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5' RACE for RNA fragments with 5' phosphate

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ABSTRACT

This protocol will allow identification of fragments of RNA that have a 5' phosphate. It relies on ligation of an RNA adapter to the 5' end of the fragment and amplification with primers that bind the adapter and gene-specific sequences ~200 nt downstream of predicted/expected fragment end. It is adapted from protocols to map mammalian transcriptional start sites, but lacks any reaction to remove the cap. We have used it to isolate fragments generated by viral RNases.

MATERIALS

Reagents

Ambion Turbo DNase (Thermofisher – Cat#AM2239)
Recombinant RNasin RNase inhibitor (Promega – cat# N2515)
Ultrapure DNase/RNase free dH_2O (Thermofisher Cat# 10-977-015)
Phenol/chloroform with acidic pH (we use Fisher cat#BP1752I-100 without adding the additional buffer, but other should work)
3 M sodium acetate (Thermofisher – Cat# AM9740)
Glycogen (Thermofisher – Cat# AM9510)
100% Ethanol (we use VWR Cat# 89125-170, but others should be fine)
T4 RNA ligase (Ambion Cat#AM2141)
Invitrogen M-MLV reverse transcriptase (Thermofisher – Cat# 28-025-013)
Random decamers (Thermofisher – Cat#AM5722G)
dNTPs (we use Thermofisher – Cat#FERR0181, but others should work; keep RNase free) – *make a working stock of 25 mM*
Taq DNA polymerase with standard Taq buffer (New England Biolabs – Cat#M0273)

RACE RNA adapter – *make working stocks of 0.3 µg/µl*

GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA

Primers – *make working stocks of 10 µM:*

5' RACE outer forward primer *(binds RNA adapter)*:
GCTGATGGCGATGAATGAACACTG

5' RACE inner forward primer *(binds RNA adapter sequences and allows for Gibson cloning of fragments into the EcoRI site of pCDNA3.1(+))*:
GGATCCACTAGTCCAGTGTGGTGGAATTCGAACACTGCGTTTGCTGGCTTTGATG

Gene-specific outer and inner reverse primers – *design ~200 nt downstream of expected cleavage site; should allow for nested PCR*.

Equipment
Thermocycler
Water bath or heat block set at 37°C
Refrigerated microcentrifuge

1 **RNA purification:**
Extract RNA and elute/resuspend in 50 µl H_2O

2 **DNase treatment:**

To each 50 µl sample, add 10 µl of DNase reaction mix containing:

- 6 µl DNase buffer
- 1.2 µl Turbo DNase
- 1.5 µl RNasin
- 1.3 µl H_2O

(total volume = 60 µl)

Incubate for 15 min at 37°C (waterbath or heatblock).

3 **RNA extraction with phenol/chloroform:**

Add 140 µl H_2O to each tube, then 200 µl phenol/chloroform.

Vortex for 15 sec.
Spin down for 5 min at 12,000 xg at room temperature.
Transfer the top aqueous phase to a new tube with 2 µl glycogen + 20 µl 3M sodium acetate.
Add 600 µl 100% ethanol to each tube, invert a few times to mix.
Incubate at -20°C for 1h (can leave it overnight if needed).
Spin down to pellet at 16,000xg at 4°C for 20 min.
Remove supernatant and wash pellet with 1ml freshly made 70% ethanol.
Spin down again at 16,000xg at 4°C for 5 min.
Remove supernatant and spin down again for 1min.
Remove all liquid and dry pellet for a few min (not too long).
Add 12 µl water and leave at RT for 10 min to resuspend.

Measure concentrations.
At this point, can set aside 250-500 ng of DNase-treated RNA for RT-qPCR to test RNA levels and/or RNase activity.

4 **RACE adaptor ligation:**

Depending on concentrations of RNA samples, try to use as much RNA as possible for the ligation (i.e. use 10 µl of the lowest sample if one sample is very low). Between 1.5 and 6 µg of RNA works well. Dilute all samples to this amount in 10 µl H_2O in PCR tubes.

To each sample add 5 µl of ligation mix containing:

- 1.5 µl 10X RNA buffer
- 1 µl T4 RNA ligase (5 U/µl)
- 1.5 µl 5' RACE adapter (0.3 µg/µl)
- 1 µl dH_2O

(total volume= 15 µl)

Incubate samples at 25°C for 2 h in a thermocycler.

5 Reverse transcription with M-MLV RT

Mix 2 µl ligated RNA with 8 µl of primer-dNTP mix containing :

- 0.4 µl dNTP (25 mM)
- 1 µl random decamers
- 6.5 µl dH_2O

Heat mixture to 65°C for 5 min in thermocycler, then chill quickly on ice and incubate on ice for at least 1 min.
Add to the reaction 7 µl of buffer-DTT mix containing :

- 4 µl RT buffer
- 2 µl DTT (0.1 M, included in kit)
- 1 µl RNasin

Mix and incubate at 37°C for 2 min in thermocycler.

Add 1 µl of M-MLV RT to each reaction and mix.

Carry out RT reaction in thermocycler:

- 25°C for 10 min
- 37°C for 50 min
- 70°C for 15 min

6 Outer PCR with Taq polymerase:

Mix 1 µl ligated cDNA with 49 µl of PCR mix containing :

- 0.4 µl dNTP (25 mM)
- 5 µl 10x Standard Taq buffer
- 0.25 µl Taq (5 U/ µl)
- 2 µl 5' RACE outer primer (10 µM)
- 2 µl Gene-specific outer primer (10 µM)
- 39.35 µl dH_2O

Run the following program on a thermocycler:
- 94°C for 3min
- 35 cycles of :
  94°C for 30 sec
  60°C for 30 sec
  72°C for 30 sec
- 72°C for 7 min

7 Inner PCR with Taq polymerase:

Mix 2 µl of outer PCR reaction with 48 µl of PCR mix containing :

- 0.4 µl dNTP (25 mM)
- 5 µl 10x Standard Taq buffer
- 0.25 µl Taq (5 U/ µl)
- 2 µl 5’ RACE inner primer v2(10 µM)
- 2 µl Gene-specific inner primer (10 µM)
- 38.35 µl dH_2O

Run the same PCR program as outer PCR.

8 Fragment analysis on agarose gel

Run inner (and if desired outer) PCR on agarose gel

We usually use a 2% agarose gel, 15 x 20 cm, and run it for 3 h at 70V

Fragment size depends on location of primers relative to cut site (remember to add adaptor/primer sequence when calculating it, since it is pretty large)