12/13/2019

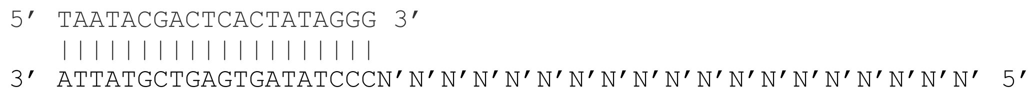
Milligan Small RNA Transcription

Adapted from Milligan, Groebe, Witherell, Uhlenbeck (1987). Oligoribonucleotide synthesis using T7 RNA Polymerase and synthetic DNA templates. *NAR* 15(21): 8783-8798

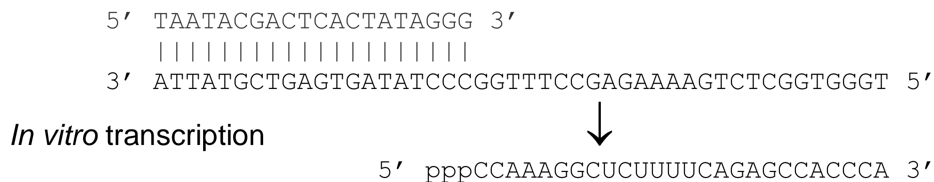
This is a relatively simple protocol for generating small RNAs suitable for electrophoretic mobility shift assays or *in vitro* processing assays. RNAs can be made unlabeled or body labeled with α_{32}P-NTPs or left unlabeled. If end labeling is desired, triphosphates must be removed first.

Template design considerations:

An oligonucleotide encoding the T7 promoter is annealed to a second oligonucleotide with the reverse complement to the desired RNA sequence and T7 promoter. Annealed oligos will be as follows:



For example, if the desired RNA sequence is: CCAAAGGCUCUUUCAGAGCCACCCA



<u>Annealing</u>

1. Mix:

10 μM Template oligonucleotide	1.0 μL
10 μM T7 primer	1.0 μL
10x annealing buffer or NEB2	0.8 μL
H_2O	5.2 μL

2. Heat at 100_oC for 10 minutes
3. Place on ice for 10 minutes

<u>*In vitro* transcription of body labeled RNA</u>

1. To the annealed oligo mix, add:

Annealed DNA	8.0 μL
10 X Transcription Buffer (NEB)	5.0 μL
10 mM rA,G,U mix (3.3 mM each)	5.0 μL
0.1 mM rCTP	12.5 μL
3.3 μM [α-$_{32}$P]-3000 Ci/mmol-CTP	5.0 μL
Ribolock (40 U/μL) (Thermo #FEREO0381)	1.0 μL
T7 RNA Polymerase (50 U/μL) (NEB M0251L)	2.0 μL
dH_2O	11.5 μL

2. Incubate at 37_oC for 2 hours

3. Add 1 µL 1U/µL of RQ1 DNase (Promega #M6101)
4. Incubate at 37$_{o}$C for 15 minutes
5. Remove unincorporated nucleotides by running total reaction through Illustra S Microspin G-25 column (GE Healthcare Life Sciences #27532501). This step is necessary to reduce the amount of radiation in downstream steps.
6. Ethanol Precipitate the eluate with 1 µL of 15 mg/mL GlycoBlue (ThermoFisher AM9515)
7. Resuspend the precipitated pellet in 20 µL of RNA loading dye
8. Run on 12% acrylamide gel (19:1 Acrylamide:Bis-acrylamide/1X TBE) until xylene cyanol is ~2/3 of way down the gel
9. Dissasemble gel apparatus and place wet (<u>very radioactive</u>) gel between cut page protector.
10. Expose to film for ~5 minutes to visualize bands
11. Excise gel band corresponding to full length transcription product
12. Cut excised band into smaller strips and place in 1.5 mL microcentrifuge tube
13. Freeze at -80$_{o}$C or on dry ice for 30 minutes
14. Add 400 µL of gel elution buffer
15. Rotate overnight at room temperature
16. The following day, remove the liquid to a new tube
17. Phenol/Chloroform extract
18. Chloroform extract
19. Ethanol precipitate with 2 volumes of 100% EtOH
20. Resuspend in 50 µL of dH$_2$O
21. Determine concentration by % incorporation of CTP via Scintillation counting

<u>*In vitro* transcription of unlabeled RNA</u>

Hi-Scribe T7 High Yield RNA Synthesis kit (NEB #E2040S) or RiboMax kit are preferred here since higher amounts of unlabeled RNA are typically needed. Adjust transcription reaction to meet your needs. Below is the protocol using Hi-Scribe

1. To the annealed oligo mix, add:

Annealed DNA	8.0 µL
100 mM ATP	2.0 µL
100 mM GTP	2.0 µL
100 mM UTP	2.0 µL
100 mM CTP	2.0 µL
10 X NEB buffer	2.0 µL
T7 Enyme Mix	2.0 µL

2. Incubate at 37$_{o}$C for 2 hours
3. Add 1 µL 1U/µL of RQ1 DNase (Promega #M6101)
4. Incubate at 37$_{o}$C for 15 minutes
5. Ethanol Precipitate the eluate with 1 µL of 15 mg/mL GlycoBlue (ThermoFisher AM9515)
6. Resuspend the precipitated pellet in 20 µL of RNA loading dye
7. Run on 12% acrylamide gel (19:1 Acrylamide:Bis-acrylamide/1X TBE) until xylene cyanol is ~2/3 of way down the gel
8. Stain gel with Ethidium Bromide or SYBR Gold in 1X TBE for 5 – 15 minutes
9. Wash for 5 minutes in water
10. Visualize by UV and excise the desired bands
11. Cut excised band into smaller strips and place in 1.5 mL microcentrifuge tube

12. Freeze at -80 ^{o}C or on dry ice for 30 minutes
13. Add 400 μL of gel elution buffer
14. Rotate overnight at room temperature
15. The following day, remove liquid from tube
16. Phenol/Chloroform extract
17. Chloroform extract
18. Ethanol precipitate with 2 volumes of 100% EtOH and 3 M Sodium Acetate. Do not use ammonium acetate at this step. Ammonium ions will inhibit T4 PNK if used for downstream applications.
19. Resuspend in 50 μL of dH_2O
20. Quantify RNA by OD_{260}.
21. If end labeled products are desired, triphosphates must be removed using a phosphatase.

RNA Loading Dye
98% Formamide
0.2 mM ETDA [pH 8.0]
0.25% SDS
0.01% Bromophenol Blue
0.01% Xylene Cyanol

Gel Elution Buffer
20 mM Tris [pH 7.5], 250 mM Sodium Acetate, 1 mM EDTA [pH 8.0], 0.25% SDS

10x DNA Annealing Buffer
100 mM Tris [pH 7.9], 500 mM NaCl, 100 mM $MgCl_2$, 10 mM DTT