OF DNA TEMPLATE

1. Following the RNA synthesis reaction described above, add RQ1™ DNase to a concentration of 1 unit/µg DNA.

2. Incubate at 37°C for 15 minutes.

3. Extract with an equal volume of phenol:chloroform.

4. Extract with an equal volume of chloroform.

5. The RNA may be recovered by sodium acetate-ethanol precipitation (add 1/10 volume of 3M NaOAc and 2.5 volumes of ethanol).

PROTOCOL #3

USE OF RIBOPROBE GEMINI™ SYSTEM PROBES IN BLOT ANALYSES

1. RNA Blot Hybridization (Northerns)

Following transcription of linearized DNA (see accompanying protocols) and removal of DNA template, the RNA to be analyzed is electorphoresed in denaturing agarose gels and blotted to nitrocellullose or DBM paper. We use nitrocellulose filters, baked in vacuo for two hours at 80°C after blotting. The filters are prehybridized at 55-60°C for 4 hours in the following buffer:

50% formamide
50mM sodium phosphate, pH 6.5
0.8M NaCl
0.05% Ficoll
0.05% PVP
250µg/ml denatured salmon sperm DNA

1 mM EDTA
0.05% BSA
500µg/ml yeast RNA
+/- poly A at 10µg/ml

This hybridization solution is rather standard, and it is not known whether all of these components are necessary.

The hybridization is carred out in the pre-hybridization buffer with the addition of probe. For the analysis of globin transcripts, hybridization is best if performed at 55-60°C for 10-24 hours, with the filters being washed in the following buffer at 60-65°C:
50mM NaCl
20mM sodium phosphate, pH 6.5
1 mM EDTA
0.1% SDS

Please note that the hybridization conditions may have to be varied depending on the specific nature of the probe and hybridizing substrate. Thus, the temperature and time of the hybridization and washes can be varied with the desired stringency of the hybridization. 3-5 washes of 20 minutes each are typically recommended.

II. DNA Blot Hybridizations (Southerns)

For DNA blot hybridizations the same buffer used for Northerns (above) is employed, except that the temperature, formamide, and/or ionic conditions are lowered an appropriate amount to compensate for the lower melting temperature of an RNA/DNA hybrid relative to an RNA/RNA hybrid. Typically the melting temperature adjustment is 5-10 Celsius degrees, depending on the probe used.

PROTOCOL #4

USE OF RIBOPROBE GEMINI™ SYSTEM PROBES IN SOLUTION HYBRIDIZING & RNASE ("S1 TYPE") MAPPING

1. The test sample is alcohol precipitated, with or without carrier RNA, as necessary.

2. The test RNA pellet is dissolved in 30 microliters of 80% formamide hybridization buffer (see below).

3. The "anti-sense" **Riboprobe** ^{32}P-RNA is dissolved in 50-100 microliters of 80% formamide hybridization buffer. One microliter is added to each hybridization reaction.

4. The hybridization mix is incubated at 85°C for 5 minutes followed by incubation at the hybridization temperature (see note 1 below) overnight (at least 8 hours).

5. Following hybridization, 300 microliters of RNase digestion buffer (see below) containing 40µg/ml of RNase A and 2µg/ml of RNase T1 is added. RNase digestion is allowed to proceed at the desired temperature (see note 2 below) for 1 hour.

6. The RNase digestion reaction is terminated by the addition of 20 microliters SDS and 50 micrograms of proteinase K, followed by an additional incubation at 37°C for 15 minutes.

7. The reaction is extracted on time with phenol/chloroform/isoamyl alcohol and the ^{32}P-RNA/RNA hybrid is precipitated by the addition of 20 micrograms tRNA carrier and 1 milliliter of ethanol.

8. The precipitate is washed with 70% ethanol, dissolved in loading buffer, denatured at 90°C for 3 minutes and analyzed on a denaturing acrylamide-8M urea gel. Alternaively, it is possible to analyze duplex RNAs in non-denaturing agarose gels.

Notes:

1. The hybridization temperature should be empirically determined, and is dependent upon parameters such as the G-C content of the test RNA and the length of the RNA/RNA hybrid. However, it has been found that 45°C is a suitable hybridization temperature for many ^{32}P-RNA/RNA hybrids.

2. Similarly, the RNase digestion temperature should be empirically determined and can significantly influence the signal to noise ratio. Here it has been found that 30°C is a useful Rnase digestion temperature for many ^{32}P-RNA/RNA hybrids.

Buffers:

<u>80% Formamide Hybridization Buffer</u>: 40mM PIPES, pH 6.7, 0.4M NaCl, 1 mM EDTA

<u>RNase Digestion Buffer</u>: 10mM Tris-HCl, pH 7.5, 5mM EDTA, 300mM NaCl