# Northern blotting for small RNAs: assessing the steady-state levels of tRNAs and determining their degree of thiolation using APM ((N-acryloylamino)phenylmercuric chloride)

Before starting: For this type of analysis we perform Laemmli gel electrophoresis using the Biorad Mini-PROTEAN II Electrophoresis Cell. Wash plates, combs, spacers and gaskets with SDS / distilled water, then DEPC-water and then 70% ethanol prepared in DEPC water. Air dry.

Casting the gel: Cast a 10% polyacrylamide / 7M urea gel. We use the SequaGel Sequencing System (National Diagnostics). To assess thiolation of tRNAs, include APM in the gel mixture at a final concentration of ~1µg/ml (adjust concentration for each lot – there will be differences between batches in terms of purity, etc.). Add TEMED (RNase-free) and 10% APS (prepared in DEPC water) as recommended.

Sample preparation: Use 5-7µg total RNA from cells or ~1.5µg total RNA from tissues. Bring all samples to the same total volume with DEPC-water. Add TBE-urea sample buffer (BioRad), 1:1 (vol:vol). Denature for 5 min at 90$^{0}$C, then cool briefly on ice (~1 min).

Running the gel: Pre-run gel at 170V for ~15 min. After the pre-run and before loading the samples, wash wells vigorously by pipetting running buffer in each individual well. Run gel in 1X TBE, at 200V, for 60 – 75 min, or until the xylene cyanol dye is ~1cm from the bottom of the gel.

Transfer: If the gel contains APM, incubate it in 0.2M ß-mercaptoethanol /1X TBE for one hour to reduce and/or break the sulfur linkages formed between the thiolated tRNA and the APM in the gel. Transfer using OWL semi-dry system (or equivalent) in 1X TBE at constant amperage (0.8mA / cm$^{2}$ of Hybond-N$^{+}$ membrane) for ~75 min. If desired, the efficiency of the transfer can be verified by staining the gel post-transfer with ethidium bromide (or equivalent) diluted in 1X TBE. Air-dry membrane for 1 hr, then UV crosslink. At this point the membrane can be stored at 4$^{0}$C until ready to hybridize.

Hybridization: To probe, use a 24bp oligonucleotide primer complementary to the 3' end of the tRNA of interest. 5'-end label 10 pmoles of the primer using 2µL T4 polynucleotide kinase (NEB) with 15µCi ATP[$\gamma^{32}$P](6,000 Ci/mmol, 10µCi/µl) in 50µl total volume. Incubate at 37$^{0}$C for 30 min, then stop the reaction at 60$^{0}$C for 15 min. Purify probe on an Illustra Microspin G-25 columns or equivalent. Prepare the pre-hybridization and hybridization solutions in ExpressHyb (Clontech), as per manufacturer's instructions. Three rinses with 2X SSC, 0.05% SDS should suffice for washing of the membrane; if necessary, continue washing in the same solution for up to 30 min at RT. Check counts every 15 min! If supplementary washing is necessary, continue for up to 30 min in 0.1X SSC, 0.1% SDS. Expose overnight to phosphoimager cassette.