Teoprinting assay for translocation

Buffers:

- **10x SB buffer**

Stock	Final conc(10x)	Final conc(1x)	volume	
			$+Mg^{2+}$	$-Mg^{2+}$
1M Tis-HCl, pH7.5	100mM	10mM	100ul	100ul
1M MgAc	100mM	10mM	100ul	-
5M NH_4Cl	600mM	60mM	120ul	120ul
14.4 β-ME	60mM	6mM	4ul	4ul
Water			676ul	776ul
			1000ul	1000ul

Make 100ul aliquot. Store at –80°C

- 1M spermidine (FW=145.2,free base, FW= 254.6 trihydrochloride, sigma, powder store at RT)
  145g in 1L=1M or 254.6 g in 1L = 1M
  145mg in 1ml=1M 254.6mg in 1ml=1M (make 200ul aliquot and store at –20°C)

- 50mM spermine(FW=348.3, powder store at –20°C)
  348.3mg in 1 ml=1M
  17.4mg in 1ml= 0.05M (make 100ul aliquot and store at –20°C)

- **5x Nierhaus(0/6/18mM $MgCl_2$) (polyamine buffer)**

1x	Stock solution	Volume			Final conc. (5x)
		1x	5x		
20mM Hepes-KOH,pH7.6	1M	1.0ml	100ul	500ul	100mM
0/6/18mM $MgCl_2$	1M	0.3ml	0/30/90ul	0/**150**/450ul	0/30/90mM
150mM NH_4Cl	5M	1.5ml	150ul	750ul	750mM
2mM spermidine	1M	0.1ml	10ul	50ul	10mM
0.05mM spermine	50mM	50ul	5ul	25ul	0.25mM
Water		47.05ml	735/705/645ul	3675/**3525**/3225ul	-
Final volume		50ml	1ml	5ml	
4mM BME	14.4M	14ul	1.4ul	7ul	20mM

Make 1ml aliquot and store at –80°C.
To prepare 12mM NHB, mix 2 volume 18mM NHB and 1 volume 0 mM NHB.

- **Standard Buffer (conventional buffer-I)**

	Stock solution	Volume(ul)	Final conc.(5X)
80mM potassium cacodylate,pH7.2			400mM
20mM magnesium acetate	1M	100ul	100mm
150 mM ammonium chloride	5M	150ul	750mM
3mM BME	14.4M		15mM
water			
		1000ul	

- **2x sequencing loading buffer**

2x	Stock solution	volume	1 x
95% Formamide	99.5%, Store at 4C	920ul	46%
20mM EDTA	0.5M	40ul	10mM
0.05% Xylene cyanol	2.5%	20ul	0.025%
0.05% Bromphenol blue	2.5%	20ul	0.025%
Final volume		1000ul	

Translocation Reaction conditions: (in 25ul before adding EF-G.GTP)

		tight-coupled	reassociated
0.4 uM 70S	10pmol	10ul	17.5ul
0.8uM gene32 mRNA	20pmol	5ul	2.5ul
0.8uM $tRNA^{fMET}$	20pmol	5ul	2.5ul
0.8uM $tRNA^{Phe}$	20pmol	5ul =25ul	2.5ul=25ul
2.0uM EF-G/GTP(1mM GTP)	50pmol	in 2.5ul	

two control reaction: no mRNA or no tRNA reaction.

AL2 primer labeling with ^{32}P

Sequence of AL2: 5' CTT TAT CTT CAG AAG AAA AAC C 3' (22 nucleotides,Tm=58°C)
Bind mRNA32 +64~+85

H_2O	
10x PNK buffer	2.5ul
100pmol/ul AL2 primer	1ul (100pmol)
T4 PNK(NEB 10u/ul)	2.0ul (<10%)
^{32}P-ATP	5.0ul
	25.0ul

- Incubate at 37°C for 15min.
- Add 10ul 3M NaAc (pH5.2) + 2ul 10mg/ml Glycogen + 65ul water (~100ul)
- 2x chloroform extraction. (~90ul)
- EtOH precipitate DNA by adding 2.5x 100% EtOH (250ul)
- 4°C for o/n.
- Spin at top speed for 30min.
- Discard radioactive EtOH to radioactive waste container.

- Dry pellet at 4°C for 2min.
- Resuspend pellet in water (mix with mRNA and 10x SB buffer)

Ribosome

70S or 30S/50S	20pmol
5x NHB(18mM Mg^{2+})	1ul
H_2O	ul
	5 ul

50S	5ul	(20pmol)
30S	5ul	(10pmol)

- Heat reactive at 42°C for 20min,then slow cool to 37°C and keep at 37°C for10min.
- 30S and 50S associated at 18mM Mg^{2+} at 37°C for 10min. Add 2 volume 1x NHB (no Mg^{2+}) adjust Mg^{2+} to 6mM.For low concentration subunist, associated at 12mM Mg^{2+} and add 1 volume of 1x HNB(-Mg^{2+})

AL2-mRNA (Tm=48C)

mRNA	40pmol
AL2-^{32}P	40pmol
10x SB (-Mg^{2+})	1.0ul
	10.0ul

- 60°C, 3min
- Dry ice /EOtH, 1min.
- Thaw on ice.
- Add 5x NHB and water
- Add to 70S complex (2 reactions and one control reaction)
- 37°C, 10min.

tRNA

H_2O	3.6ul
(100pmol/ul) tRNA	0.4ul
5x NHB	1.0ul
	5.0ul

- Add $tRNA^{fMet}$ to 70S-mRNA complex.
- 37°C, 30min.
- Add $tRNA^{Phe}$ to 70S-mRNA-$tRNA^{fMet}$ complex.
- 37°C, 30min.

EF-G.GTP

(>25pmol/ul) EF-G	ul	(50pmol/rx)
100mM GTP	0.25ul	(0.3-1mM final conc.)
5x NHB	0.5ul	
	2.5ul	

- 25°C, 10min before use.

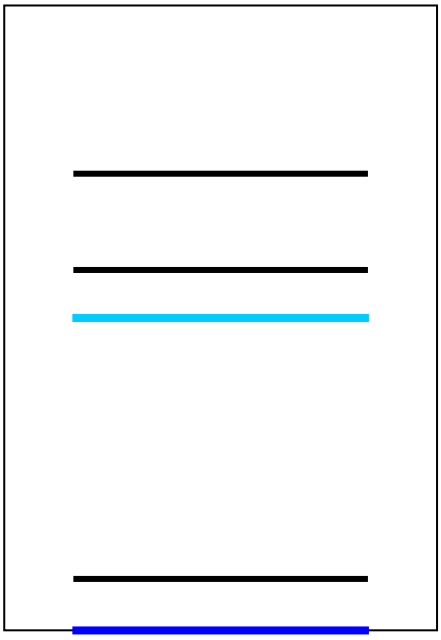
- Add 2.5ul to pre-translocation complex. Add 1x HNB to control
- 37°C, 10min. or select time point at 0.5,1,2,5,10min.
- Remove 1ul for reverse transcription reaction. Place on ice before adding reverse buffer.

Reverse transcription

3.75mM dNTP:

	5	6	7	8	9	10	11	12	13	14
H_2O	34.5	41.5	48.5	55	62	69	76	83	90	97
10x SB	5	6	7	8	9	10	11	12	13	14
3.75mM dNTP	5	6	7	8	9	10	11	12	13	14
15u/ul AMV-USB	0.5	0.5	0.5	1	1	1	1	1	1	1
Total volume	45ul	54ul	63ul	72ul	81ul	90ul	99ul	108ul	117ul	126ul

- 9ul Reaction buffer + 1ul sample
- 37°C 15min
- Add 10ul 2x SLB
- Heat at 90°C for 2min
- Load 5ul/lane onto 10% sequencing dPAGE gel (0.4mm thick)
- pre-run >30min, Run gel in TBE buffer at 50W for 2h 15min.
- Dry gel (store gel at –80C o/n if you can not dry gel immediately)
- Expose on phosphor screen at higher resolution (50microns).



Free mRNA

Ribosome complex (>4k)

Dye-xylene cyanole

Free primer

Dye-BPB