6.2 Pause Assay

Buffers and Reagents

CBnuc

100mM KAc	25 uL of 4M stock
10mM Tris-Cl pH 7.5	5 uL of 2M
8mM CaCl2	8 uL of 1M
6mM MgCl2	6 uL of 1M
956 uL water--> 1mL	

5x BT

750mM KAc	187.5 uL of 4M
100mM Hepes-K pH 7.5	100 uL of1M
50mM MgCl2	50 uL of 1M
25mM EGTA	25 uL of 1M
10mM DTT	10 uLof 1M
627.5 uL water--> 1 mL	

BTS0.25 ,/b>

1X BT	200uL of 5X
0.25M sucrose	125uL of 2M
675 uL water---> 1mL	

BPD

50mM NaCl	12.5 uL of 4M
50mM Tris-Cl pH 7.5	25 uL or 2M

5mM EDTA	10 uL of 0.5M
0.5% SDS	25 uL of 20%
928 uL water---> 1mL	

1X BT

- 20 mM cycloheximide
- 15 u/uL Staphylococcal nuclease (Boehringer) see section 3.2 for handling
- 0.1 M EGTA Note: very labile. store frozen, make often.
- 0.2 M ribonucleoside-vanadyl complex (RVC; NEB). Note: heat to 55øC for 10 min before use to dissolve
- 4 mg/mL proteinase K (Boehringer) see section 5.2 a. for handling
- 20 mg/mL yeast tRNA
- 7.5 M NH4Ac

Isolation of RPFs

1. Set up a 25 uL translation reaction (RRL or WG) and let it proceed 15 to 25 min. (a)
2. Place reaction on ice. Add 1.25 uL of 20 mM cycloheximide, mix. Remove sample for SDS-PAGE.
3. Add 15 uL CBnuc, then 2 uL of 15 u/uL Staph nuclease. Incubate 3 min at 0øC then 30 min at 24øC. (b)
4. Place reaction on ice. Add 2 uL of 0.1 M EGTA and 2 uL of 0.2 M RVC to stop digestion.
5. Add 60 uL of BT. Layer mixture over 60 uL of BTS0.25 in a 200 uL polyallomer airfuge tube. Centrifuge at 30 psi (180,000 x g?) for 30 min at 4øC.
6. Remove top 120 uL and discard. To the lower 40 uL and pellet, add 100 uL BPD prewarmed to 37øC and resuspend pellet. Keep airfuge tube inside a microfuge tube for handling. Add 7 uL of 4 mg/mL Proteinase K, and incubate 30 min at 37øC.(c)
7. Transfer reaction to a microfuge tube and extract with 140 uL of buffered phenol. Transfer aqueous supernatant to a fresh tube.
8. Ethanol precipitate by adding 14 uL of 7.5 M NH4Ac, 2 uL of 10 mg/mL tRNA, 380 uL 100% ethanol and spin in a microfuge at full speed 15 min at 4øC. Wash pellet with 95% ethanol, dry pellet and resuspend in 10 uL of water. Store at -70øC.

Notes:

(a) The cell-free reaction should ideally be terminated at steady-state translation. The incorporation of 35S-met into full-length protein product should increase linearly with time at this point. For SRà (plasmid #191) this occurs at roughly 25 min of reaction. The translation of other proteins may differ and should be tested beforehand with a time course.

(b) Higher concentrations of nuclease may be used. However, there is some nuclease remaining from the treatment of the RRL, about 0.5 u/uL final, which will be reactivated by the Ca ions in the CBnuc. The conditions here add 0.75 u/uL nuclease for about 1.25 u/uL final. Check the mRNA digestion products on a gel and adjust the nuclease conditions accordingly. The nuclease digestion may also be supplemented with V1 nuclease at 0.025 u/uL by adding 1.4 uL of 700 u/mL stock (Pharmacia) to digest double stranded RNA. The termination conditions are the same.

(c) The SDS in BPD will precipitate with the K+ ions in the reaction, but will redissolve at 37øC. I have also tried supplementing BPD with 0.5% CHAPS which prevents the precipitate and appears not to affect the rpfs.

Electrophoresis of RPFs

1. Radiolabel the mRNA by adding 1 uL of à[35S]-UTP to a 10 uL transcription reaction (see section 1). Use this transcript in the translation reaction and isolate rpfs as above.
2. Add 1 uL of labelled rpfs to 9 uL of deionized formamide with 0.01% xylene cyanol and 0.01% bromophenol blue. Store at -20øC.
3. For size markers, I use radiolabelled transcripts of plasmid #369 truncated with SacI, NcoI and AvaII to provide RNA of 23 b, 50 b and 110 b in length. Mix 0.5 uL of each with 10 uL formamide to load on gel.
4. Pour a 12% acrylamide, 1xTBE, 8 M urea gel in a BioRad mini protein gel apparatus. The sequencing gel acrylamide stock will do.
5. Pre-run gel about 5 min at 25 W constant power (temperature of gel about 50øC). Heat samples to 72øC for 2 min before loading. Load 10 uL samples and run at 25 W constant power until bromophenol blue is about 2/3 down gel.
6. Fix gel 10 min in destain (35% MeOH, 10% HAC) and dry.
7. Autoradiograph gel. Rpfs from RRL are about 33 nucleotides long, from WG about 28 nucleotides long. A band migrating at about 60 b is usually also present, and is probably 35S Met from the translation reaction.

End-Labelling Primers

Set up reaction:

Primer at 10ng/uL	1 uL
10X buffer	1
[32P]-ATP	1
T4 PNK (10 U/uL	1

water	6
Total	10 uL

Incubate 1 h at 37øC.

Terminate reaction with 1 uL of 0.5 M EDTA.

Ethanol precipitate, wash with 95% ethanol, dry pellet and resusupend in 100 uL water (0.1 ng/uL final).

T4 polynucleotide kinase and buffer are purchased from NEB.

Toeprinting Primer Extension

Prepare single stranded DNA from M13 phage infected cells (see BUGS section). Set up reaction both with and without rpfs. For a negative control, substitute tRNA at 2 mg/mL.

Annealing:

10X buffer	2 uL
BSA (1mg/mL)	2
ssDNA (50ng/uL)	1
hot primer	5
rpfs	1
water	6
Total	17 uL

Incubate: 65øC for 5 min 42øC for 10 min.

Add:

3 mM dNTPs	2 uL
T4 pol (3U/uL)	1
Total	20 uL

Incubate: 37øC for 30 min.

Ethanol precipitate with 10 ug tRNA as carrier. Resuspend in 10 uL of formamide.

Run 2 uL on a standard sequencing gel beside a sequencing reaction from the same primer (see DNA section).

Note: 1 uL of rpfs isolated as above usually provides a good pattern of toeprints. However, it may be necessary to test different amounts of rpfs (see introduction), in which case adjust the reaction to maintain the same final volumes.

I use T4 polymerase and buffer from NEB with no ill effects. T7 polymerase works less well and Klenow fragment not at all.