^{35}S IN SITU PROTOCOL

A. PREPARATION OF TEMPLATE

1. Digest 10 μg DNA with an enzyme to linearize
2. Extract with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1)
   then with an equal volume of chloroform/isoamyl alcohol
3. EtOH precipitate (1/10 vol. 3M NaAc pH 4.8, 2.5 vol. 100% EtOH)
4. Spin 14K for 10 min, wash with 75% EtOH, aspirate and air dry
5. Resuspend in 20 μL (0.5 μg/μL)
6. Run 1 μL on 0.7% agarose gel

B. IN VITRO TRANSCRIPTION

Transcription Reaction	Full Reaction	Half Reaction
ddH_20 (DEPC)	1 μL	7 μL
5X Transcription Buffer	5 μL	5 μL
DNA Template (1 μg)	4 μL	2 μL
100mM DTT	1 μL	1 μL
10mM rATP	1 μL	1 μL
10mM rCTP	1 μL	1 μL
10mM rGTP	1 μL	1 μL
RNase Block (40 units/μL)	1 μL	1 μL
^{35}S rUTP	8 μL	4 μL
RNA Polymerase (T3,T7 or SP6)	2 μL	2 μL
	25 μL	25 μL

CAUTION: Everything from this point forward is *radioactive*, please dispose of the waste properly!

1. Set up transcription reaction
   Incubate at 37˚C for 1 hour
2. Add 0.5 μL RNA Polymerase
   Incubate at 37˚C for 30 min
3. Add 1 μL RNase-free DNase I (10 units/μL)
   Incubate at 37˚C for 10 min
   (You can also bring volume up to 100 μl by adding 75 μL, skip to C2
   and double all volumes in steps C2-4)
4. Extract with phenol/chloroform
5. EtOH precipitate (1/10 vol. 3M NaAc pH 4.8, 2.5 vol. 100% EtOH)
6. Spin 14K for 10 min, wash with 75% EtOH, aspirate and air dry
7. Resuspend in 50 μL 10mM DTT

C. HYDROLYSIS OF RIBOPROBE

1. Hydrolyze riboprobe to ~ 300 bp to improve penetration

$$T\ (\text{min}) = \frac{L_o - L_f}{k \cdot L_o \cdot L_f}$$

L_o = Length of original probe in kb
L_f = Length of desired length in kb
k = 0.11 cuts/kb/min

For example, L_o =2.8 kb, L_f = 0.3 kb

$$T\ (\text{min}) = \frac{2.8 - 0.3}{(0.11)(2.8)(0.3)} = 25\ \text{min}$$

Length	Time	Length	Time	Length	Time	Length	Time	Length	Time
0.4 kb	7 min	1.0 kb	21 min	1.6 kb	24 min	2.2 kb	26 min	2.8 kb	27 min
0.5 kb	12 min	1.1 kb	22 min	1.7 kb	25 min	2.3 kb	26 min	2.9 kb	27 min
0.6 kb	15 min	1.2 kb	22 min	1.8 kb	25 min	2.4 kb	26 min	3.0 kb	27 min
0.7 kb	17 min	1.3 kb	23 min	1.9 kb	25 min	2.5 kb	26 min	3.1 kb	27 min
0.8 kb	19 min	1.4 kb	23 min	2.0 kb	25 min	2.6 kb	26 min	3.2 kb	27 min
0.9 kb	20 min	1.5 kb	24 min	2.1 kb	25 min	2.7 kb	27 min	3.3 kb	27 min

2. Add 50 μL 2X Carbonate Buffer (25 μL 1M Na_2C0_3 and 25 μL 1M $NaHC0_3$, pH 10)
   Incubate at 65˚C for calculated time
3. Add 10 μL 10% acetic acid
4. Add 100 μL ddH_20 (DEPC)
   20 μL 3M NaAc
   400 μL 100% EtOH
   Mix, incubate at -20˚C for 30 min
5. Wash with 70% EtOH
6. Aspirate, air dry
7. Resuspend in 50 μL 10mM DTT
8. Count 1 μL (activity should be ~$1x10^6$ cpm/μL)

D. PREHYBRIDIZATION

FIXATION

1. Pick out sections and lay them flat to dry (20 min to 3 days)
2. Fix in 4% PFA in 1X PBS for 20 min at RT

200 mL	10% PFA	80 mL
50 mL	10X PBS	20 mL
250 mL	ddH_20 (DEPC)	100 mL

3. Wash in 3X PBS for 4 min at RT

150 mL	10X PBS	60 mL
350 mL	ddH_20 (DEPC)	140 mL

4. Wash in 1X PBS for 4 min at RT

50 mL	10X PBS	20 mL
450 mL	ddH_20 (DEPC)	180 mL

5. Wash in 1X PBS for 4 min at RT
6. Rinse in ddH_20 (DEPC) for 15 sec at RT
7. Air dry briefly

PROTEINASE K DIGESTION

1. Incubate slides with proteinase K (1μg/mL) for 20 min at RT in

50 mL	1M Tris-HCL pH 8	20 mL
50 mL	0.5M EDTA pH 8	20 mL
400 mL	ddH$_2$0 (DEPC)	160 mL
25 μL	Proteinase K	10 μL

2. Rinse in 1X PBS for 15 sec at RT

POSTFIXATION

1. Fix in 4% PFA in 1X PBS for 5 min at RT
2. Repeat steps 3 to 7

ACETILATION

1. Add slides while stirring 0.1M Triethanolamine (TEA)

6.5 mL	7.5M TEA	2.6 mL
500 mL	ddH$_2$0 (DEPC)	200 mL

2. Add acetic anhydride dropwise while stirring

1250 μL	Acetic Anhydride	500 μL

Incubate for 10 min while stirring

3. Rinse in ddH$_2$0 (DEPC) for 15 sec at RT
4. Rinse in ddH$_2$0 (DEPC) for 15 sec at RT
5. Dehydrate in 50% EtOH for 2 min at RT

250 mL	100% EtOH	100 mL
250 mL	ddH$_2$0 (DEPC)	100 mL

6. Dehydrate in 80% EtOH for 2 min at RT

400 mL	100% EtOH	160 mL
100 mL	ddH$_2$0 (DEPC)	40 mL

7. Dehydrate in 95% EtOH for 2 min at RT

475 mL	100% EtOH	190 mL
25 mL	ddH$_2$0 (DEPC)	10 mL

8. Air dry slides

E.HYBRIDIZATION

1. Place hybridization chamber into hybridization oven at 55˚C to prewarm trays
2. Cut parafilm into appropriate sizes for use as coverslips (cut off side edges)
3. Warm hybridization buffer up to 55˚C

	Final Concentration	Volume
Formamide	50%	50 mL
Dextran Sulfate (50%)	10%	20 mL
50X Denhardt's	2X	4 mL
20X SSC	5X	25 mL
ß-Mercaptoethanol	10mM	73 μL
Yeast tRNA (25 mg/mL)	250 μg/mL	1 mL
Salmon Sperm DNA (10 mg/mL)	500 μg/mL	5 mL
		100 mL

4. Heat ~$2x10^6$ cpm of labeled riboprobe at 65˚C for 5 min, place immediately on ice
5. Mix probes with hybridization mix at 55˚C (approximately 100 μL per slide)
6. Place hybridization mix on parafilm cover slip and ensure that sections are evenly coated by the hybridization mix (and make sure it does not touch the edge of the slide)
7. Place slides into humidified hybridization chamber (filled with ddH_20 in bottom)
8. Wrap hybridization chamber with Saran Wrap and seal with tape
9. Incubate at 55˚C overnight

F. POSTHYBRIDIZATION WASH

WARM ALL SOLUTIONS TO TEMPERATURE BEFORE PROCEEDING

1. Remove parafilm coverslip by dipping slides into 2X SSC, 10mM ß-ME at 55˚C
   25 mL 20X SSC
   183 μLß-ME
   225 mL ddH_20
2. Wash in 2X SSC, 10mM ß-ME for 30 min at 37˚C
3. Wash in 50% Formamide, 2X SSC, 1mM EDTA, 10mM ß-ME for 30 min at 55˚C
   125 mL Formamide
   25 mL 20X SSC
   500 μL0.5M EDTA pH 8.0
   183 μLß-ME
   100 mL ddH_20
4. Wash in 2X SSC for 30 min at 37˚C
5. RNase treat slides in 2X SSC, 1mM EDTA, 20 μg/mL RNase A, 1 U/mL RNase T1 for 1 hour at RT
   25 mL 20X SSC
   500 μL0.5M EDTA pH 8.0
   250 μLRNase A (20 mg/mL)
   1 μLRNase T1 (254 U/μL)
   225 mL ddH_20
6. Wash in 50% Formamide, 2X SSC, 1mM EDTA, 10mM ß-ME for 30 min at 55˚C
7. Wash in 50% Formamide, 2X SSC, 1mM EDTA, 10mM ß-ME for 30 min at 55˚C
8. Wash in 0.2X SSC for 5 min at RT
   2.5 mL 20X SSC
   248 mL ddH_20
9. Dehydrate in 50% EtOH for 2 min at RT
10. Dehydrate in 80% EtOH for 2 min at RT
11. Dehydrate in 95% EtOH for 2 min at RT
12. Air dry
13. Expose slides to x-ray film overnight

(DO NOT LET CASSETTE FELT TOUCH SLIDES AT ANY TIME!)

G. AUTORADIOGRAPHY

MAKE SURE TO PLACE SIGN ON DARKROOM ALLOWING NO ONE TO ENTER

1. IN DARKROOM ON DAY OF USE, remove ~10-15 mL of NTB2 emulsion with plastic spoon, place into a 50 mL Falcon tube and incubate at 40˚C for 30 min

2. Dilute 1:1 with prewarmed 1% glycerol in ddH_20 and incubate at 40˚C until emulsion is completely dissolved.
3. Check that emulsion mix is bubble-free by dipping several clean test slides and examine them for an even coating.
4. Dip slides one by one, wiping the back and sides of each, and place them horizontally in a box which can be covered.
5. Let slides dry for 2 hours.
6. Place slides into small black boxes with dessicant, seal them with tape, then aluminum foil and further tape in a ziplock bag with more dessicants at 4˚C for usually no longer than 2 weeks

H. DEVELOPING

1. Remove slide box from fridge and allow to warm to RT (> 2 hours)
2. Prepare

    Developing Solution: Heat 500mL ddH_2O to not more than 52˚C and add 78.28 g of developer (D-19 Developer Kodak #146 4593) Stir until dissolved and chill to 16˚C in ice water.

    Fixer Solution: Add 89.47g of fixer (Fixer Kodak #197 1746) to 500mL ddH_20 while stirring until dissolved and chill to 16˚C in ice water.
3. In the darkroom, put slides into staining racks and develop in Developer diluted 1:1 with ddH_20 for 5 min
4. Transfer to ddH_20 for 30 sec with minimal agitation.
5. Put slides into Fixer for 8 min
6. Rinse slides gently in running tap water for 20 min
7. Scrap backs of slides with razor blade to remove excess emulsion.
8. Stain slides with 0.05% Toludine Blue containing 10mM NaAc pH 4.7 for 1 min
9. Rinse briefly in ddH_20
10. Dehydrate 2 min each in graded ethanols (50%, 75%, 85%, 95%, 100%, 100%)
11. Dehydrate 3 X 2 min in Xylene
12. Mount using Permount