# Ribosome Filter Binding Assay (Xinying Shi)

- **tRNA Dephosphorylation:**

| | | |
|---|---|---|
| (100pmol/ul) tRNA | 25ul | (2500pmol) |
| 10x buffer 3(NEB) | 50ul | |
| (1u/ul) CIP AK Phosphatase (Amersham) | 25ul | (1u/100pmol) |
| water | 400ul | |
| | 500ul | x2 |

1. 37C 60min,
2. Add 50ul 3M NaOAc,pH 5.2
3. 1x phenol extraction
4. 2x chloroform extraction
5. 2.5x EtOH precipitate
6. 4C 30min,
7. 16000g 30min
8. Resuspend in 40ul water

- **tRNA Labeling**

| | |
|---|---|
| (100pmol/ul) Dephosphorylated $tRNA^{Phe}$ | 5.0ul |
| $^{32}$P-γ-ATP | 5.0ul |
| 10 x PNK | 2.5ul |
| (10u/ul) PNK(NEB) | 2.0ul |
| water | 10.5ul |
| | 25.0ul |

1. 37C 30min,
2. Add 25ul 2x sequence loading buffer
3. Hear at 90C for 2min,then place on ice for 10min
4. Purify on 10% dPAGE gel, 30W 2h 30min
5. Exposure on film for 5min and develop for 2min (place four fluorescence marker on each conner of the gel)
6. Put the film on the gel and cut out the band
7. Add 300ul RNA elution buffer and shake O/N
8. Transfer buffer to a new tube and wash gel with 300ul elution buffer.
9. 2x chloroform extraction (something white in the interface)
10. Add 2x 750ul EtOH, -20C o/n or 1h
11. 16000g, 30min
12. Resuspend in 30ul water.
13. Add cold $tRNA^{Phe}$
14. Measure $A_{260}$ of hot&cold tRNA
15. Measure radioactive intensity by scintillation counting.
16. Adjust to 1pmol= 1000cpm by adding cold tRNA.

Note: Store labeled tRNA at –20C for at most 1 week! Use fresh ATP!

**Buffer: $C_{80}M_{10}N_{150}$ or $C_{80}M_{20}N_{150}$**

| | Stock solution | 1x | 5x |
|---|---|---|---|
| 80mM K-cacodylate | 1M,pH7.5 | 4.0ml | 400ul |
| 10mM $MgCl_2$ | 1M | 0.5ml | 50ul |
| 150mM $NH_4Cl$ | 5M | 1.5ml | 150ul |
| water | | 44.0ml | 400ul |
| | | 50.0ml | 1000ul |

- Prepare labeled tRNA.
- Prepare 50ml 1x CMN buffer and store at 4C o/n
- Clean and set up filtration apparatus(Millipore), adjust flow rate (drip, not in a line)
- Refold tRNA in 10mM $MgCl_2$ at 60C for 3min,then slowly cool down to RT and stay at RT for 5min, then add 5x CMN and dilute to 1x
- Soak nitrocellulose filter(2.5cm,0.45uM, HWAP, Millipore, cat# ) in 1x CMN for at least 1h at 4C

Note: Don't use buffer containing polyamine. It will increase the background.

**Reaction condition for P site binding:**

| | | | |
|---|---|---|---|
| 30s or 70s | 10pmol | 10ul | reactive at 42C,10min---37C---37C,10min |
| mRNA | 20pmol | 5ul | |
| $tRNA^{Phe}$ | 40pmol | 10ul | refolded |
| | total volume =25ul | | |

Do duplicate for each reaction.
Need negative control (no ribosome)

1. Refold tRNA at 60C for 3min, slowly cool to RT, and stay for 5min.
2. Reactive at 42C 10min, slowly cool to 37C, stay at 37C for10min.
3. Associate ribosome, tRNA and mRNA at 37C, 30min.
4. Place on ice.
5. Wash nitrocellulose filter with 1ml cold CMN buffer.
6. Dilute 25ul sample to 1ml with 1x CMN and add to the filter immediately.
7. Wash filter with 2ml buffer.
8. Place filter in order and dry under the heating lamp for 3 min.
9. For counts of each pmol, add 2ul of $tRNA^{Phe}$ to Whatman filter circle (2.3mm, grade 3)
10. Place filter into a marked scintillation vial.
11. Add 4ml scintillation solution (Fisher).
12. Determine radioactivity by scintillation counting.
13. Calculate the binding efficiency:

$$\frac{\text{counting - background}}{\text{counts per pmol}}$$

Note: About 10% background.