RIBONUCLEASE PROTECTION ASSAY

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Background:

The RNase protection assay is a highly-sensitive and specific method for the quantitation of mRNA species. The assay was made possible by the discovery, isolation, and characterization of DNA-dependent RNA polymerases from the *S. typhimurium* bacteriophage SP6 and the *E. coli* bacteriophages T7 and T3, and the elucidation of their cognate promoter sequences. The following properties of the bacteriophage RNA polymerases make them well-suited for the synthesis of high-specific-activity RNA probes: i) they exist as single subunit enzymes and are stable and relatively easy to purify; ii) they exhibit a high degree of fidelity for their cognate promoters, and the promoter sequences are long and are thus unlikely to appear fortuitously; and iii) they polymerize RNA at a very high rate (200-300 nt/min), efficiently transcribe long segments, and do not require high concentrations of rNTPs. Several plasmid vectors are available that contain two different bacteriophage promoters arranged in opposite orientation and flanking the polycloning site. Thus, a DNA fragment of interest can be subcloned into the vector and used as a template for anti-sense or sense RNA synthesis depending on promoter usage. The strategy for development of a multi-probe RNase protection assay is to generate a series of such subclones representing sequences in mature mRNA species of interest. The subclones must be of distinct length (≥15 nt apart) and should be oriented to allow anti-sense RNA synthesis from the same promoter. To generate templates, the plasmids are linearized with an appropriate restriction enzyme (ie, at a unique site downstream from the promoter and insert), are purified, and are combined in a set. The templates are used for polymerase-directed synthesis of high-specific-activity, [^{32}P]-labeled anti-sense RNA. The probes are purified and hybridized (at probe excess) to target RNA, after which free probe and other ssRNA are digested with RNases. The remaining "RNase-protected" probes are purified, are resolved on denaturing polyacrylamide gels, and are quantified by autoradiography or phosphorimaging. The quantity of each mRNA species in the original RNA sample can then be determined based on the intensity of the appropriately-sized probe.

References:

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Reagents:

5'-[α-^{32}P]UTP (3000 Ci/mmol, 10 mCi/ml)
UTP (10 μM)
GTP, ATP, CTP pool (2.5 mM each)
RNase-free dH_2O
DTT (100 mM)
Transcription buffer (5X): 30 mM $MgCl_2$, 50 mM NaCl, 10 mM spermidine, 200 mM Tris, pH 7.9
RNAsin (40 U/μl)
Linearized DNA templates (50-100 μg/ml)
T7, T3, and SP6 polymerases (20 U/μl)
RQ1-DNase (1 U/μl)
EDTA (20 mM and 0.5 M, pH 8.0)
Tris-saturated phenol
Chloroform:isoamyl alcohol (50:1)
Yeast tRNA (2 mg/ml)
Ammonium acetate (4 M)
Ethanol (100% and 90%)
Hybridization buffer (1X): 80% formamide, 1 mM EDTA, 400 mM NaCl, 40 mM PIPES, pH 6.7
Mineral oil
Tris (1 M, pH 7.5)
NaCl (5 M)
RNase A (100 μg/ml)
RNase T1 (1500 U/μl)
SDS (10%)
Proteinase K (10 mg/ml)
Loading buffer (1X): 80% formamide, 1 mM EDTA, 50 mM Tris-borate, pH 8.3, 0.05% (w/v) xylene cyanole and bromophenol blue
TBE (10X)
Acrylamide solution: 5% acrylamide (5% C), 8 M urea, 1X TBE
Ammonium persulfate (10%)
TEMED
Rain-X

Probe Synthesis:

1. Thaw the nucleotide preparations at room temp. For each probe, add the following to a 1.5 ml Eppendorf tube:
   7.3 μl UTP (10 μM)
   1.1 μl GTP, ATP, CTP pool (2.5 mM each)
   12 μl [α-^{32}P]UTP (3000 Ci/mmol, 10 mCi/ml)
   Cover with a temporary, perforated cap and freeze for 10 m, -70°C. Lyophilize in a speed vac for 45 m (no heat).

2. To the dried nucleotides, add the following reagents (all brought to room temp):
   5.2 μl dH_2O
   1 μl DTT (100 mM)
   2 μl transcription buffer (5X)
   Mix by vortexing (~15 s) and quick μfuge.

3. Add the following:
   0.3 μl RNasin (40 U/μl)
   1 μl linearized DNA templates (50-100 μg/ml)
   Mix by gentle flicking and quick μfuge.

4. Initiate the reaction by adding 0.5 μl of the appropriate bacteriophage polymerase (T7, T3, or SP6 polymerase, ~20 U/μl). Mix by gentle flicking and quick μfuge. Incubate for 1 h, 37°C, in a heat block.

5. Terminate the reaction by adding 2 μl of RQ1-DNase (~1 U/μl). Mix by gentle flicking and quick μfuge. Incubate for 30 m, 37°C.

6. Add in order:
   36 µl EDTA (20 mM, pH 8.0)
   25 µl Tris-saturated phenol
   25 µl chloroform:isoamyl alcohol (50:1)
   2 µl yeast tRNA (2 mg/ml)
   Mix by vortexing and µfuge 5 m, room temp.

7. Transfer the upper aqueous phase to a new Eppendorf tube and add 50 µl chloroform:isoamyl alcohol (50:1). Mix by vortexing and µfuge 5 m, room temp.

8. Transfer the upper aqueous phase to a new Eppendorf tube and add 50 µl 4 M ammonium acetate and 250 µl ice cold 100% ethanol. Invert several times and incubate for 15 m, -70°C. µfuge for 15 m, 4°C.

9. Carefully remove the supernatant and add 100 µl of ice cold 90% ethanol to the pellet. µfuge for 5 m, 4°C.

10. Carefully remove all of the supernatant and add 50 µl of hybridization buffer to the pellet. Solubilize by gently vortexing for 20 s and quick µfuge.

11. Quantitate duplicate 1 µl samples in the scintillation counter (open channel [9]). Expect a maximum yield of ~3 x 10^6 Cherenkov counts/µl, with an acceptable lower limit of ~2.5 x 10^5 counts/µl. Store the probe at -20°C; use for two sequential assays at most.

Hybridizations:

12. Add the desired amount of target RNA to Eppendorf tubes. Bring the total amount of RNA in all tubes to weight equivalence (15 µg maximum) by addition of yeast tRNA (alternatively, samples can be set up at equivalent input cell numbers). Include a degradation control (tRNA-only) and a positive hybridization control (~0.1 fmol sense RNA + tRNA).

13. Cover the samples with temporary, perforated caps and freeze for 15 m, -70°C. Dry completely (~1 h) in the speed vac (no heat).

14. Add 8 µl of hybridization buffer to each sample. Solubilize RNA by gently vortexing (Vortex-Genie-2) for 3-4 m and quick µfuge.

15. Dilute the probe in hybridization buffer to the desired concentration (generally 500 cpm/µl for each uridine residue in the probe set). Add 2 µl to each RNA sample; quick vortex and quick µfuge. Add a drop of mineral oil and µfuge 10 s.

16. Place samples in a heat block pre-warmed to 90°C. Immediately turn the temp to 56°C and incubate for 12-16 h (overnight).

RNase Treatments:

17. Turn the heat block to 37°C and prepare the RNase cocktail (per 20 samples):
    2.3 ml dH_2O
    25 µl Tris (1 M, pH 7.5)
    150 µl NaCl (5 M)
    25 µl EDTA (0.5 M, pH 8.0)
    5 µl RNase A (100 µg/ml)
    1 µl RNase T_1 (~1500 U/µl)
    Remove the RNA samples from the heat block and jet 100 µl of the RNase cocktail underneath the oil. µfuge 10 s and incubate for 45 m, 30°C, in a water bath.

18. Before the RNase digestion is completed, prepare the proteinase K cocktail (per 20 samples):
    235 µl dH_2O
    155 µl SDS (10%)
    30 µl proteinase K (10 mg/ml)
    30 µl yeast tRNA (2 mg/ml)
    Mix and add 18 µl aliquots of the cocktail to new Eppendorf tubes.

19. Using a thin pipet tip, extract the RNase digests from underneath the oil (avoid the oil) and transfer to the proteinase K solution. Quick vortex, quick µfuge, and incubate for 15 m, 37°C.

20. Add 65 µl Tris-saturated phenol, 65 µl chloroform:isoamyl alcohol (50:1). Vortex vigorously and µfuge 5 m, room temp.

21. Carefully extract the upper aqueous phase (set the Pipetman at 120 µl and totally avoid the organic interface) and transfer to a new tube. Add 120 µl 4 M ammonium acetate and 650 µl ice cold 100% ethanol. Invert several times and incubate 15 m, -70°C. µfuge 15 m, 4°C.

22. Carefully remove the supernatant and add 100 µl ice cold 90% ethanol. µfuge 5 m, 4°C.

23. Carefully remove the supernatant and air dry the pellet completely. Add 5 µl of loading buffer, vortex (Vortex-Genie-2) for 3-4 m, and quick µfuge. Prior to loading on the gel, heat the samples for 3 m, 90°C, and place immediately in an ice bath.

Gel Resolution of Protected Probes

24. Clean the gel plates with dH_2O and wipe dry. Silanize the short plate by adding Rain-X, buffing dry, and cleaning again with dH_2O. Clean both plates with ethanol/razor blade, wipe dry, and assemble, tape, and clamp the gel mold (≥40 cm length, 0.4 mm spacers).

25. Combine the following:
    74.5 ml acrylamide solution (5% acrylamide [5% C]/8 M urea/1X TBE)
    450 µl ammonium persulfate (10%)
    60 µl TEMED
    Pour into the gel mold, add an appropriate comb (5 mm well width), and clamp.

26. After polymerization (≥1 h), remove the comb and flush the wells with 0.5X TBE. Place the gel in a vertical rig (with an aluminum heat disperser) and prerun at 40 watts constant, ≥45 m, with 0.5X TBE as the running buffer.

27. Flush the wells with 0.5X TBE and add the samples. Run the gel at 50 watts constant until the leading edge of the BPB (front dye) reaches 30 cm.

28. Disassemble the gel mold, remove the short plate, and adsorb the gel to filter paper. Cover the gel with saran wrap and layer between two additional pieces of filter paper. Place in the gel dryer in vacuo for ~1 h, 80°C. Place the dried gel on film in a cassette with intensifying screens, and develop at -70°C.

Radioactive Waste Disposal

Per single batch probe synthesis:

60 μCi in dry waste, decay group I (tips, tubes, gel, gloves, etc)
60 μCi in ethanol waste, decay group I (all ethanol)

Reagent Preparation:

All purchased reagents should be Molecular Biology Grade, and all solutions should be prepared and handled such that nuclease contamination is minimized. Gloves should be warn at all times. All glassware and spatulas should be baked (300°F, 4 h). Commercial, sterilized plasticware is generally RNase-free and should be used whenever possible. Bottle tops and stir bars should be cleaned thoroughly, rinsed with chloroform, and autoclaved. All tips and tubes should come from dedicated packages and should be autoclaved.

5'-[α-^{32}P]UTP, triethylammonium salt (3000 Ci/mmol, 10 mCi/ml, 3.33 x 10^{-6} mmol/ml)
Amersham #PB.10203 ($43.00/25 µl aliquot)
Fresh label is ~1/3 hot UTP and ~2/3 cold UTP. Store at -20°C and use within 2 wk (avoid repeated freeze-thaw).

Riboprobe Gemini System II Buffers
Promega #P1121 ($56.00/kit)
10 mM ATP, pH 7 (50 µl)
10 mM CTP, pH 7 (50 µl)
10 mM GTP, pH 7 (50 µl)
10 mM UTP, pH 7 (50 µl)
100 mM DTT (100 µl)
Nuclease-free dH_2O (1.25 ml)
5X Transcription Buffer (200 µl): 200 mM Tris-HCl, pH 7.9, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl
Store all main stocks at -70°C. Maintain small aliquots at -20°C for frequent use. All reagents are stable for ≤1 yr (outdated DTT is a common culprit in failed transcriptions).

HPLC-grade dH_2O (for all uses other than transcription reactions)
Baker #4218-03 ($39.00/4 L). Handle aseptically and store aliquots at rt.

10 µM UTP
Combine 1 µl 10 mM UTP and 999 µl dH_2O. Store 100 µl aliquots at -20°C.

GTP, ATP, CTP pool (2.5 mM each)
Combine 10 µl each of 10 mM GTP, 10 mM ATP, 10 mM CTP, and dH_2O. Store 10 µl aliquots at -20°C.

Recombinant RNasin (40 U/µl)
Promega #N2511 ($75.00/2,500 U). 1 U inhibits 50% of the activity of 5 ng RNase A under SC. RNasin inhibits RNases A, B, and C, but not RNases 1, T1, and H. Maintain at -20°C in the Stratacooler.

RQ1 RNase-free DNase (1 U/µl)
Promega #M6101 ($49.00/1000 U). 1 U degrades 1 µg DNA in 10 m, 37°C, under SC. Store main stock at -70°C. Maintain an aliquot in the -20°C Stratacooler for frequent use.

Bacteriophage RNA polymerases (20 U/µl)
T7: Promega #P2075 ($30.00/1000 U)
T3: Promega #P2083 ($26.00/1000 U)
SP6: Promega #P1085 ($120.00/1000 U)
1U catalyzes incorporation of 1 nmol rNTP in 60 m, 37°C, under SC. Store main stocks at -70°C. Maintain small aliquots in the -20°C Stratacooler for frequent use.

DNA templates for anti-sense RNA synthesis:

DNA fragments corresponding to regions of mature mRNA species are subcloned into a suitable transcription vector (eg, the pGEM series from Promega). Insert length should range from 100-350 bp and each should differ in length (≥15 bp) from all other subclones to be used in the template set. When choosing regions for subcloning, areas with a high probability of secondary structure or with runs of A residues should be avoided (these can result in probe nicking during RNase treatments). If desired, the subclones can span intron-intron boundaries to help ensure detection of mature mRNA. For linearization of individual plasmids, choose a restriction enzyme that does not cut within the insert and that does not leave a 3' overhang (increases the chance of sense RNA synthesis from the opposite strand). Gel purification of linearized templates generally yields the best results. After assembling the template set, check whether the transcription rates are balanced (if not, adjust each template concentration accordingly). Store the template stocks (50-100 µg/ml) at -20°C.

Ethylenediaminetetraacetic acid, disodium, dihydrate ($Na_2EDTA{\cdot}2H_2O$; FW 372.2)
Sigma #E 5134 ($47.70/500 gm). Store at rt.

Sodium Hydroxide, pellets (NaOH; FW 40.0)
FisherBiotech #BP 359-500 ($11.15/500 gm). Store dessicated at rt.

10 M NaOH
Dissolve 40 gm NaOH (FW 40.0) in 60 ml dH_2O. Bring to 100 ml with dH_2O. Store in polyprop tubes at rt..

0.5 M EDTA, pH 8.0
Dissolve 93 gm $Na_2EDTA{\cdot}2H_2O$ (FW 372.2) and 10 gm NaOH (FW 40.0) in 350 ml dH_2O. Adjust pH to 8.0 with 10 N NaOH, and bring to 500 ml with dH_2O. 0.2 µ filter and store at rt.

0.5 M EDTA, pH 8.0 *(commercially prepared)*
Sigma #E-7889 ($16.25/100 ml). Store at rt.

Trizma Base (FW 121.1)
Sigma #T 8524 ($31.40/500 gm). Store dessicated at rt.

Trizma Hydrochloride (FW 157.6)
Sigma #T 7149 ($64.00/500 gm). Store dessicated at rt.

1 M Tris, pH 7.5 (at 37°C)
Dissolve 19.7 gm Trizma base (FW 121.1) and 53.2 gm Trizma HCl (FW 157.6) in 350 ml dH_2O. Check the pH (7.8 at rt), and bring to 500 ml with dH_2O. 0.2 µ filter and store at 4°C.

1 M Tris solutions *(commercially prepared)*
pH 7.2 (at 25°C): GIBCO BRL #5566 UA ($17.00/1 L)
pH 7.5 (at 25°C): GIBCO BRL #5566 UA ($17.00/1 L)
pH 8.0 (at 25°C): GIBCO BRL #5566 UA ($17.00/1 L)

Phenol, redistilled, crystalline (FW 94.11)
GIBCO BRL #5509 UA ($32.00/100 gm). Store at -20°C in the dark.

H_2O-saturated phenol

Loosen the cap on a 100 gm bottle of phenol and bring to rt. Melt the phenol crystals in a 65°C water bath. Add dH_2O to the bottle top and shake to mix the phases to a fine emulsion. Let the phases separate overnight at 4°C. Aspirate off the upper aqueous phase and replace with enough dH_2O to cover the phenol. Store at 4°C in the dark; the solution is stable for several mos.

Tris-saturated phenol

Add an equal volume of 0.5 M Tris, pH 8.0, to an aliquot of H_2O-saturated phenol. Mix well and allow the phases to separate at room temp. Aspirate off the upper aqueous phase and add an equal volume of 0.1 M Tris, pH 8.0. Mix well and allow the phases to separate at room temp. Aspirate off the upper aqueous phase and add an equal volume of 10 mM Tris, 1 mM EDTA, pH 8.0. Store at 4°C in the dark; the solution is stable for ~1 mo.

Tris-saturated phenol *(commercially prepared)*

GIBCO BRL #5513 UA ($50.00/100 ml). Store at 4°C in the dark; after opening, the solution is stable for ~1 mo.

Chloroform, nanograde ($CHCl_3$; FW 119.4)

Mallinckrodt #2175 ($30.20/L). Store at rt.

Isoamyl acohol, 99% purity (FW 88.2)

Sigma #I-1885 ($8.00/100 ml). Store at rt.

Chloroform:isoamyl alcohol (50:1)

Combine 98 ml chloroform and 2 ml isoamyl alcohol. Store at rt.

Yeast tRNA, nuclease-free, lyophilized

GIBCO BRL #5401 SA ($35.00/25 mg)

Add 5 ml cold dH_2O and allow to solubilize for 30 m on ice. Dilute to 2 mg/ml with dH_2O, aliquot, and store at -20°C.

Ammonium acetate, anhydrous (FW 77.1)

Sigma #A-1542 ($21.25/500 gm). Store dessicated at 4°C.

4 M ammonium acetate

Dissolve 77.1 gm ammonium acetate (FW 77.1) in 150 ml dH_2O. Bring to 250 ml with dH_2O. 0.2 μ filter and store at rt.

Ethanol, punctilious, dehydrated (200 proof)

Quantum Chemical Corp. (interdepartmental requisition, per pint). Store as 100% and 90% stocks at -20°C.

Formamide, redistilled (FW 45.0)

GIBCO BRL #5515 UA ($14.00/100 gm). Store at -20°C.

Deionized formamide

Thaw the formamide and combine 2.5 ml with 2.5 gm of mixed bed resin (AG 501-X8, 20-50 mesh; Bio-Rad #143-6424, $85.00/100 gm) in a 50 ml polyprop tube. Swirl, quick spin at low rpm, and discard the formamide. Top off the tube with new formamide, cap and seal with parafilm, and rotate for 1 h. Quick spin and recover the formamide. Use fresh or store for short periods at -20°C in the dark.

Sodium chloride, crystalline (NaCl, FW 58.44)
Sigma #S-3014 ($19.60/500 gm). Store at rt.

5 M NaCl
Dissolve 146.1 gm NaCl (FW 58.44) in 350 ml dH_2O. Bring to 500 ml with dH_2O. 0.2 µ filter and store at rt.

5 M NaCl *(commercially prepared)*
Sigma #S-5150 ($21.50/L). Store at rt.

PIPES (FW 302.4)
Sigma #S-9291 ($52.95/100 gm). Store at rt.

0.5 M PIPES, pH 6.7
Dissolve 30.2 gm PIPES (FW 302.4) and ~50 pellets NaOH in 150 ml dH_2O. Adjust the pH to 6.7 with 10 M NaOH, and bring to 200 ml with dH_2O. 0.2 µ filter and store at 4°C. The solution is stable for ≤1 yr.

Hybridization buffer (1X):
80% formamide, 1 mM EDTA, 400 mM NaCl, 40 mM PIPES, pH 6.7
In a 50 ml polyprop tube, combine:
Deionized formamide (fresh): 16 ml
0.5 M EDTA, pH 8.0: 40 µl
5 M NaCl: 1.6 ml
0.5 M PIPES, pH 6.7: 1.6 ml
dH_2O: 0.76 ml
Mix and add aliquots to 2 ml, screw cap polyprop tubes. Store main stocks at -70°C, with an aliquot of working stock kept at -20°C.

Mineral oil (heavy white)
Sigma #400-5 ($9.75/100 ml). Store at rt.

RNase A, lyophilized, ultra-pure (100 Kunitz U/mg protein)
Sigma #R-5500 ($12.30/10 mg). Store dessicated at -20°C.
RNase A (MW 13,700 daltons), isolated from bovine pancreas, is an endoribonuclease that cleaves at the 3' end of U and C residues (Pyp↓N). The pH optimum is 7.0-7.5.
To prepare a 2 mg/ml stock solution, combine:
RNase A: 10 mg
1 M Tris, pH 7.5: 50 µl
5 M NaCl: 15 µl
dH_2O: 4.92 ml
Mix and place in a boiling water bath for 15 m (destroys contaminating nucleases). Cool slowly to room temp. and add aliquots to 2 ml, screw cap polyprop tubes. Store main stocks at -70°C. For the RNase protection assay stock (100 µg/ml), dilute 1:20 with 15 mM NaCl, 10 mM Tris, pH 7.5, and aliquot and store at -20°C.

RNase T1 (2000 U/µl in 10 mM phosphate, pH 6.7, 50% glycerol)
GIBCO BRL #8030 SA ($47.00/125,000 U)
RNase T1 (MW 11,000 daltons), isolated from *Aspergillus oryzae*, is an endoribonuclease that cleaves at the 3' end of G residues (Gp↓N). The pH optimum is 7.5. Store in -20°C Stratacooler.

Lauryl sulfate, sodium salt (SDS, FW 288.4)
Sigma #L-4390 ($30.20/100 gm). Store at rt.

10% SDS

Add 10 gm SDS (FW 288.4) to 80 ml dH_2O in a sterile flask. Heat to 68°C with gentle swirling. Add 2 drops of 1 M HCl and bring to 100 ml with dH_2O. 0.2 µ filter and store at rt.

10% SDS *(commercially prepared)*

Sigma #L-4522 ($21.95/100 ml). Store at rt.

Calcium chloride, dihydrate ($CaCl_2 \cdot 2H_2O$, FW 147.0)

Sigma #C-3306 ($6.00/100 gm). Store at rt.

Proteinase K, lyophilized, nuclease-free

Promega #V-3021 ($75.00/100 mg). Store dessicated at 4°C.

Proteinase K, isolated from *Tritirachium album*, hydrolyzes proteins (incuding nucleases) over a pH range of 4-12.5. To prepare a 10 mg/ml stock solution, combine 100 mg with 9.5 ml dH_2O, 0.5 ml 1 M Tris, pH 7.5, and 14.7 mg $CaCl_2.2H_2O$ (final: 50 mM Tris, 10 mM $CaCl_2$). Mix by inversion and add aliquots to 2 ml, screw cap polyprop tubes. Store main stocks at -70°C. Store small aliquots at -20°C for frequent use.

Boric acid, anhydrous (FW 61.8)

Sigma #B-6768 ($17.00/500 gm). Store at rt.

1 M Tris-borate, pH 8.3

Dissolve 60.6 gm Trizma-base (FW 121.1) and 30.9 gm boric acid (FW 61.8) in 375 ml dH_2O. Adjust the pH to 8.3 and bring to 500 ml with dH_2O. 0.2 µ filter and store at rt.

Gel electophoresis dyes

Xylene cyanole FF (XC, FW 538.6), Sigma #X-4126 ($18.50/10 gm), store at rt.

Bromophenol blue (BPB, FW 691.9), Sigma #B-5525 ($18.75/10 gm), store at rt.

Loading buffer (1X)

80% formamide, 1 mM EDTA, 50 mM Tris-borate, pH 8.3, 0.05% (w/v) XC and BPB

In a 50 ml polyprop tube, combine:

- Deionized formamide (fresh): 16 ml
- 1 M Tris-borate, pH 8.3: 1 ml
- 0.5 M EDTA, pH 8.0: 40 µl
- XC: 10 mg
- BPB: 10 mg
- dH_2O: 2.94 ml

Mix and add aliquots to 2 ml, screw cap polyprop tubes. Store main stocks at -70°C, with an aliquot of working stock kept at -20°C.

10X Tris-borate-EDTA (10X TBE)

0.89 M Tris, 0.89 M boric acid, 20 mM EDTA, pH 8.3

Dissolve 108 gm Trizma-base (FW 121.1) and 55 gm boric acid (FW 61.8) in 700 ml dH_2O. Add 40 ml 0.5 M EDTA, pH 8.0, check the pH (8.3), and bring to 1 L with dH_2O. 0.2 µ filter and store at rt.

10X TBE *(commercially prepared)*

Boehringer Mannheim #100-957 ($95.00/4 L), store at rt.

Acrylamide, 99% purity

Biorad #161-0101 ($82.00/500 gm), store at rt.

Bis

Biorad #161-0201 ($24.00/50 gm), store at rt.

40% acrylamide (5% Bis)

Dissolve 190 gm acrylamide and 10 gm Bis in 250 ml dH_2O (warm to ≤50°C if necessary). Bring the volume to 500 ml with dH_2O and 0.2 µ filter. Store covered at 4°C (stable for ≤4 mo).

40% acrylamide (5% Bis) mixture *(commercially prepared)*

Biorad #161-0123 ($46.00/150 gm), store at rt.
Add 237 ml dH_2O (375 ml final volume), mix, and 0.2 µ filter. Store covered at 4°C (stable for ≤4 mo).

Urea (FW 60.1)

Biorad #161-0731 ($47.00/1 kg), store at rt.

5% acrylamide (5% Bis), 8 M urea, 1X TBE

Dissolve 120 gm urea in 104 ml dH_2O and 25 ml 10X TBE (warm to ≤50°C if necessary). Add 31.3 ml 40% acrylamide (5% Bis) solution, mix, and 0.2 µ filter. Store covered at rt (make fresh every 3 d).

Ammonium persulfate, 98% purity (FW 228.2)

Biorad #161-0700 ($7.75/10 gm), store covered at 4°C.

0

10% ammonium persulfate

Dissolve 1 gm ammonium persulfate (FW 228.2) in a final volume of 10 ml dH_2O. Store covered at 4°C (make fresh weekly).

TEMED (FW 116.2)

Biorad #161-0801 ($28.50/50 ml), store covered at 4°C.

Acrylamide gel solution

Add in order:

5% acrylamide (5% Bis), 8 M urea, 1X TBE: 74.5 ml
10% ammonium persulfate: 450 µl
TEMED: 60 µl

Swirl and pour immediately into the gel mold. Polymerize for ≥1 h.

Rain-X

Unelko Corp. (~$4.00/100 ml). Store at rt.

Dimethyldichlorosilane, liquid (silane, FW 129.1) *(alternative to Rain-X)*

Sigma #D-3879 ($5.90/50 ml), store covered at 4°C.
Prepare a 2.5% stock (for silanizing gel plates) by combining 1.25 ml silane and 48.75 ml 100% ethanol. Store covered at rt.

40% Acrylamide Solution 19:1 (5%)
500 ml bottle BioRad 161-0144