Primer Extension

Reagents

- Promega Primer Extension system – AMV Reverse Transcriptase Cat.# E 3030. Includes PNK buffer (500mM Tris-HCl (pH 7.5), 100mM $MgCl_2$, 50mM DTT, 1mM spermidine).
- [γ-^{32}P]ATP (6,000 Ci/mmol)
- 8% acrylamide (19:1 acrylamide:bis acrylamide)
- 7 M urea
- 10x TBE buffer
- 10% Ammonium Persulfate (APS)
- TEMED
- Primer (ideally, the primer should hybridize ~100 bases downstream from RNA 5´ end).

Primer Labeling

- Add to Eppendorf tube:

Primer 10 pmoles	2 μl
T4 PNK 10x Buffer	1 μl
[γ-^{32}P]ATP (6,000 Ci/mmol)	3 μl
T4 Polynucleotide Kinase (PNK)	1 μl
Nuclease-Free Water	3 μl

- Incubate at 37°C for 10 minutes.
- Heat-inactivate at 90°C for 2 min.

DNA Marker Labeling

- Add to Eppendorf tube:

X174 (HinfI) marker	5 μl
T4 PNK 10x Buffer	1 μl
[γ-^{32}P]ATP (6,000 Ci/mmol)	3 μl
T4 Polynucleotide Kinase (PNK)	1 μl

- Incubate at 37°C for 10 min.
- Heat-inactivate at 90°C for 2 min.

RNA preparation

To enrich for mRNA (only necessary for low-copy-number RNA, not for tubulin, for example) use the Rneasy Kit (Qiagen). You can follow instructions provided with kit but titrations suggest it works fine with ~$2x10^8$ trypanosomes. Kit gives 100 μg RNA as maximum column capacity.

Primer Extension

- Warm 40 mM Sodium Pyrophosphate, Nuclease-Free Water, and AMV Primer Extension 2x Buffer, to RT or 37°C.
- Add to Eppendorf tubes:

	Primer extension	No RNA
Sample or Control RNA	5 µl	—
Nuclease-Free Water	—	5µl
Primer	1 µl	1 µl
AMV Primer Extension 2x Buffer	5µl	5µl

- Anneal primer and RNA by heating tubes at 58°C for 20 min and then place tubes at RT to cool for 10 min.
- To another Eppendorf tube add:

	Per reaction	6 reaction
AMV Primer Extension 2x Buffer	5 µl	30 µl
Sodium Pyrophosphate, 40 mM	1.4 µl	8.4 µl
AMV reverse transcriptase	1 µl	6 µl
Nuclease-Free Water	1.6 µl	9.6 µl

- Immediately dispense 9 µl of the reverse transcriptase mix into each reaction tube containing annealed primer/RNA.
- Incubate at 41–42°C for 30 min.
- Add 20 µl of Loading Dye to each tube.
- Prepare DNA marker by adding 10 µl of loading dye to a tube containing 1 µl of labeled DNA marker and 9 µl of TE.
- Heat tubes (including the marker) at 90°C for 10 min.
- Load 20 to 40 µl of sample per lane.
- Run gel at 250 volts for 2.5–3 h (until the bromophenol blue dye is at most 2 cm from the bottom of the gel).
- Carefully (gel breaks very easily) place gel on a number of wet Whatman papers and transfer to gel drier for about 45 min.
- Use Phosphorimager to quantify signal.

Polyacrylamide gel

Use glass plates 7x12 inch and 1 mm comb of 1 mm thickness.
Clean glass plates very thoroughly and tape edges using yellow electrical tape from Scotch. The system is very prone to leakage and it is important to tape and clamp the plates well.

7 M urea	33.63 g
10x TBE	8 ml
19:1 acrylamide:bis)	16 ml
10% APS	600 µl
TEMED	60 µl
water	To 80 ml total