I, Mohamed M. Marei, hereby submit this original work as part of the requirements for the degree of:

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Student Signature: Mohamed M. Marei

This work and its defense approved by:

Committee Chair: Pearl Tsang
Albert Bobst
Patrick Limbach

Approval of the electronic document:
I have reviewed the Thesis/Dissertation in its final electronic format and certify that it is an accurate copy of the document reviewed and approved by the committee.

Committee Chair signature: Pearl Tsang
The use of pLysB19, a new plasmid, for in vitro transcription of milligram quantities of human lysyl tRNA and purification by urea denaturing PAGE

A thesis submitted to the
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of the University of Cincinnati
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by
Mohamed M. Marei
B.S. University of Cincinnati
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Committee Chair: Pearl Tsang, Ph.D.
Abstract

A method for production of milligrams of hKtRNA, using new template plasmid pLysB19, by in vitro transcription is presented and compared to methods in use elsewhere. The in vitro transcription yield was found to be almost exclusively dependent on Mg$^{2+}$ and template concentration. Currently, production of milligrams of hKtRNA is possible but only by use of higher template concentrations than reported elsewhere (7μg vs. 1μg per 10μL reaction). The typical yield of hKtRNA is ≥10μg per 10μl of transcription reaction mixture, of which approximately 6.5μg is recovered after gel purification. Up to 75% of the in vitro transcribed hKtRNA can be aminoacylated by human lysyl tRNA synthetase on acidic urea denaturing PAGE. The requirement of higher template concentrations than reported elsewhere to achieve similar yields is probably due to reduced T7RNAP activity; but the exact cause cannot be stated with absolute certainty due to multiple variations between the in vitro transcription reaction described here and reactions previously described elsewhere. However, recent results by others in this group show lower template concentrations used in the system described here can produce higher yields than shown here when different batches of T7RNAP are used. This is very supportive of conclusions here that reduced T7RNAP activity was the basis for the requirement of higher template concentrations than reported elsewhere.

Large-scale production of new template plasmid, pLysB19, is achieved by regeneration and re-use of silica membrane and ion exchange columns provided in commercial kits. Design and construction of pLysB19, done elsewhere, is also described. The transcribed hKtRNA is purified to ~95% on denaturing urea PAGE. The cause of the ~5% degradation products is explored through incubations of hKtRNA in high and low magnesium, as well as high and low pH buffers to determine whether these factors contribute to transcript degradation. The transcript degradation product pattern did not appear to be affected at Mg$^{2+}$ concentrations as high as 207mM, or during incubations in pH 6 gel elution buffer. These results suggest that degradation under the purification conditions (37°C for 12 hours at pH8) are not due to the reported instability of RNA in the presence of Mg$^{2+}$ at elevated temperature or pH.

A method is also adapted for full resolution of the aminoacylated hKtRNA band from non-aminoacylated hKtRNA on longer acidic urea denaturing PAGE than previously described. A method for comparing the activities of hKRS using a 3H lysine charging assay is also presented and preliminary results are shown. In summary, the results and methods described here may serve as a guide for future experiments which require preparation of milligrams of in vitro transcribed hKtRNA, or the resolution of aminoacylated hKtRNA from non-aminoacylated hKtRNA on acidic urea PAGE.
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<th>Definition</th>
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<tbody>
<tr>
<td>3’CCA</td>
<td>Refers to acceptor stem of tRNA</td>
</tr>
<tr>
<td>BMB</td>
<td>Bromophenol Blue</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
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<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>HIV1</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>hKRS</td>
<td>Human lysyl tRNA synthetase</td>
</tr>
<tr>
<td>hKtRNA</td>
<td>Refers to non post-transcriptionally modified <em>in vitro</em> transcript</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>MALDI-MS</td>
<td>Matrix assisted laser desorption ionization mass spectrometry</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>NTP</td>
<td>Ribonucleotide triphosphate</td>
</tr>
<tr>
<td>OSU</td>
<td>Ohio State University</td>
</tr>
<tr>
<td>OSU-hKRS</td>
<td>Human lysyl tRNA synthetase provided by OSU (22)</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol (8000g/mol)</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>SUU</td>
<td>Refers to tRNA_{3}^{Lys} anticodon</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetate EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris Borate EDTA buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’,N’-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>T7RNAP</td>
<td>Bacteriophage T7 RNA polymerase</td>
</tr>
<tr>
<td>XCFF</td>
<td>Xylene Cyanol FF</td>
</tr>
<tr>
<td>yFtRNA</td>
<td>Yeast phenylalanine tRNA</td>
</tr>
</tbody>
</table>
1. Background
1.1 Structure and Function of tRNA\textsubscript{3}\textsuperscript{Lys}

Natural human tRNA\textsubscript{3}\textsuperscript{Lys}, along with isoacceptors tRNA\textsubscript{1}\textsuperscript{Lys} and tRNA\textsubscript{2}\textsuperscript{Lys}, are part of the family of tRNA’s which are responsible for accurate translation of mRNA into polypeptides by the ribosome (Figure 1). Each tRNA is specifically recognized and aminoacylated by a dedicated aminoacyl tRNA synthetase. The aminoacyl tRNA synthetase appends the cognate amino acid to tRNA’s 3’CCA acceptor stem (Figure 2). The three base anticodon of aminoacylated tRNA allows addition of amino acids in a specific order as mRNA translocates on the ribosome.

![Figure 1: Structure of natural human tRNA\textsubscript{3}\textsuperscript{Lys}, including post-transcriptional modifications (Adapted from reference 1).](image1)

![Figure 2: Schematic of the two step tRNA aminoacylation reaction by an aminoacyl tRNA synthetase (Adapted from reference 2).](image2)
1.2 Function of tRNA_{Lys} Post-transcriptional Modifications

Natural tRNA_{Lys} has been extensively studied due to two unique functions. These unique functions are a result of the hyper modification, mcm^5s^2U (5-methoxycarbonylmethyl- 2-thiouridine) and mmn^5s^2U (5-methylaminomethyl- 2-thiouridine) at position 34 of the anticodon for mammalian tRNA_{Lys} and E.coli tRNA_{Lys}, respectively (Figure 3A and 3B). Bacterial tRNA_{Lys}SUU is essential for accurate translation of the E.coli DNA polymerase γ subunit, due to a -1 frame shift caused by the modified anticodon (3). Mammalian tRNA_{Lys} also causes an essential -1 frame shift during translation of retroviral gag-pol junctions (4). Human tRNA_{Lys} is also the primer for reverse transcription in the HIV1 lifecycle (5). The 2-thio group at position 34 of human tRNA_{Lys} is required for efficient priming of reverse transcription in HIV1 (1).

**Figure 3:** Modified anticodon position 34 of A: Human tRNA_{Lys} and B: E.coli tRNA_{Lys}SUU (Adapted from reference 7).

Human tRNA_{Lys}^1, tRNA_{Lys}^2, and tRNA_{Lys}^3 comprise 60% of all small RNA’s in the HIV1 particle, but only constitute 6% of small RNA’s in the cytoplasm (6). The packaging of tRNA_{Lys}^1, tRNA_{Lys}^2, and tRNA_{Lys}^3 is mediated by hKRS (12). A non causal correlation between aminoacylation efficiency and HIV1 packaging of tRNA_{Lys} has been reported (11). However, aminoacylation of the tRNA 3’CCA is detrimental to primer extension which begins from the same 3’end. The packaging of tRNA_{Lys}^1 and tRNA_{Lys}^2, which have no known function in HIV1, suggests that hKRS recognition of tRNA is the common specificity determinant of both aminoacylation and viral packaging (12).
The non post-transcriptionally modified hKtRNA (Figure 4) is not a suitable model for natural tRNA\textsubscript{3}Lys in cases where modifications are important. However, hKtRNA is useful for studying interactions with hKRS. The \( k_m \) of aminoacylation by similar hamster-KRS of the non post-transcriptionally modified in vitro transcript (hKtRNA) and natural tRNA\textsubscript{3}Lys by hKRS is 3\( \mu \)M vs. 1.7\( \mu \)M (8, 9). By contrast, aminoacylation efficiency of cognate vs. non-cognate tRNA’s by hKRS varies by several orders of magnitude (10). Advantages of in vitro transcripts are the relative ease of obtaining large quantities and flexibility to synthesize variants and partial transcripts, through appropriately designed DNA templates.

\textbf{Figure 4:} Structure of non-post transcriptionally modified in vitro transcript, hKtRNA (Adapted from reference 10).
2. Preparation of In Vitro Transcription Template

2.1 Introduction

2.1.1 Previously used templates for in vitro transcription of hKtRNA

The most commonly employed procedure for in vitro production of hKtRNA calls for the amplification of a 181bp fragment of a plasmid known as pLysF119 (22), containing the DNA template for hKtRNA downstream of the T7 promoter sequence (Figure 5). Plasmid pLysF119 is digested by FokI to produce six fragments of length 1271bp, 720bp, 643bp, 287bp, 181bp, and 162bp (Figure 5). Independent digestions of plasmids pLysF119 and pUC19 by FokI both resulted in smeared indiscernible bands (Figure 6B and 6C). The smearing could either be attributed to a problem with the plasmids or with the enzyme. Due to the suboptimal results in the FokI digests, plasmid pUC19 and restriction enzyme HinfI were used as a control.

Figure 5: Map of plasmid pLysF119 indicating locations of FokI restriction sites, ampicillin resistance, and fragment with T7 promoter and hKtRNA encoding sequence (DNA Clone™).
The 2686bp pUC19 is recognized by FokI at positions 77, 321, 1679, 1860, and 2147; and by HinfI at positions 427, 641, 706, 781, 1177, and 1694 (Figure 7) (13). Based on these positions, digestions of pUC19 by FokI should yield five fragments 1358bp, 616bp, 287bp, 244bp, and 181bp in length; and digestion by HinfI should yield six fragments 1419bp, 517bp, 396bp, 214bp, 75bp, and 65bp in length. These trials confirmed that the problem is indeed FokI. Clean well separated bands of the correct size were achieved by HinfI digests, but not FokI digests, of plasmid pUC19 (Figure 6C and 6D). FokI is not an ideal restriction enzyme for several reasons. FokI requires the formation of a dimer in order to cut dsDNA. It has been reported that when presented with a plasmid containing two restriction sites, the ratio of the relative rates at which those sites are cut by FokI is $>>1$ (44).

Figure 6: Digestions of pLysF119 by FokI and digestions of pUC19 by FokI and HinfI analyzed on 2% agarose gel visualized with EtBr. **Legend A:** Lane 1: Bio-Nexus Hi-Low Marker. **Legend B:** Lane 1: FokI digest of pLysF119. Lane 2: Intact undigested pLysF119. **Legend C:** Lane 1: FokI digest of pUC19. Lane 2: Intact undigested pUC19. **Legend D:** Lane 1: HinfI digest of pUC19. Lane 2: Bio-Nexus Hi-Low Marker.
Incubation for longer periods to overcome the difference in FokI restriction rates introduces star activity, defined as restrictions at non-specific sites, and over digestion according to several suppliers of this enzyme. Clean digestions using FokI have been found to be difficult to reproduce here and elsewhere (22). Since large scale production of hKtRNA is our goal, it was desirable to develop an alternative template which utilizes a more consistent restriction enzyme to generate the hKtRNA template. The template for in vitro transcription of hKtRNA described here is based on new plasmid pLysB19 (designed and constructed by Protein Express, Inc.). The process is fully described in the next section (42).
2.1.2 Design and construction of a new in vitro transcription template

PCR primers were used to amplify a portion of pLysF119, containing the hKtRNA template and upstream T7RNAP promoter sequence (Figure 9). The PCR primer sequences and amplified regions of pLysF119 are shown in Figure 9. The PCR amplified fragment was digested by restriction enzymes BamHI and NdeI at the sites indicated in Figure 9. Plasmid pUC19, which was also digested with BamHI and NdeI, only has one restriction site for each endonuclease. This allowed directionally controlled ligation of the PCR fragment, which had BamHI and NdeI overhangs on its two ends, into similarly linearized pUC19 to generate new plasmid pLysB19 (Figures 7,8).

**Figure 7:** Map of pUC19 showing locations of the origin, ampicillin resistance gene and FokI, HinfI, NdeI, and BamHI restriction sites (DNA Clone Tm).
Figure 8: Schematic of A: sub-clone procedure to generate pLysB19 performed by Mike Howell (Protein Express, Inc.), B: BglI digestion of pLysB19 to produce hKtRNA template (DNA Clone Tm). Step 1: Fragment removed from pLysF119 is inserted into pUC19 w/ BamHI/NdeI to generate pLysB19. Step 2: pLysB19 is expressed under ampicillin selection and digested with BglI to generate three fragments, including template fragment shown here.

Figure 9: PCR amplified fragment of pLysF119 containing T7RNAP promoter, hKtRNA template and BamHI/NdeI restriction sites. Primer sequence underlined and restriction sequences in bold. NdeI restriction site ^, and BamHI restriction site # (DNA Clone Tm).
Plasmid pLysB19 is amplified in E.coli under ampicillin selection. Large quantities of high quality plasmid are obtainable from many commercial kits, although the costs are high. Methods were adapted for re-use of DNA purification columns supplied in the 5 Prime and Purelink commercial kits (14-16, 42). The 5 Prime kit utilizes a silica membrane column, which binds DNA in high concentrations of chaotropic salts such as guanidine HCl (14). Silica columns are regenerated with 1M HCl to break down remaining nucleic acids and the HCl rinsed with water (15). The Purelink columns are DEAE coated Silica and bind DNA through ionic interactions at low salt concentrations (14). DEAE columns are regenerated with high salt buffers to disrupt ionic interactions of un-eluted nucleic acids followed by water rinse to remove excess salt (16). The regeneration method is further detailed in the Experimental section and directions for preparation of additional amounts of purification buffers are given in Appendix B.

The isolated pLysB19 is digested by BglI into fragments that are 1400, 1118, and 252bp with the 252bp fragment being the transcription template (Figure 8). The BglI site at the 5’ end of the bottom strand (coding strand where transcription terminates by runoff) is situated so BglI cleaves after the final GGT, corresponding to 3’CCA acceptor terminus of hKtRNA transcript (Figure 10). The BglI site at the 5’end of the top (non-coding) strand results in a conveniently sized 252bp template fragment (Figure 10). When BglI digestion is complete, there is no danger of longer fragments having functional T7RNAP promoter regions. Thus, it is unnecessary to remove non-template fragments (1400bp and 1118bp) and the entire digest can be purified and used as an in vitro transcription template.

Figure 10: 252bp fragment of pLysB19 after BglI digestion containing T7RNAP promoter and hKtRNA template sequences. T7RNAP promoter sequence is italicized, hKtRNA template strand is underlined, BglI recognition sequence is bold, and restriction site indicated by *.
2.2 Experimental

This section describes the process of preparing the in vitro transcription template. The entire process is summarized in Chart 1. There are two essential quality control points which were found to be necessary for ensuring the final template is suitable for use in in vitro transcriptions. The quick preps ensure that the transformed E.coli. colonies express the correct plasmid. Comparison to reference pLysB19 on 0.7% agarose gels visualized with EtBr stain allows for a fast visual evaluation of whether there may be other plasmids expressed by the colony in question. The second quality control point is during the BglII digests. A small amount of the purified pLysB19 was digested with BglII on a trial scale to ensure that the digestion resulted in the expected three distinct bands. The correct digestion pattern indicates plasmid purity while the lack of streaking can be used to affirm the lack of DNAse contamination.

Chart 1: Overview of Production of hKtRNA transcription template from pLysB19.

2.2.1 Transformation

A 50μL aliquot of DH5α E.coli cells is thawed on wet ice from -70°C. The cells are gently mixed by flicking the tube. Plasmid pLysB19 is then added to the cell mixture. The amount of plasmid added is 50pg in a volume not exceeding one tenth of the cell mixture volume (5μL). The cell and plasmid mixture is incubated on ice for 30 minutes, then incubated at 42°C in a water bath for 45 seconds, followed by an additional 2 minutes on ice. The mixture is then diluted with 950μL of SOC buffer (2% Tryptone, 0.5% Yeast Extract, 0.4% glucose, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄) to a final volume of 1mL. The diluted mixture is incubated in a shaking incubator set to 200rpm for 60 minutes at 37°C. Meanwhile, ampicillin is spread onto LB agar plates to a final concentration of 100μg/mL and the plates are
incubated for 30 minutes at 37°C. Several ampicillin plates are then plated with 5-200μL of the SOC mixture containing the transformed cells. The plates are incubated at 37°C for 12-18 hours until the colonies reach approximately 1mm in diameter. The plates can then be refrigerated for up to seven days.

2.2.2 Quick preps to test for successful transformation

Sterile loops are used to inoculate 4-6 separate 5mL LB mini-cultures containing 100μg/mL ampicillin with one colony each from the LB agar plates. The mini-cultures are allowed to grow in a shaking incubator (200rpm at 37°C) to an OD600 of 1.0-1.5. The mini-cultures are refrigerated and a 1.5mL aliquot of each mini-culture is centrifuged at 13,000G for 5minutes and the supernatant is discarded. The cell pellets are re-suspended in 300μL TENS buffer (50mM Tris HCl, 20mM EDTA, 100mM NaCl, 1% wt/vol. SDS), followed by addition of 150μL 3M NaOAc pH 5.2 and vortexing to mix. The resulting mixture is centrifuged at 13,000G for 2minutes to collect cell debris into a pellet. The resulting supernatant is transferred to a fresh tube and the plasmid is precipitated from the supernatant with 2.5 volumes 100% EtOH at -20°C for at least 2 hours. The plasmid is pelleted by centrifugation at 13,000G for 30 minutes, and the ethanol supernatant is discarded. The pelleted plasmid is rinsed with 1 volume 70% EtOH at room temperature for 5minutes, followed by a spin at 13,000G for 5minutes. The resulting pellet is air-dried to remove residual ethanol and re-suspended in 50μL TE. A small amount of plasmid obtained from each colony is run alongside reference pLysB19 on 0.7% agarose gel containing 0.75μg/mL EtBr for visualization.

2.2.3 Plasmid amplification, isolation, and quantifying yields

Based on the results of the quick preps, a mini-culture is chosen to inoculate a 500mL to 1L LB culture containing 100μg/mL ampicillin. The culture is grown to an OD600 of 1.5-1.8. The plasmid is isolated using one of two commercial kits (5 Prime or Purelink) according to the manufacturer’s instructions. The plasmid purification columns supplied with the 5 Prime and Purelink kits were regenerated and re-used with the solutions provided in Appendix B. The quality of each batch of isolated plasmid is estimated by analysis on 0.7% agarose gel next to known pLysB19 plasmid. Quantifying the product by densitometry was based on comparing band intensities to reference pLysB19 of known concentration (Protein Express, Inc).
2.2.4 Regeneration of columns

It has been found that columns from both kits can be re-used after appropriate washing and re-generation (14-16). The silica resin columns from the 5 Prime kit are washed with 1M HCl, followed by 5-10 washes with autoclaved de-ionized water to remove residual HCl. The columns can be air dried and stored for re-use. The columns may also be stored in 1M HCl for several days before being rinsed with water. Re-use of the columns is simply a matter of following the kit instructions, using the solutions provided in Appendix B in place of buffers supplied with the kit. Regeneration of the DEAE ion exchange columns found in the Purelink kit is achieved by rinsing with 30mL of a high salt wash buffer (2.5M NaCl, 0.15% vol/vol triton X-100, pH 8.5) to elute residual nucleic acids, followed by a 50mL autoclaved de-ionized water rinse to remove excess salt. These columns can also be re-used as directed by the kit instructions using the solutions provided in Appendix B in place of the buffers supplied with the kit. The DEAE coated silica particles in the gravity powered Purelink columns become settled with repeated use and the flow rate tends to decrease. This can be remedied by flushing the regenerating buffer from the bottom into an inverted column (16).

2.2.5 Plasmid Sequencing

The pLysB19 plasmid containing the template for the hKtRNA transcript was sent out for commercial sequencing by Genewiz Inc using standard M13 Forward and Reverse primers to sequence both strands of the pLysB19 region containing the hKtRNA template fragment.

2.2.6 Restriction Digests

BglII (NEB) was the restriction enzyme used to isolate the template fragment coding for hKtRNA from the pLysB19 plasmid. The digests were assembled per the instructions provided by NEB, using the supplied 10X reaction buffer and up to 1 unit of BglII per 1μg of plasmid. Incubation was at 37ºC for 5 to 24 hours. The progress of each digest was checked on 2% agarose gels with EtBr for visualization to ensure that digestion was complete. The reaction was determined to be complete when the intact pLysB19 plasmid band was absent, and the distinct three band pattern of the digest became apparent. The crude digest was extracted with an equal volume 5:1 TE buffered phenol; chloroform to remove BglII and any contaminating RNAs, followed by a chloroform extraction to remove traces of phenol. The extracted digested plasmid
was then precipitated with 2.5 volumes EtOH for at least 2 hours at -20°C. The DNA fragments were pelleted by centrifugation at 15,000G for 30 minutes at 4°C. The DNA pellet was washed with 70% EtOH at room temperature for 5 minutes, followed by centrifugation at 15000G for 5 minutes at 4°C. The DNA pellet was air-dried and resuspended in TE buffer. The re-suspension’s concentration was determined by densitometric comparison to BglI digests of reference pLysB19 (Protein Express, Inc.). RNAsure reagent (Ambion) was used to treat the re-suspended pure template which was then ready for use as an in vitro transcription template.
2.3 Results & Discussion

2.3.1 Preparation of pLysB19

pLysB19 was isolated using the 5 Prime and Purelink kits. Subsequent isolations from regenerated columns are pooled and analyzed below. Plasmid confirmed and quantified by comparing band intensity to reference pLysB19 (protein Express, Inc.) (Figure 11).

![Image](Image)

**Figure 11:** Identifying and quantifying pLysB19 obtained by subsequent isolations on regenerated 5 Prime and Purelink columns. Analysis on 0.7% agarose gel visualized with EtBr

**Legend:** Lane 1: Bio-Nexus Hi-Low Marker. Lanes 2,3,4: 250ng, 500ng, 750ng Reference standard pLysB19. Lanes 5,6,7: 0.25μL, 0.5μL, 0.75μL pooled Purelink pLysB19 (3 preps). Lanes 8,9,10: 0.25μL, 0.5μL, 0.75μL pooled 5 Prime pLysB19 (4 preps).
2.3.2 Re-use of Plasmid Purification Columns

Regeneration and re-use of 5 Prime and Purelink columns allows isolation of large quantities of plasmid, but it was necessary to check quality of subsequent preparations. The 5 Prime and Purelink columns yield identical results for subsequent preparations (Figure 12). Gravity flow ion exchange columns like those in the Purelink kit can be re-used up to 20 times with no deterioration (16). The silica membrane in the 5 Prime columns is fragile and usually leaks after 5-6 uses, at which point it must be discarded.

Figure 12: Analysis of subsequent pLysB19 preparations using re-generated 5 Prime and Purelink columns on 0.7% agarose gel visualized with EtBr. **Legend A:** Lane 1: Bio-Nexus Hi-Low Marker. Lanes 2,3: 2μL each of subsequent preparations on 5 Prime regenerated column. **Legend B:** Lane 1,2,3: 8μL each of subsequent preparations on Purelink regenerated column.
2.3.3 Sequencing of pLysB19

Sequencing was done by Genewiz Inc. using standard M13 Forward and Reverse primers to sequence both strands of the pLysB19 region containing BglII restriction sites on either end of template fragment and T7RNAP promoter. The sequencing results were an exact match for this region, shown in figure 10, on both strands (Figure 13).

Figure 13: Sequencing Results for pLysB19 using M13 forward and reverse primers (Genewiz Inc.)
2.3.4 Digesting pLysB19 with BglI

pLysB19 is digested with restriction enzyme, BglI, to produce three distinct fragments. The digest is purified as described in the Experimental section. At this stage, DNAse contamination is readily apparent if it is present (Figure 14B). It is also apparent whether the plasmid preparation contains undesirable DNA as indicated by the presence of undigested bands. In cases where the digest produced three distinct fragments with no streaking or undigested bands, it was purified for use as an in vitro transcription template. The purified digest products were quantified by comparing band intensity to standards made by digesting reference pLysB19 with BglI (Protein Express, Inc) on 2% agarose gels (Figure 14A).

Figure 14: Confirming and quantifying purified BglI digests of pLysB19 on 2% agarose gel visualized with EtBr **Legend A:** Lane 1: Bio-Nexus Hi-Low Marker. Lane 2: 250ng Reference standard pLysB19. Lanes 3,4,5: 350ng, 700ng, 1000ng Reference standard BglI digested pLysB19. Lanes 6,7: 0.25μL, 0.5μL purified BglI digest of Prime pLysB19. Lanes 8,9: 0.25μL, 0.5μL purified BglI digest of Purelink pLysB19. **Legend B:** Lane 1: DNAse contaminated BglI digest of pLysB19.
2.4 Conclusions

In conclusion, production of large quantities of pLysB19 is possible by re-use of regenerated commercial purification columns. It is essential to employ several checks throughout the plasmid production process to ensure the correct plasmid is amplified and the resulting BglII digest is suitable as an in vitro transcription template. It is useful to conduct quick small scale plasmid preparations, as described in the Experimental section, to ensure transformed cells express the correct plasmid prior to large scale culture growth. A small scale BglII digest of a portion of each pLysB19 preparation should be conducted for two reasons. The first is to ensure all bands can be digested to rule out contamination with undesirable plasmids. The second reason is BglII digestion conditions (37°C and 10mM MgCl₂) are useful for detecting DNAse activity, since DNAse is active at 37°C when Mg²⁺ is present (17, 18).
3. Production of hKtRNA by In Vitro Transcription

3.1 Introduction

3.1.1 Transcription Buffer

In vitro transcriptions using T7RNAP, DNA template, NTPs, and a transcription buffer have been widely described (Table 1). It has also been reported that addition of BSA to a final concentration of 50-100μg/mL or PEG8000 to 80mg/mL may enhance the transcription reaction yield (23). The replenishment of MgCl₂ midway through the reaction has also been reported to increase yield (21).

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl</td>
<td>40-80mM</td>
</tr>
<tr>
<td>Spermidine</td>
<td>1-2mM</td>
</tr>
<tr>
<td>DTT</td>
<td>5-40mM</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.01%-0.1%</td>
</tr>
<tr>
<td>NTPs</td>
<td>0.5mM-10mM each</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>5-50mM (Depends on NTP and template concentration)</td>
</tr>
<tr>
<td>DNA template</td>
<td>(5nM to 2μM)</td>
</tr>
<tr>
<td>T7RNAP</td>
<td>(0.01mg/mL to 0.2mg/mL)</td>
</tr>
</tbody>
</table>

Table 1: Summary of transcription reaction conditions reported in the literature (19-23).

One particular protocol for the synthesis of short transcripts by Milligan et al. has been widely used and cited (23). The 1X transcription buffer consists of 40mM Tris, 6mM MgCl₂, 5mM DTT, 1mM spermidine, 0.01% triton X-100, and 50μg/mL BSA (23). In this protocol, a 10X stock buffer is prepared by mixing autoclaved MgCl₂ and Tris-HCl with DTT, MgCl₂, and triton X-100 and sterilizing through a 0.25μm filter (23). BSA (fraction V powder) may be added up to 50μg/mL, although this may be omitted for large scale transcriptions, and the buffer is brought up to final volume after adjusting pH to 8. The buffer is aliquoted and stored at -20ºC (23). Since NTP concentrations vary between reactions, a 0.5M stock solution of MgCl₂ is used to keep the Mg²⁺ concentration higher than the total NTP concentration (23).

The transcription reactions are assembled by addition of 10X buffer (diluted to 1X with water), followed by NTPs, DNA template, and mixed at room temperature to avoid precipitating the template by spermidine and MgCl₂ (23). The T7RNAP is thawed on ice and is the final ingredient added and the reaction is incubated at 37ºC for 1-2 hours (23). Our colleagues at OSU use the conditions described above to produce the same transcript which we seek to make from a similar, related template (22). The buffer is prepared and stored as described above (22,
The specific conditions used at OSU are: 40mM Tris, 20mM MgCl$_2$, 5mM DTT, 1mM spermidine, .01% triton X-100, 50μg/mL BSA, 4mM each NTP, 44-55nM template (8-10μg per 100μL reaction), and incubated at 37°C for 3-4 hours (Table 2) (22). The reaction is then quenched with 0.5M EDTA to a final concentration of 0.05M and purified by urea denaturing PAGE as described here. The EDTA is added to chelate Mg$^{2+}$ which causes the transcription reaction to stop and also removes soluble and insoluble Mg$^{2+}$ (left over soluble Mg$^{2+}$ and insoluble magnesium pyrophosphate byproduct).

The buffer described here was made by mixing Tris HCl, MgCl$_2$, DTT, spermidine, triton X-100 in RNAse free water, adjusting pH and volume. The buffer was then aliquoted and stored at -20°C. Transcription reactions described here were assembled by the addition of water, 10X buffer, NTP, and DNA template in that order. The final conditions were 40mM Tris, 36mM MgCl$_2$, 10mM DTT, 2mM spermidine, 0.01% triton X-100, 7.5mM each NTP, 385nM template (7μg per 10μL reaction), 0.64mg/mL T7RNAP, and pH8. The ingredients are combined at room temperature with mixing after addition of each ingredient as detailed in the Experimental section. The T7RNAP was then added after thawing on ice from -70°C, and the reaction mixture was incubated at 37°C for 3-4 hours. The EDTA quenching step is omitted here, and the magnesium pyrophosphate precipitate is removed by centrifugation instead (19).

The transcription system herein described is a hybrid system derived from three sources. The NTP concentration of 7.5mM was based on the initial use of the Ambion Mega Short Script Kit, which is optimized to transcribe short RNAs (24). The T7RNAP concentration and buffer composition in the Ambion kit is proprietary and thus unknown, although the template was made in house as described above. The chief expenses of the in vitro transcription reaction are the T7RNAP and the DNA template. We were kindly provided T7RNAP by Dr Stuart Le Grice at NCI. Thus, all the ingredients were at hand with the exception of a suitable transcription buffer. Since pLysB19 is a new template, it was necessary to determine the most effective conditions using this hybrid system. The experiments were performed and quantitatively analyzed as described in the experimental section.
Table 2: Transcription of transcript yields under a variety of conditions. Reaction 10 conditions are 40mM Tris, 20mM MgCl₂, 5mM DTT, 1mM spermidine, 0.01% triton, 50μg/mL BSA, 4mM each NTP, and 50nM template incubated at 37°C for 3-4 hours (Adapted from reference 23)
3.1.2 T7 RNA Polymerase

The key to the in vitro transcriptions described here is the T7 RNA polymerase enzyme, due to its role in the T7 bacteriophage lifecycle. During the early stages of infection the bacteriophage relies on E.coli RNA polymerase to transcribe early mRNAs, including the mRNA responsible for T7RNAP (25). During later stages of infection the E.coli. polymerase is deactivated and the T7 RNA polymerase is used to transcribe only viral DNA, which it recognizes using a very specific promoter sequence shown in Figure 15 (25). Besides its high specificity, T7RNA polymerase is a convenient single peptide chain which can be obtained by recombinant expression and purification from E.coli. (26). T7RNAP enzyme requires a double stranded promoter sequence, while the coding region can be either single or double stranded (23). Additionally, both synthetic DNA constructs and plasmid based DNA templates can be used (23).

Figure 15: Sequence of the T7RNAP promoter. The +1 position indicated is the first base incorporated (Ambion).

Although T7RNAP is somewhat flexible with template choice, there are several important constraints. It has been reported that double stranded templates with 3’ overhangs may result in the polymerase doubling back to transcribe the top strand rather than dissociating by runoff (27). For this reason, most published protocols suggest the use of 5’ overhang or blunt end double stranded templates. T7 RNAP has also been shown to non-specifically append 1-3 bases to the 3’ end of a transcript, a trait it shares with other polymerases lacking an exonuclease
function (23). The addition of these bases may be dependent on the entire template sequence since two templates with BstnI terminated ends have been shown to yield transcripts with significantly different 3’ end heterogeneity (28, 29). One last consideration is the transition between initiation and elongation phases of T7RNAP. This transition is the rate limiting step and results in many abortive transcripts, but the enzyme is very processive after the transition and will transcribe around a plasmid many times before dissociating (23). For this reason, plasmid DNA should be subjected to complete linearization or digestion since failure to do this will exhaust the NTP supply in a given reaction (23).
3.2 Experimental

This section describes the procedure by which the optimal in vitro transcription conditions were determined. The assembly of in vitro transcription reactions was always performed in the order shown in Chart 2. The reaction is assembled at room temperature since the template may precipitate in the presence of spermidine at low temperature. It is also critical to ensure complete mixing by pipetting or flicking the tube after addition of each ingredient. T7RNAP was thawed on ice and was always the last ingredient added. It is also essential to use RNAse free water (MP Biomedical, LLC) and RNAse Free certified pipette tips and tubes. All transcriptions were incubated at 37°C for 3-4 hours. An aliquot was then removed for analysis on a 10cm 8M urea denaturing 12%PAGE and the rest of the reaction stored at -20°C. Commercially obtained yFtRNA, which has identical mobility under these electrophoresis conditions, was used as molecular weight marker and intensity standard for densitometric analysis. The NCI T7RNAP polymerase (50mM NaPO₄, 300mM NaCl, 250mM imidazole, 10% glycerol, pH 6.0) was aliquoted into single use portions and stored at -70°C upon receipt to avoid repeated freeze thaw cycles.

![Chart 2: Overview of in vitro transcription reaction assembly and quantitative analysis. All ingredients combined once they reach room temperature, except for T7RNAP which is thawed from -70°C on ice until use.](image)

3.2.1 Determination of optimal Mg²⁺ concentration

A 10X transcription buffer containing 400mM Tris, 60mM MgCl₂, 100mM DTT, 20mM spermidine, and 0.1% vol/vol triton X-100 was used to test the effect of Mg concentration on transcription yield. The final desired Mg²⁺ concentration was obtained by the addition of a suitable amount of 1M MgCl₂ to each reaction. The template concentration was 190nM (3.5μg in 10μL reaction), T7RNAP concentration was 0.64mg/mL, and 7.5mM of each NTP were used. Reaction assembly and quantitative analysis were conducted as described above.
3.2.2 Determination of optimal Template concentration

The experiment probing the effect of template concentration on transcript yield was conducted using a 10X transcription buffer containing 400mM Tris, 360mM MgCl₂, 100mM DTT, 20mM spermidine, and 0.1% vol/vol triton X-100. The template concentrations tested were 190nM, 385nM, 575nM, and 625nM (3.5μg, 7 μg, 10.5 μg, 11.5 μg, respectively per 10μL reaction). T7RNAP concentration was 0.64mg/mL and 7.5mM of each NTP was used. Reaction assembly and quantitative analysis were conducted as described above.

3.2.3 Determination of whether Mg²⁺ replenishment increases transcript yield

The experiment probing whether the replenishment of Mg²⁺ midway through the transcription reaction increased yield was performed using a 10X transcription buffer containing 400mM Tris, 360mM MgCl₂, 100mM DTT, 20mM spermidine, and 0.1% vol/vol triton X-100. The template concentration was 190nM (3.5μg in 10μL reaction), T7RNAP concentration was 0.64mg/mL, and 7.5mM of each NTP was used. The reaction was centrifuged after a 1.5 hours incubation at 37°C to collect the magnesium pyrophosphate precipitate into a pellet, followed by the addition of 1M MgCl₂ to a final concentration of 36mM MgCl₂. The reaction was incubated an additional 1.5 hours at 37°C before quantitative analysis, alongside a control which was continuously incubated at 37°C for 3hours as previously described.

3.2.4 Determination of whether BSA or PEG8000 improve transcript yield

The experiments probing the effect of transcription buffer additives such as 80mg/mL PEG8000 or 50μg/mL BSA were conducted using a 10X transcription buffer containing 400mM Tris, 360mM MgCl₂, 100mM DTT, 20mM spermidine, and 0.1% vol/vol triton X-100. The BSA experiment utilized a template concentration of 100nM (1.85μg in 10μL reaction), while the PEG8000 experiment was conducted using a template concentration of 190nM (3.5μg in 10μL reaction). The T7RNAP concentration was 0.64mg/mL, and 7.5mM of each NTP was used and in both cases. The additives were added from stock solutions consisting of 5X PEG8000 (400mg/mL) or 10X BSA (0.5mg/mL) prepared in RNAse free water and treated with RNAsure. The reactions were analyzed alongside control in vitro transcription reactions in which no additive was used. Reaction assembly and quantitative analysis were otherwise conducted as described above.
3.2.5 Determination of whether reaction scale affects yield

All of the experiments were conducted with NCI T7RNAP, with the exception of these scale experiments. These experiments were conducted using OSU T7RNAP and OSU 10X transcription buffer, which were kindly provided. The 10X OSU transcription buffer consisted of 40mM Tris, 6mM MgCl$_2$, 5mM DTT, 1mM spermidine, .01% triton X-100, 50μg/mL BSA. The NTP’s were used at a concentration of 4mM each and the MgCl$_2$ concentration supplemented to 20mM in accordance with the OSU procedure. The template concentration was 55nM (8-10μg per 100μL reaction) and the provided OSU T7RNAP was used at the recommended 4% vol/vol of the total reaction volume. The reactions were incubated at 37°C for 3 hours, and the results analyzed in the usual manner.

3.2.6 Demonstrative large scale transcriptions

Two large scale transcriptions were assembled using 350μg of purified template derived from 5 Prime pLysB19, and 310μg of purified template derived from Purelink pLysB19. The transcriptions were assembled to a total volume of 500μL and 440μL, respectively so that template concentration in each was 380nM (7μg per 10μL reaction). The reactions were assembled using a 10X transcription buffer containing 400mM Tris, 360mM MgCl$_2$, 100mM DTT, 20mM spermidine, and 0.1% vol/vol triton X-100. The T7RNAP was 0.64mg/mL and 7.5mM of each NTP was used. Reaction assembly and quantitative analysis were conducted as described above.
3.3 Results & Discussion

3.3.1 Effect of Mg\(^{2+}\) Concentration on hKtRNA Yield

A series of in vitro transcription reactions with MgCl\(_2\) concentrations from 25-50mM were attempted (details are described in the experimental section). The optimum hKtRNA transcript yield occurred at 36mM (Figure 16). However, these trials were only conducted once and the standard deviation could not be calculated. The error bars are estimated based on a standard error of +/-5% for calibrated pipettes, since these were used to prepare the samples for analysis. The findings are consistent with literature reports that the Mg\(^{2+}\) concentration has a large effect on transcription yield. In general, the Mg\(^{2+}\) concentration should be greater than the total NTP concentration, and should be optimized for each transcription system (23).

![Transcription Yield vs. Mg\(^{2+}\) Concentration](image)

**Figure 16:** hKtRNA yield in micrograms vs. total Mg\(^{2+}\) concentration in a 10μL reaction. (Reaction conditions: 40mM Tris, 10mM DTT, 2mM spermidine, 0.01% triton X-100, 7.5mM each NTP, 190nM template (3.5μg per 10μL reaction), 0.64mg/mL T7RNAP, and 25-50mM MgCl\(_2\), pH8). Error bars estimated based on +/-5% pipette calibration error.
3.3.2 Effect of Template Concentration on hKtRNA Yield

Once the optimal Mg\(^{2+}\) concentration was determined, it was hypothesized that the transcription yield could be further enhanced through the use of higher template concentrations. The yields from transcriptions with template concentrations of 190nM, 385nM, 575nM, and 625nM (3.5μg, 7 μg, 10.5 μg, 11.5 μg, respectively per 10μL reaction) are summarized in Figure 17. The best transcription yield in quantity is achieved at a template concentration of 385nM, and the yield levels off at higher template concentrations (Figure 17). However, the plotted results represent a single trial and the standard deviation could not be calculated. The error bars shown in figures 17 and 18 are based on a standard error of +/-5% for calibrated pipettes. The fold amplification (moles hKtRNA transcribed per mol template) is 23 fold at a template concentration of 385nM and 33 fold at 190nM (Figure 18). The transcription is clearly more efficient at a template concentration of 190nM, but the overall yield is higher at a template concentration of 385nM. Since large quantities of template are obtainable, the optimal template concentration for maximum transcription yield is 385nM (Figures 17, 18).

![Transcription Yield vs. Template Concentration](image)

**Figure 17:** Transcription yield of hKtRNA (μg) vs. template concentration (nM). (Reaction conditions: 40mM Tris, 36mM MgCl\(_2\), 10mM DTT, 2mM spermidine, 0.01% triton X-100, 7.5mM each NTP, 0.64mg/mL T7RNAP, 190-625nM Template (3.5μg to 11.5 μg per 10μL reaction), pH8). Error bars estimated based on +/-5% pipette calibration error.
Figure 18: Transcription efficiency defined as moles hKtRNA transcribed per mole template (y-axis), vs. template concentration (x-axis). (Reaction conditions: 40mM Tris, 36mM MgCl₂, 10mM DTT, 2mM spermidine, 0.01% triton X-100, 7.5mM each NTP, 0.64mg/mL T7RNAP, 190-575nM Template (3.5μg to 10.5 μg per 10μL reaction), pH8). Error bars estimated based on +/-5% pipette calibration error.
3.3.3 Effect of BSA, PEG8000, and Mg\(^{2+}\) Replenishment on hKtRNA Yield

The use of BSA to a final concentration of 50μg/mL as a component of the transcription buffer has been reported to increase in vitro transcription yields (23). This is presumably due to BSA coating the reaction vessel walls in order to minimize loss of T7RNAP during the course of the reaction. It has also been reported that addition of PEG8000 to a final concentration of 80mg/mL as a component of the transcription buffer may enhance in vitro transcription yields (23). The PEG8000 may act as a crowding agent, promoting formation of more T7RNAP/DNA template initiation complexes (30). This may in turn help drive the transition of T7RNAP from the rate limiting initiation step to the productive elongation step. Another reported modification involves brief centrifugation to collect the precipitate followed by the replenishment of Mg\(^{2+}\) (21). The Mg\(^{2+}\) is depleted throughout the reaction as it forms insoluble magnesium pyrophosphate with the inorganic phosphate byproduct of the polymerization reaction (31). The effect of each of these additives on transcription yield was found to be negligible when compared with an identical reaction lacking these additives, as summarized in Table 3.

<table>
<thead>
<tr>
<th>Modification</th>
<th>Reaction Yield</th>
<th>Control Reaction Yield</th>
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<tbody>
<tr>
<td>Addition of BSA to 50μg/mL</td>
<td>4.5μg</td>
<td>5.0μg</td>
</tr>
<tr>
<td>Addition of PEG8000 to 80mg/mL</td>
<td>8.4μg</td>
<td>8.0μg</td>
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<tr>
<td>Replenishment of Mg(^{2+})</td>
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<td>8.0μg</td>
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</table>

Table 3: Transcription buffer additives effects on hKtRNA yield. Each reaction quantitatively analyzed on urea denaturing PAGE alongside a non-modified control transcription reaction, as described in the Experimental section. **BSA experiment conditions:** 50μg/mL BSA 40mM Tris, 36mM MgCl\(_2\), 10mM DTT, 2mM spermidine, 0.01% vol/vol triton X-100, template concentration 100nM (1.85μg in 10μL reaction), T7RNAP concentration 0.64mg/mL, and 7.5mM of each NTP. **PEG8000 experiment conditions:** 80mg/mL PEG8000 40mM Tris, 36mM MgCl\(_2\), 10mM DTT, 2mM spermidine, 0.01% vol/vol triton X-100, template concentration 190nM (3.5μg per 10μL reaction), T7RNAP concentration 0.64mg/mL, and 7.5mM of each NTP. **Mg\(^{2+}\) replenishment experiment conditions:** 40mM Tris, 36mM MgCl\(_2\), 10mM DTT, 2mM spermidine, 0.01% vol/vol triton X-100, template concentration 190nM (3.5μg per 10μL reaction), T7RNAP concentration 0.64mg/mL, and 7.5mM of each NTP. Magnesium pyrophosphate precipitate removed by centrifugation and MgCl\(_2\) added to 36mM after 1.5 hours.
3.3.4 Effect of Reaction Scale on hKtRNA Yield

To test the effect of scale on transcript yield, three in vitro transcription reactions of varying scale were assembled, as described in the experimental section. The only variable was the total reaction volumes, which were 10μL, 50μL, and 100μL. The T7 RNA polymerase used in these reactions was kindly provided by the Karin Musier-Forsyth Group (OSU). Based on the intensity of yFtRNA marker bands, the overall hKtRNA yields from each transcription reaction were 100ng per μL of reaction volume for the 10μL and 50μL, while the 100μL reaction yielded approximately 150ng hKtRNA per μL of reaction volume (Figure 19). The yields from the reactions were lower than expected, presumably due to reduced enzyme activity as compared to previous transcription reactions using NCI T7RNAP (Figure 19 Lane 4), however the yield of the reaction was largely unaffected by scale (Figure 19, Lanes 5-7). The slight increase of yield per unit volume as the reaction volume increased can be rationalized in one of two ways. One is that even minor pipette error, whether human or due to calibration limits, is more detrimental to smaller reactions than larger ones. A second explanation is that loss of water due to evaporation results in a more significant change in conditions for smaller volume reactions.

**Figure 19:** Effect of reaction scale on hKtRNA yield analyzed on 8M urea denaturing 12%PAGE visualized with sybr gold (negative shown for better visualization of faint bands).

**Legend:** Lanes 1,2,3: 100ng, 200ng, 300ng yFtRNA standard, Lane 4: 0.33μL of 10μL scale transcription w/NCI T7 RNAP, Lane 5: 0.33μL of 10μL scale transcription w/OSU T7 RNAP, Lane 6: 0.33μL of 50μL scale transcription w/OSU T7 RNAP, Lane 7: 0.33μL of 100μL scale transcription w/OSU T7 RNAP.
3.3.5 Large Scale Transcriptions of Milligram Quantities of hKtRNA

Two large scale in vitro transcriptions were assembled using 350μg of purified template derived from 5 Prime kit pLysB19, and 310μg of purified template derived from the Purelink kit. The transcriptions were assembled to a total volume of 500μL and 440μL, respectively so that template concentration in each was 380nM (7μg per 10μL reaction volume), as described in the Experimental section. The hKtRNA transcript was quantitatively analyzed on 8M urea denaturing 12%PAGE (Figure 20). The total yield of in vitro transcribed hKtRNA from 5 Prime pLysB19 was 550μg, while 484μg hKtRNA were obtained from the in vitro transcription using Purelink pLysB19. This translates to 1.1μg of transcript obtained per μL of reaction volume.

Figure 20: Large scale in vitro transcription of hKtRNA quantitatively analyzed on 8M urea denaturing 12%PAGE visualized with sybr gold. Legend: Lanes 1,2,3,4,5: 10ng, 30ng, 50ng, 100ng, 200ng yFtRNA standard, Lane 6,7,8: 0.25μL, 0.5μL, 0.75μL of 1 in 10 dilution of transcription reaction using 5 Prime pLysB19 based template. Lane 9,10,11: 0.25μL, 0.5μL, 0.75μL of 1 in 10 dilution of transcription reaction using Purelink pLysB19 based template.
3.4 Conclusions

The in vitro transcription yield was found to be almost exclusively dependent on Mg\textsuperscript{2+} and template concentration. Additives to the transcription buffer, such as BSA and PEG8000 were found to have a negligible impact on hKtRNA yield. The optimal transcription conditions were found to be 40mM Tris, 36mM MgCl\textsubscript{2}, 10mM DTT, 2mM Spermidine, 0.01% triton X-100, 7.5mM each NTP, 385nM Template (7μg in a 10μL reaction), 0.64mg/mL T7RNAP, pH8. It was found that all parameters fell within the ranges found in the literature, with the exception of the T7RNAP concentration as shown in Table 1 (19-23). The optimal T7RNAP concentration has been reported to fall within the range of 0.01mg/mL to 0.2mg/mL for most transcription reactions (23). The T7RNAP provided by NCI was used at a concentration of 0.64mg/mL as suggested.

We observed approximately 15-33 transcript copies per template copy, depending on template concentration. The yields described by Milligan et al show that up to 250 copies of transcript can be produced from each template copy (Table 2, Reaction 10). T7RNAP activity has been reported to be particularly sensitive to the temperature and length of storage of T7RNAP (23, 26). The T7 RNA polymerase enzyme is subject to oxidative inactivation when stored for extended periods (23). In our system, reduced enzyme activity may explain the observed need for higher T7RNAp and template concentrations than reported elsewhere to achieve a similar yield per unit volume of the reaction.

Comparison to the transcription system used by our colleagues at OSU suggests that reduced enzyme activity may account for the large difference in template concentrations to achieve comparable yields (7μg vs. 1μg per 10μL reaction). Although significant differences exist between the OSU reaction and the one described here, some limited comparisons can be made. The yields from the OSU system are comparable to ours despite their use of lower template, NTP, and Mg\textsuperscript{2+} concentrations. The variation in the Mg\textsuperscript{2+} concentration can be largely attributed to the difference in NTP concentrations between the two systems. The large difference in efficiency is likely, although not conclusively, due to a difference in the activity of the T7RNAP used.

The best evidence that NCI T7RNAP is sub-optimally stored is comparing yields of identical transcription reactions conducted 14 months apart. The hKtRNA yields observed for the Mg\textsuperscript{2+} optimization experiments were ≥1.5μg per μL of reaction volume. Recent
transcriptions have yielded only 1.1μg per μL of reaction volume. It is also possible that the difference in yield between the reaction described here and the reaction at OSU may be due to the employment of different templates. This is considered somewhat unlikely since the template in use at OSU is based on plasmid pLysF119. This template, like pLysB19, is based on the pUC19 vector (22).

Results of others in this group show hKtRNA yields as high as 1.6μg per μL of reaction volume with template concentrations as low as 55nM (2μg pLysB19 in 20μL transcription reaction) using the TranscriptAid™ T7 High Yield Transcription Kit (Fermentas, Inc. Cat#K0441) which comes with T7RNAP. Additionally, recent results by others in this group show hKtRNA yields of ≥1.6μg per μL of reaction volume at template concentrations as low as 50nM (1μg per 10μL reaction) using the system described here, but only after the NCI T7RNAP has been exchanged into pH 8 50% glycerol buffer containing DTT.

It is possible that an alternative means of storing the enzyme may help to preserve activity over a period of years. T7RNAP is stable and can be preserved at pH 8 in a 50% glycerol storage buffer at -20ºC for several months (19, 23). Addition of DTT to prevent oxidative inactivation is recommended every 6 months (23). Alternatively it has been reported that the enzyme may be stored long term by flash freezing and storing at -80ºC (19). The NCI T7RNAP used here was aliquoted into single use portions and stored at -70ºC, as instructed upon receipt (32). The NCI T7RNAP was left in the elution buffer used to purify it from a nickel column (50mM NaPO₄, 300mM NaCl, 250mM imidazole, 10% glycerol, pH 6.0) (32).

In summary, the purification of milligram quantities of hKtRNA transcript is possible with the method herein described, but it is dependent on use of much higher template concentrations than previously reported elsewhere and more recently by others in this group. The requirement of higher template concentrations than found elsewhere is very likely due to reduced enzyme activity of the NCI T7RNAP in the experiments described here. The very recent finding that hKtRNA yields can be maintained with lower template concentrations (after exchange of the NCI T7RNAP buffer) is also very supportive of this conclusion.
4. Purification of hKtRNA Transcript by Urea Denaturing PAGE

4.1 Introduction

The hKtRNA transcript was purified from the crude transcription reaction by urea denaturing PAGE. Urea denaturing PAGE is performed by running the transcript on 20cm long 8M urea 12% PAGE, followed by UV shadowing to identify and cut out the band corresponding to the desired transcript. The transcript is then removed from the gel slice by ‘crushing and soaking’ in gel elution buffer as detailed in the Experimental section. The transcript is isolated from the gel elution buffer by phenol: chloroform extraction and ethanol precipitation. The chief advantage of urea denaturing PAGE purification is the availability of all the required equipment and materials. Since this method relies on denaturing the transcript during purification, problems have been reported due aggregation of denatured RNAs, especially for large transcripts (20, 21). These aggregates are usually visible as hang up in the well of the gel or slow migrating smears relative to the desired transcript (21, 22).

Other methods reported in the literature to purify in vitro transcribed RNA’s include size exclusion chromatography, and high pressure liquid chromatography (20, 21, and 23). High pressure liquid chromatography methods have not been widely developed due to incomplete denaturation and formation of aggregates by partially denatured RNA’s (33). Size exclusion chromatography seems to be the best method and has been described on simple gravity flow columns as well as fast performance liquid chromatography columns (20, 21). The advantages of size exclusion chromatography are the transcript is never denatured, and recovery from the column can be as high as 99%, although there are losses in the steps prior to loading the column (20, 21). Size exclusion chromatography is also particularly useful for plasmid based transcription systems since there is often a large size difference between the transcript and other molecules in the transcription reaction (T7RNAP, DNA template, transcript, small molecules and NTP’s) (21). The final advantage of size exclusion chromatography is the lack of small water soluble acrylamide contaminants which have been shown to interfere in NMR experiments (21). Size exclusion chromatography was not used here since it required time consuming equipment set-up and method development. It may however prove to be useful once a method is established, and is recommended for future purifications.
4.2 Experimental

This section describes the process by which the hKtRNA transcript is isolated from the crude in vitro transcription reaction. Commercially obtained yFtRNA, which has identical mobility to hKtRNA under these electrophoresis conditions, was used as molecular weight marker and intensity standard for densitometric quantitative analysis. The purification procedure is summarized below in Chart 3.

![Chart 3: Overview of transcript purification procedure by urea denaturing PAGE.](chart3)

4.2.1 Purification of hKtRNA on 20cm 8M urea denaturing 12%PAGE

Crude transcription reaction mixtures were centrifuged to precipitate the magnesium pyrophosphate, and the liquid portions pooled into one tube and combined with 5X gel loading buffer (.004g BPB, .004g XCFF, .25g Ficol, .48g Urea, 500μL TBE in 1mL RNAse Free water), and run on a 20cm long 8M urea denaturing 12%PAGE at 10-15V/cm. Once the bromophenol blue tracking dye reached the bottom edge of the gel, the bands were excised by UV shadowing. UV shadowing is performed by laying the gel atop a TLC plate wrapped with clear plastic wrap. A metal spatula, treated with RNAse Erase spray and baked at 180ºC for at least 4 hours then cooled to room temperature, is used to excise the hKtRNA transcript band with the aid of a handheld UV lamp. The recovered gel slices are crushed and immersed in 5mL gel elution buffer (1mM EDTA, 0.2% SDS, 0.5M Ammonium Acetate) and left to shake at 37ºC overnight. The elution buffer was passed through a sterile 0.45μm filter to remove insoluble gel remnants, and then extracted with an equal volume 5:1 acidic phenol: chloroform to remove proteins and free nucleotides. A second extraction with an equal volume of chloroform was done to remove traces of phenol. The resulting sample was butanol concentrated to ~400μL and then precipitated with 2.5 volumes EtOH at -20ºC overnight. In the case of the transcript obtained from the 5 Prime pLysB19 template, a second 5mL aliquot of gel elution buffer was used to rinse the crushed gel slice. This rinse was processed separately and re-suspended in a separate aliquot.
of 50μL RNAse free water as described below. The sample was centrifuged at 15000G for 30 minutes at 4°C. The pellet is washed with 70% ethanol for 5 minutes at room temperature to remove excess salt followed by centrifugation at 15000G for 5 minutes at 4°C. The hKtRNA pellet is then re-suspended in 50μL RNAse free water. The transcript obtained from the Purelink pLysB19 based template also had a separate 5mL gel extraction buffer rinse, but the pellets were resuspended sequentially in the same 50μL RNAse Free water aliquot. An aliquot of each sample was removed and diluted for analysis by UV absorption spectrometry and comparison to yFtRNA on 8M urea denaturing 12% PAGE as shown in the Results.

4.2.2 MALDI-MS

Mass spectrometry analysis was done by the UC mass spectrometry facility to determine the purified hKtRNA transcript molecular weight.

4.2.3 Incubation tests

The incubation tests were assembled using 1μL of the crude in vitro transcription reaction in a total volume of 5μL and incubated at 37°C overnight to simulate the incubation step during the gel purification procedure described above. Five incubations were performed to determine the extent of transcript degradation in a variety of conditions as described in Table 4. The solutions used to assemble these tests were RNAse Free water, gel elution buffer (1mM EDTA, 0.2% SDS, 0.5M Ammonium Acetate), gel elution buffer acidified to pH 6 with glacial acetic acid, 1M MgCl₂, or 200mM EDTA. In order to probe whether any degradation could be attributed to trace RNAse contamination, the same five incubations were also repeated using RNASecure treated solutions to assemble the incubations (RNAse Free water, gel extraction buffer, acidified gel extraction buffer, 1M MgCl₂, and 200mM EDTA were each treated with RNASecure prior to use in assembling the incubation). A portion of each of the incubations was analyzed on 8M urea denaturing 12% PAGE, alongside a control non-incubated aliquot of the crude in vitro transcription reaction.
<table>
<thead>
<tr>
<th>Incubation</th>
<th>Assembly</th>
<th>Conditions</th>
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</thead>
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<tr>
<td>Water</td>
<td>1μL Crude transcript,</td>
<td>8mM Tris, 7.2mM MgCl₂, 2mM DTT, 0.4mM spermidine, 0.002% triton X-100, pH 8</td>
</tr>
<tr>
<td></td>
<td>4μL RNase free water</td>
<td></td>
</tr>
<tr>
<td>Gel extraction buffer</td>
<td>1μL Crude transcript,</td>
<td>8mM Tris, 7.2mM MgCl₂, 2mM DTT, 0.4mM spermidine, 0.002% triton X-100, 0.8mM EDTA, 0.16% SDS, 0.4mM Ammonium Acetate, pH 8</td>
</tr>
<tr>
<td></td>
<td>4μL Gel elution buffer</td>
<td></td>
</tr>
<tr>
<td>Acidified gel extraction</td>
<td>1μL Crude transcript,</td>
<td>8mM Tris, 7.2mM MgCl₂, 2mM DTT, 0.4mM spermidine, 0.002% triton X-100, 0.8mM EDTA, 0.16% SDS, 0.4mM Ammonium Acetate, pH 8</td>
</tr>
<tr>
<td>buffer</td>
<td>4μL Acidified gel elution buffer</td>
<td></td>
</tr>
<tr>
<td>High MgCl₂</td>
<td>1μL Crude transcript,</td>
<td>8mM Tris, 207.2mM MgCl₂, 2mM DTT, 0.4mM spermidine, 0.002% triton X-100, 0.6mM EDTA, 0.12% SDS, 0.3mM Ammonium Acetate, pH 8</td>
</tr>
<tr>
<td></td>
<td>3μL Gel elution buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1μL 1M MgCl₂</td>
<td></td>
</tr>
<tr>
<td>High EDTA (Low Mg²⁺)</td>
<td>1μL Crude transcript,</td>
<td>8mM Tris, 7.2mM MgCl₂, 2mM DTT, 0.4mM spermidine, 0.002% triton X-100, 40.6mM EDTA, 0.12% SDS, 0.3mM Ammonium Acetate, pH 8</td>
</tr>
<tr>
<td></td>
<td>3μL Gel elution buffer</td>
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<tr>
<td></td>
<td>1μL 200mM EDTA</td>
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</tbody>
</table>

**Table 4:** Summary of incubation test conditions probing effect of Mg²⁺ concentration and pH on hKtRNA degradation
4.3 Results & Discussion

4.3.1 UV Shadowing to Isolate hKtRNA

The crude transcription reaction is shown on a representative 20cm 8M urea denaturing 12%PAGE, imaged by UV shadowing (Figures 21). After excision of the hKtRNA band, the remainder of the gel is stained with EtBr (Figure 22).

Figure 21: Preparative scale purification of hKtRNA on 20cm long 8M urea denaturing 12% PAGE visualized by UV shadowing. The pLysB19 fragments, hKtRNA transcript and bromophenol blue dye fronts are indicated.
Figure 22: Purification gel shown in Figure 21 after excision of hKtRNA band and EtBr staining to visualize remaining nucleic acids. The pLysB19 fragments, removed gel slice containing hKtRNA transcript and bromophenol blue dye fronts are indicated.
4.3.2 Analysis of purified hKtRNA by UV Spectrometry and Urea Denaturing PAGE

The gel slice is crushed and the hKtRNA transcript is recovered as described in the experimental section. A portion of the purified hKtRNA transcript is diluted and analyzed by UV absorption spectroscopy. The concentrations are calculated using an ε_{260} of 604,000mol^{-1}cm^{-1}L and a molecular weight of 24,500g/mol (22, 34). The UV absorption spectrum of purified hKtRNA transcribed from 5 Prime pLysB19 suggests a total concentration of 6.5μg/μL with an A_{260}:A_{280} of 1.89, and A_{260}:A_{230} of 2.18 (Figure 23). As explained in the Experimental section, the gel remnants were rinsed with an additional volume of gel extraction buffer to determine whether the yield was significantly enhanced by recovering RNA bound to the crushed gel or whether this step could be omitted. The UV spectrum shows the concentration of this rinse was 1.0μg/μL by UV, with A_{260}:A_{280} of 1.94 and A_{260}:A_{230} is 2.27 (Figure 23). For the hKtRNA transcribed from Purelink pLysB19, The UV spectrum shows a concentration of 6.1μg/μL with A_{260}:A_{280} at 2.03 and A_{260}:A_{230} at 2.20 (Figure 23). A rinse was also performed with this purification, but the two pellets were sequentially re-suspended in a single aliquot of 50μL RNAse free water as described in the Experimental section.

The purified hKtRNA transcripts were also analyzed for quality and quantity on 8M urea denaturing 12% PAGE (Figure 24). Comparison to yFtRNA standards suggests a concentration of 6.2μg/μL for the 5 Prime based hKtRNA transcript, 0.94μg/μL for the rinse and 6.0μg/μL for the Purelink based hKtRNA transcript. Using the concentrations obtained by urea denaturing PAGE, the yield of purified hKtRNA transcribed from 5 Prime pLysB19 was 310μg for the main extraction and 47μg for the rinse, for a total of 357μg purified hKtRNA from 550μg in the crude transcription reaction, making the percent recovery 65%. The purified hKtRNA transcribed from Purelink pLysB19 had a total yield of 300μg from an initial 484μg in the crude transcription reaction for a 62% recovery.
Figure 23: UV absorption spectra of purified hKtRNA transcripts. **Legend A:** 1 in 410 dilution of purified hKtRNA transcript from 5 Prime kit. **Legend B:** 1 in 110 dilution of gel rinse for extraction A. **Legend C:** 1 in 410 dilution of purified hKtRNA transcript from Purelink template (pooled extract and rinse).

Figure 24: Analysis of purified hKtRNA transcript on 8M urea denaturing 12% PAGE visualized with sybr gold. **Legend:** Lanes 1,2,3,4: 25ng, 50ng, 75ng, 100ng, yFtRNA standard, Lane 5,6: 0.25μL, 0.5μL of 1 in 60 dilution of purified hKtRNA transcript from 5 Prime pLysB19 based template (1st extraction of crushed gel slice). Lanes 7,8: 0.25μL, 0.5μL, of 1 in 10 dilution of purified hKtRNA transcript using 5 Prime based pLysB19 template (rinse of crushed gel slice). Purelink pLysB19 based template. Lanes 9,10: 0.25μL, 0.5μL, of 1 in 10 dilution of purified hKtRNA transcript using Purelink based pLysB19 template (1st extraction and rinse pooled).
4.3.3 Effects of Mg$^{2+}$ Concentration and pH on Degradation of hKtRNA

It was noted that some degradation appears during PAGE analysis of final purified transcript, which is consistent with the difference in the quantitative results between the UV and densitometry methods (Figures 23, 24). It was hypothesized that these degradation products developed during the incubation step of the purification process. It is known that incubation of RNA in the presence of Mg$^{2+}$ at high temperatures and/or elevated pH can contribute to degradation (37). Overnight incubations modeling the incubation step during the gel purification process were conducted to test the effects of Mg$^{2+}$ concentration and pH on hKtRNA degradation (Figure 25). No difference was observed between overnight incubations at 37ºC in high and low Mg$^{2+}$ concentrations (Figure 25, lane 5&11 incubations contained 207.2mM MgCl$_2$ and 0.6mM EDTA, lane 6&11 incubations contained 7.2mM MgCl$_2$ and 40.6 mM EDTA). No difference was observed between overnight 37ºC incubations at acidic and slightly basic pH (Figure 25, lanes 3&8 incubations were pH 8, and lanes 4&9 incubations were pH6). No benefit was gained by RNASecure treating solutions used in the incubations since no difference in the degradation pattern is observed when this treatment is omitted (Figure 25, Box A is not RNASecure treated, Box B is RNASecure treated). These observations suggest the observed hKtRNA degradation is not due to Mg$^{2+}$ concentration, pH or RNAse contamination in the incubation step. However, using EDTA to quench the reaction and chelate Mg$^{2+}$ as described elsewhere is a convenient and simple step that circumvents concerns of Mg$^{2+}$ playing a role in transcript degradation.
Figure 25: Analysis of overnight 37°C incubation tests on 8M Urea denaturing 12% PAGE visualized with sybr gold. **Legend A:** Lane 1: 250ng Control un-incubated crude transcription reaction. Lane 2: 250ng crude transcript in 8mM Tris, 7.2mM MgCl₂, 2mM DTT, 0.4mM spermidine, 0.002% triton X-100, pH 8. Lane 3: 250ng crude transcript in 8mM Tris, 7.2mM MgCl₂, 2mM DTT, 0.4mM spermidine, 0.002% triton X-100, 0.8mM EDTA, 0.16% SDS, 0.4mM Ammonium Acetate, pH 8. Lane 4: 250ng crude transcript in 8mM Tris, 7.2mM MgCl₂, 2mM DTT, 0.4mM spermidine, 0.002% triton X-100, 0.8mM EDTA, 0.16% SDS, 0.4mM Ammonium Acetate, pH 6. Lane 5: 250ng crude transcript in 8mM Tris, 207.2mM MgCl₂, 2mM DTT, 0.4mM spermidine, 0.002% triton X-100, 0.6mM EDTA, 0.12% SDS, 0.3mM Ammonium Acetate, pH 8. Lane 6: 250ng crude transcript in 8mM Tris, 7.2mM MgCl₂, 2mM DTT, 0.4mM spermidine, 0.002% triton X-100, 40.6mM EDTA, 0.12% SDS, 0.3mM Ammonium Acetate, pH 8. **Legend B:** Lanes 7-11: Same order as lanes 2-5 except that all solutions were individually treated with RNAsure prior to use.
4.3.4 Analysis of Purified hKtRNA by MALDI-MS

A MALDI-MS peak at 25.1kDa was obtained for the purified hKtRNA transcript whereas the calculated molecular weight is 24.5kDa (Figure 26). The molecular weight is larger than expected, but is within expected error range for MALDI-MS for this particular RNA (+/- 5% or 1kDa).

Figure 26: MALDI-MS of purified hKtRNA shows peak at 25.1kDa
4.4 Conclusions

The reported aggregation of denatured RNA in the wells of purification gels was observed, but was not especially problematic with this hKtRNA transcript (Figure 22). The hKtRNA transcript was purified from the crude transcription reaction to better than 60% yield. The A\textsubscript{260}:A\textsubscript{280} ratios were $\geq 1.9$ suggesting a pure sample, since A\textsubscript{260}:A\textsubscript{280} ratio of 1.8 or higher indicates protein free RNA (Figure 23). The UV quantitative values reflect the amount in the total absorption due to all the RNA’s present, while the densitometric values reflect the amount in the hKtRNA band. This difference was used to estimate the purity of the transcript. The purity was found to range between 94 and 98% for the purifications shown in this section. The estimated 2-6% impurities are visible as bands below the transcript band on urea denaturing PAGE (Figure 24).

The incubation tests suggest that some degradation is inevitable when working with RNA in aqueous solutions, since the degradation pattern was not significantly affected by variations in Mg\textsuperscript{2+} concentration or pH (Figure 25). This is supported by the observation that a similar amount of degradation was observed in commercially obtained yFtRNA (Figure 24). However, quenching with EDTA to a final concentration of 0.05M is a convenient way to sidestep concerns of Mg\textsuperscript{2+} catalyzed hKtRNA degradation, and is recommended for future purifications. Additionally, it is generally recommended that RNA should be stored as an ethanol precipitate for long term storage, since it is subject to hydrolysis in aqueous buffers (35). It is unknown at this time whether this purified transcript is useful for NMR studies due to the reported interference of water soluble acrylamide contaminants in such experiments. In the event that significant interference is observed, it may be possible to remove NMR interfering species by micro-dialysis. The mass spectrometry shows a molecular weight that is ~600Da larger than expected, but is within the +/-1kDa error expected for MALDI-MS of large nucleic acids. A more accurate mass for the hKtRNA transcript has yet to be determined. It will be necessary to use alternative mass spectrometry methods such as electro-spray with special sample preparation steps to remove cations (45).
5. Aminoacylation

5.1 Introduction

This section focuses on describing the method by which optimal resolution of aminoacylated and non-aminoacylated hKtRNA can be achieved on acidic urea denaturing PAGE. The aminoacylation assays analyzed by urea denaturing PAGE were focused on determining the total percentage of tRNA aminoacylated after 30 minutes. Preliminary results for these assays will also be presented, which will hopefully serve as a guide for future experiments. Analysis of aminoacylation assays on acidic urea PAGE is dependent on the differential mobility of acylated vs. non-acylated (36). The mobility differential is largely due to the extra positive charge of the attached amino acid (37). The primary differences from more routine urea denaturing PAGE are the length of the gel to allow for sufficient resolution of the closely migrating species, as well as use of low wattage, acidic pH, and low temperature. These precautions help to preserve the fragile aminoacyl bond which is susceptible to alkaline hydrolysis (36). Aminoacyl bond stability is low, like other ester bonds with neighboring hydroxyl groups, but the electron-withdrawing effect of the lysine side group also causes tRNA^Lys to be one of the least stable aminoacylated tRNA’s (36, 38-40).

An alternative aminoacylation assay based on detection of 3H labeled lysine will also be presented. This assay aims to measure the initial aminoacylation reaction velocity (first 160 seconds). The technique is dependent on TCA/ethanol precipitation of tRNA onto cellulose membranes which are then analyzed using a liquid scintillation counter. The data shown from this ‘charging assay’ is not directly comparable to the aminoacylation assays on acidic urea PAGE, but may prove useful to either complement or confirm future aminoacylation results.
5.2 Experimental

Analytical scale aminoacylation assays were performed as described by Walker & Fredrick (36). The procedure and analysis are summarized below (Chart 4).

**Chart 4:** Summary of aminoacylation assay procedure and analysis on acidic urea denaturing PAGE.

### 5.2.1 hKRS preparation

A recombinant fusion hKRS protein comprising a TEV cleavage site and N-terminal histidine tag was expressed in BL21 strain E.coli. The protein was purified by nickel column affinity chromatography, followed by TEV cleavage and dialysis to remove the histidine tag. The TEV, which has a histidine tag, was removed by purification through a second nickel column which allows now cleaved hKRS to pass through. Each step was confirmed by SDS-PAGE and the final concentration was determined by UV. Protein concentrations were determined by UV after dialysis into 100mM HEPES-KOH pH 7.6, using an extinction coefficient of 0.643(mg/mL)^-1 cm^-1 and a molecular weight of 68,048g/mol, and checked for degradation by SDS-PAGE.

### 5.2.2 tRNA preparation

The hKtRNA transcript, or yFtRNA in the case of one assay, were renatured by heating to 80°C for 2 minutes, 60°C for an additional 2 minutes, followed by addition of 10mM MgCl₂.
from a 10X stock in RNAse Free water, and stored on ice until use (22). It is recommended that tRNA is buffered with 0.05M HEPES or phosphate buffer prior to heating and MgCl\textsubscript{2} addition to stabilize pH during renaturation. The ramification of omitting this step is further discussed in the conclusion of this section.

**5.2.3 Aminoacylation Assays for Analysis on Acidic Urea PAGE**

The reactions were assembled by mixing water, aminoacylation buffer from a 10X stock to the following final concentrations (100mM HEPES-KOH pH7.6, 100μM Lysine, 1mM DTT, 10mM KCl, 20mM MgCl\textsubscript{2}, 10mM ATP (from freshly dissolved 10X stock in RNAse Free water), 1μg (8.1μM) hKtRNA transcript, and hKRS diluted to a convenient 5X stock with 100mM HEPES-KOH pH7.6. The total assay volume was 5μL. A “mock” aminoacylation assay with no hKRS was performed alongside each aminoacylation assay as a control. The assays were then incubated at 37°C for 30 minutes, and precipitated by addition of 1/10th volume sodium acetate pH5.2, and 2.5 volumes of ethanol. The precipitation was allowed to proceed for 2-12 hours, and then centrifuged at 10,000G for 30 minutes at 4°C. The pellets were rinsed with 70% ethanol, followed by a brief centrifugation at 10,000G for 5 minutes at 4°C. The air dried pellets were directly resuspended in 20μL of acidic gel loading buffer (100mM NaOAc pH5.2, 7M urea, 0.05% bromophenol blue). Thus, the approximate concentration of the sample was 50ng/μL and this was used to estimate the amount loaded on each aminoacylation gel. The gels were prepared and run as described in Appendix A.

**5.2.4 tRNA Charging Assays with 3H Labeled Lysine**

The tRNA charging assays using 3H labeled lysine were performed in collaboration with the Dr. Karin Musier-Forsyth group (OSU). The procedure is briefly described below. A 2X Mix is prepared (0.2mg/mL BSA, 40mM KCl, 40mM BME, 18mM MgCl\textsubscript{2}, 100mM HEPES, 8mM ATP, 40μM lysine, and a negligible concentration of 3H lysine). The reaction mix is then assembled (23μL of 2X mix, 1μM tRNA, RNAse free water to a total volume of 46μL, the mix is incubated at 37°C and a 8μL aliquot is removed for determining baseline counts per minute, and the desired concentration of hKRS is then added to a new total volume of 40μL). The aliquot for determination of baseline serves to determine how much lysine remains on the filter pads in the absence of aminoacylation. Cellulose filter papers pre-wetted with 5% TCA in EtOH are
used to absorb all aliquots. Once the hKRS is added, 8μL aliquots are removed at several time points and applied to the pre-wetted filter paper. The filter paper is treated with several rinses of 5% TCA/EtOH to remove free lysine followed by one rinse with pure EtOH. The filter papers are then allowed to dry and inserted into counting tubes and immersed in scintillation fluid. A liquid scintillation counting machine is used to obtain counts per minute (CPMs). CPM’s are baseline corrected and the pmol of lysine on each filter is calculated using the specific activity. The specific activity is determined using three 8μL aliquots of prepared ‘reaction mix’ on filter paper which is not rinsed with 5% TCA in EtOH to wash away free lysine (allows determination of CPMs per pmol lysine present). Since aminoacylation stoichiometry is 1:1 lysine: tRNA, the pmols of lysine on the filter paper directly represent pmols of tRNA aminoacylated at each time point.
5.3 Results & Discussion

5.3.1 Quantifying Aminoacylation Extents by Line Densitometry

The method of quantifying aminoacylation extents shown here is based on the 1-D Multi line densitometry analysis tool in the AlphaEase FC gel imaging software (Alpha-inotech). The tool generates a trace where two peaks correspond to two bands in one lane of the gel (Figure 27). The total area under the peaks is considered 100% and the area under each peak is used to estimate the relative percentage of tRNA in each band. It was desirable to gauge the accuracy of this method, since it will be employed to determine aminoacylation extents in several assays. This was accomplished through analysis of mixtures of known composition. These mixtures were composed of hKtRNA and yFtRNA, since the differential mobility of these two species on acid urea PAGE was close to that observed for aminoacylated and non-aminoacylated hKtRNA. The 1D-Multi quantifying tool with a ‘rubber-band’ baseline gave values within 5% of the expected values when the peaks were manually determined (Figure 27). This method was used to quantify aminoacylation extents and estimate the error in each of the aminoacylation assays described in this section.

![Figure 27: Legend A: Extent of ‘aminoacylation’ standards using various known mixtures of yFtRNA and hKtRNA on 29cm long acidic 8M urea denaturing 6.5%PAGE visualized with EtBr. Legend B,C,D,E: traces correspond to the four lanes in A. The actual composition of sample is shown at top of each trace, and amount estimated by line densitometry is shown below. The estimated error of the line densitometry method is less than 5%.](image)
5.3.2 Results of Several Aminoacylation Assays

Aminoacylation reactions were assembled and analyzed as described in the Experimental section. Aminoacylation controls containing either BSA in place of hKRS, or yFtRNA in place of hKtRNA showed no detectable aminoacylation (Figure 28). Aminoacylation was only observed in assays that contained both hKRS and its cognate hKtRNA transcript (Figures 28, 29). Similar aminoacylation extents were found for our hKRS (70-74%) and a similar hKRS construct kindly provided by Dr. Karin Musier-Forsyth, OSU-hKRS (67-76%), even when 3μM of OSU-hKRS was used as compared to 1μM of hKRS made in this lab (Figures 29, 30). An assay using 0-1μM OSU-hKRS shows aminoacylation is visually observed at hKRS concentrations as low as 150nM (Figure 31). However, the observed aminoacylation extent did not increase over the hKRS range of 500nM-1μM (Figures 31, 32).

![Figure 28: Control aminoacylation assays using yFtRNA in place of hKtRNA, and BSA in place of hKRS on acidic 29cm long 8M urea 6.5% PAGE visualized with EtBr. Legend: Each box represents a separate assay with lane 1 containing a 1:1 mixture of actual and mock assays, Lane 2 contains mock assay (no protein), and Lane 3 actual assay. Reaction conditions are indicated at the top of each box. hKtRNA and yFtRNA are shown in outside lanes for reference.](image-url)
**Figure 29:** Comparing aminoacylation extents of OSU-hKRS to hKRS made in house on acidic 29cm long 8M urea denaturing 6.5% PAGE visualized with EtBr. **Legend:** Each box represents a separate assay with lane 1 containing a 1:1 mixture of actual and mock assays, Lane 2 contains mock assay (no protein), and Lane 3 actual assay. Reaction conditions are indicated at the top of each box. hKtRNA and yFtRNA are shown in outside lanes for reference.

**Figure 30:** Summary and comparison of aminoacylation extents for some assays in figures 28, 29, 31 as determined by line densitometry. hKtRNA concentration was 8.1μM in all cases. PTS hKRS refers to hKRS made in house. Error bars estimated as described previously in figure 27.
**Figure 31:** Aminoacylation assay of 8.1μM hKtRNA by varying amounts of OSU-hKRS on acidic 29cm long 8M urea denaturing 6.5% PAGE visualized with EtBr. The OSU-hKRS concentration is indicated above each band.

**Figure 32:** Summary of aminoacylation extents for figure 31 as determined by line densitometry. Error bars estimated as described previously in figure 27.
5.3.3 Improving Detection and Resolution on Acidic Urea PAGE

The method for estimation of aminoacylation extents on acidic urea page in assays thus far shown has two problems. The aminoacylated hKtRNA band is not fully resolved from the non-aminoacylated hKtRNA band. Also, the individual bands corresponding to aminoacylated and non-aminoacylated hKtRNA are diffuse (Figures 28, 29, 31). These diffuse poorly resolved bands resulted in poorly resolved peaks when analyzed by line densitometry using the AlphaEase FC software (Figure 33). One approach to improve band resolution is to increase percentage acrylamide of the acid urea gel. Another approach is to simply increase the gel length in order to allow more space for resolution. In addition, sybr gold may increase band sharpness and definition due to its superior sensitivity and lower background as compared to ethidium bromide. Sharp well-defined bands are expected to contribute to resolving closely migrating bands.

Figure 33: Typical line densitometry peaks obtained for the aminoacylated and non-aminoacylated hKtRNA bands shown in figures 28, 29, and 31. Peak 1 is aminoacylated hKtRNA, and Peak 2 is non-aminoacylated hKtRNA. Actual bands analyzed are shown as horizontal strip at bottom. (AlphaEase FC, Alpha-inotech Corp.)
An acidic 8M urea 8% PAGE was run to test if aminoacylated vs. non-aminoacylated tRNA bands could be better resolved from each other. The 29cm long gel was electrophoresed at 10V/cm (9-10 Watts) for a total of 15 hours (samples were actually on the gel 22hr). Identically loaded halves of the gel were stained with either ethidium bromide or sybr gold. The hKtRNA bands traveled 10.5cm into the 8% gel after 15 hours of electrophoresis (Figure 34). By comparison, hKtRNA bands travel ~25cm after 12 hours of electrophoresis on 6.5% PAGE run at 10V/cm. At this rate, it would take approximately 36 hours for the bands to migrate 25cm on 8% PAGE. Analysis of aminoacylation on 8% PAGE acid urea is impractical due to the amount of time required to obtain full resolution and concerns of spontaneous de-acylation on the gel. The aminoacylation extent could not be estimated due to the extremely poor resolution of the bands, although it is visually apparent that significantly less than 50% of the hKtRNA was aminoacylated, as compared to previous findings of ~70% on 6.5% PAGE (Figure 34). It is not known whether this decrease in aminoacylation extent is due to the amount of time the samples spent on the gel or whether hKRS lost activity during storage. It was, however, observed that tRNA bands were sharper when visualized with sybr gold. Sybr gold is more sensitive and has lower background than EtBr allowing less tRNA to be loaded which should allow better resolution of closely migrating bands.

Figure 34: Aminoacylation assay (8.1μM hKtRNA and 1μM hKRS) on acidic 29cm long 8M urea denaturing 8% PAGE visualized with ethidium bromide or sybr gold. **Legend from left to right for both halves:** Lane 1: 250ng hKtRNA negative control aminoacylation assay, Lane 2: 250ng of aminoacylation assay, Lane 3: Combination of lanes 1&2 (500ng total hKtRNA), Lanes 4: Reference hKtRNA, Lane 5: Reference yFtRNA.
A longer gel (37cm) in conjunction with more defined bands due to the sensitivity and lower background of Sybr gold allows near-full resolution of aminoacylated hKtRNA from non- aminoacylated hKtRNA as shown below (Figure 35). The 37cm gel is run at the same V/cm and wattage as the previously used 29cm gels (10V/cm, 9-10W). The electrophoresis time was increased from 12 hours to 18 hours, however. A modification to the gel casting method allowed the reclamation of 3-4cm at the bottom of the gel that were previously unusable, in addition to the 8cm gained by using 37cm long plates in place of 29cm plates. This minor improvement is fully described in Appendix A. The resolution between the line densitometry peaks is much improved, permitting more definitive determination of relative area under each peak (Figure 36).

**Figure 35:** Aminoacylation assay on acidic 37cm long 8M urea denaturing 6.5% PAGE visualized with sybr gold. **Legend from left to right:** Lane 1: 150ng yFtRNA standard, Lane 2: 150ng Free hKtRNA, Lane 3: 150ng hKtRNA-Lysine.
**Figure 36:** Line densitometry peaks obtained for the aminoacylated and non-aminoacylated hKtRNA bands shown in figure 35. Estimated aminoacylation extent is 74%. Peak 1 is aminoacylated hKtRNA, and Peak 2 is non-aminoacylated hKtRNA. Actual bands analyzed are shown as horizontal strip at bottom. (Alphaease FC, Alpha-inotech Corp.)
5.3.4 Results of hKtRNA Charging Assays with 3H Lysine

The pmols of tRNA aminoacylated with 3H lysine at several time points using a variety of hKRS batches and concentrations is shown (Figure 37A). The initial velocity of each assay is also shown (Figure 37B). The highest initial velocity per concentration unit of hKRS (maximum activity) was observed for freshly prepared OSU-hKRS. The remaining velocities were divided by concentration of hKRS in a given assay and expressed as a percentage of the maximum activity to enable comparison (Far right column of Table 5). For ‘old’ hKRS (TEV cleaved and dialyzed into 100mM HEPES-KOH pH 7.6 four months prior to the experiment), the initial velocity increased 11 fold over the enzyme range of 25nM to 275nM (also an 11 fold increase). Thus ‘old’ hKRS had approximately 8.33-8.44% of the activity of freshly prepared OSU-hKRS, regardless of concentration. For ‘new’ hKRS the initial velocity only increased 2 fold over the enzyme range of 25nM to 340nM, a 13.6 fold increase in concentration. This translates to 8 and 51% activity of the fresh made OSU-hKRS (for 340nM and 25nM ‘new’ hKRS respectively). This variation in activity after correction for hKRS concentration suggests there was an excess of hKRS at 340nM. These results, although preliminary, show that hKRS activity is a variable which must be considered not only from batch to batch of hKRS, but also within each batch over time. The results for a freshly prepared and dialyzed hKRS batch (less than one month old) show 31 to 37% activity of the freshly prepared OSU-hKRS, however this low activity may be due to over-estimation of the enzyme concentration.
Figure 37: Charging assays of 1μM hKtRNA with 3H lysine. **Legend A:** pmols tRNA charged over the course of the 160 second assay. **Legend B:** Expanded view of first 40 seconds used to determine initial velocity. **Legend for A and B:** Control charging assay with no hKRS (blue diamond), Control charging assay with no hKtRNA (red square). **KMF LysRS** refers to freshly prepared OSU-hKRS, **GST LysRS** refers to GST-tagged OSU-hKRS, **Tsang Old** refers to 4month old hKRS, **Tsang New** refers to freshly dialyzed 4month old hKRS, **Tsang Cleaved** refers to freshly dialyzed 1month old hKRS

<table>
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<tr>
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<td></td>
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</tr>
<tr>
<td>no Enzyme</td>
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</tr>
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</tbody>
</table>

Table 5: Initial velocity of hKtRNA charging assays shown in figure 37. Activity is defined as initial velocity over hKRS concentration. The activity is also expressed as a percentage of the most active hKRS in this experiment.
5.4 Conclusions

It has been shown here that aminoacylated hKtRNA can be fully resolved from non-aminoacylated hKtRNA by acid urea denaturing PAGE. Although it is possible to make only very limited comparisons between the assays performed so far, it may prove to be useful for establishing the maximum aminoacylation extent possible for hKtRNA. Since the 3’CCA tRNA acceptor terminus is required for recognition and aminoacylation by hKRS, the results found here that ~70% of the transcript can be aminoacylated suggests the primary transcript population has the correct 3’CCA terminus (41). The finding that hKtRNA aminoacylation extent was dependent on hKRS concentration may also allow evaluation and comparison of activity for hKRS.

It is not known why ~70% appears to be the upper limit of aminoacylation extent possible, even with hKRS concentrations as high as 3μM. Aminoacylation extents ≥90% are routinely observed elsewhere (36). There is a possibility that a population of transcripts cannot be aminoacylated due to incorrect sequence or even mis-folding. The presence of mis-folded tRNA’s may be a consequence of the omission of buffer to adjust pH during tRNA renaturation. The 75% limit on aminoacylation extent observed here may also reflect a post aminoacylation reaction equilibrium where up to 25% of hKtRNA is spontaneously de-acylated. This is a possibility since aminoacylated tRNA^Lys is among the least stable aminoacylated tRNA’s due to the electron-withdrawing effect of the lysine side group and inherent instability of the aminoacyl bond (36, 38–40).

The aminoacylation assays analyzed on acidic urea PAGE shown here focus on the determination of the maximum percentage of hKtRNA which can be aminoacylated. By contrast, the tRNA charging assay using 3H lysine focuses on comparing the activity of several hKRS batches. Although the results for each of these methods are not directly comparable, they are complementary and may prove to be a means of evaluating hKRS activity and hKtRNA ‘aminoacyl-ability’ for future experiments.
Materials

The following chemicals were obtained from Fisher Scientific: Luria broth (cat# BP1426), EDTA (cat# S311), sodium chloride(cat# S271), sodium acetate (cat# S210), glacial acetic acid (cat# A38), phenol (cat# BP1751), chloroform (cat# C298), Boric Acid (cat# A74), XCFF (cat# BP565), ammonium acetate (cat# A637), butanol (cat# A399), potassium hydroxide (cat# P250), sodium hydroxide (cat# S318), potassium acetate (cat# P171), sodium chloride (cat# P217), triton X-100 (cat# BP151), acrylamide (cat# BP170), bis-acrylamide (cat# BP171), tryptone (cat# BP1421), yeast extract (cat# BP1422), magnesium sulfate (cat# M65).

The following chemicals were obtained from RPI Corporation: Ampicillin (cat# A40040), Tris-HCl (cat# T60040), urea (cat# U20200), HEPES (cat# H75030), DTT (cat# 26344), lysine (cat# L37040), ATP (cat# 28112)

The following chemicals were obtained from MP Biomedicals, LLC: SDS (cat# 811034), RNAse Free Water (cat# MP821793), RNAse Erase (cat# 821682)

The following chemicals were obtained from Acros Organics: Ethidium bromide (cat# 170960010), spermidine (cat# 132740010), TEMED (cat# 420580500), APS (cat# 327081000)

The following chemicals were obtained from Sigma Aldrich: yeast phenylalanine tRNA (cat# R4018), bromophenol blue (cat# B-7021), guanidine HCl (cat# G4505), BSA (cat# A2153)

The Purelink HiPure Plasmid Filter Kit (cat# 1147565) and sybr gold (cat# S11494) were obtained from Invitrogen Corporation. Agarose (cat# 5510UB) was obtained from BRL. Ribonucleotide triphosphates (set of ATP, CTP, GTP, UTP) (cat# E6000) were obtained together from Promega Corporation. The Perfectprep Endofree Maxi Kit (cat# 2300120) was obtained from 5 Prime Products. DH5α Strain E.coli (cat# 961) were obtained from Protein Express, Inc. Ethanol (cat# E200) was obtained from Aaper Alcohol Co. HCl (cat# 284000ACS) was obtained from Pharmco Products, Inc. Restriction enzyme, BglII and its digestion buffer (cat# R0143S) were obtained from New England Biolabs. RNASecure (cat# AM7005) was obtained from Ambion, Inc. Magnesium chloride (cat# MX0045-2) was obtained from EM Chemicals. TLC plates (cat# 5735) were obtained from Merck Pharmaceuticals.
Future Work

The overall goal in this lab is to study interactions between hKtRNA and a variety of hKRS constructs with a special emphasis on the N-terminal extension of hKRS. Currently, these interactions are studied using techniques such as electrophoretic mobility shift assays, aminoacylation assays on acidic urea PAGE, and circular dichroism to a lesser extent. The production and purification of milligram quantities of hKtRNA, described here, is one step towards expanding use of techniques which require large amounts of hKtRNA such as circular dichroism, and possibly other methods such as calorimetry or NMR.

In the future, it may also be advantageous to study hKRS interactions with hKtRNA partial transcripts and/or mutant variations. The process described here may serve as a blue print for the optimization of transcription conditions to new and as yet undeveloped templates. The conclusion that reduced T7RNAP activity was responsible for requirement of higher template concentrations than previously reported shows that improvements to this component of the transcription reaction may substantially reduce the amounts of template required. The reduction of required template amounts together with the regeneration and re-use of commercial plasmid purification columns described here should allow for the production of ever greater quantities of a wide variety of RNA’s at substantially reduced costs. It may also be possible to pack plasmid purification columns in house to replace the columns from commercial kits, thereby further reducing cost.

The aminoacylation assays described here may also be used as a guide for testing and comparing the activity of various hKRS constructs. It may also be possible to determine some enzymatic kinetics from the acidic urea PAGE aminoacylation assays. Currently, full resolution of aminoacylated hKtRNA from non-aminoacylated hKtRNA has been demonstrated. It may however be necessary to use lower concentrations of hKtRNA and possibly lowering the detection limits in order to maximize the information obtainable from aminoacylation assays analyzed on acidic urea PAGE. The use of tRNA charging assays with 3H may also be used to complement or confirm results determined by acidic urea PAGE.
References

2) Web: http://tainano.com/chin/Molecular%20Biology%20Glossary.htm
14) Promega website: http://www.promega.com/education/unit004/DNA_Purification_Final.ppt
17) Ambion's TechNotes Newsletter 8:4, © 2001
34) University of North Carolina Website: http://www.unc.edu/~cail/biotool/oligo/
42) Unpublished Communications. Mike Howell. Protein Express, Inc., Cincinnati, Ohio 45241.
Appendix A: Electrophoresis Methods

Agarose gel electrophoresis

Gels were prepared by addition of 210mg or 600mg agarose to 30mL 1X TAE for 0.7% or 2% gels, respectively. The mixture is boiled briefly while stirring until agarose is completely dissolved. The mixture is then cooled to 50°C-60°C, and EtBr is added to a total concentration of 0.75μg/μL and mixed thoroughly (A 15mg/mL ethidium bromide stock is helpful). The mixture is poured into a slab, the comb placed, and the gel is left to set at room temperature for 30 minutes. The comb can then be removed and the gel is submerged in 1X TAE in a horizontal electrophoresis chamber. Samples are prepared by mixing with 1-2μL of 5X loading buffer (0.25% BMB, 0.25% XCFF, 30% glycerol in TE) and brought up to a final volume of 5-10μL with water, if necessary. The samples are loaded on the gel and run at 100V constant voltage. The progress of sample migration can be monitored by the tracking dyes, but the incorporation of EtBr in the gel also allows direct tracking of the bands with a handheld UV lamp. Once acceptable resolution is achieved, the gel is photographed using the Alpha-inotech gel imager.

Urea denaturing polyacrylamide gel electrophoresis

A gel mixture containing 12% acrylamide (19:1 acrylamide: bis) and 8M urea in 1X TBE buffer pH8 is made (4.5mL 40% 19:1 acrylamide: bis, 7.2g urea, 7.5mL 2X TBE pH 8 in a total volume of 15mL). Immediately prior to casting, 8μL of TEMED and 80μL of 10% APS are added for every 10mL of gel mixture and mixed thoroughly. The gels were cast into 10cm long glass plates using 1mm spacers and allowed to polymerize with a comb in place for 45 minutes at room temperature. The gels were pre-run for 30-45 minutes in a 1X TBE running buffer at 25mA constant current. Samples were prepared by mixing with 2μL 5X gel loading buffer (0.004g BPB, 0.004g XCFF, 0.25g ficoll, 0.48g urea, 5XTBE in a total volume of 1mL), and the total sample volume is adjusted to a total volume of 10μL with RNase free water. The samples are run at 25mA constant current until the bromophenol blue tracking dye reached the bottom third of the gel. The gels are then stained in 1X TBE containing sybr gold (Invitrogen) for 30-45 minutes, and photographed on the Alpha-inotech gel imager. Purification gels required 50mL of gel mixture, were poured into 20cm long plates with 1.5mm spacers, and imaged by UV shadowing as previously described.
Acidic urea denaturing polyacrylamide gel electrophoresis

Acidic urea gels used for analysis of aminoacylation assays were either 29cm or 37cm long. The spacers and combs were cut from 0.8mm thick Teflon sheets, and the spacers were greased to provide a seal against the glass plates which were obtained from Southern Glass, Inc. The glass plates were held together using large binder clips. The gel mix was prepared by dilution of 3M NaOAc to 100mM final concentration (3.33mL) along with 8M urea (48g), and 6.5% 19:1 acrylamide (16mL of 40% 19:1 acrylamide: bis stock), in a total volume of 100mL adjusted to pH 5.2. The 8% gel used 20mL of 40% 19:1 acrylamide: bis, but was otherwise identical. Immediately before gel casting, 8μL of TEMED and 80μL of 10% APS were added and mixed for every 10mL of gel mixture. Usually, a 10mL portion of the gel mix was used to pour the bottom 3-4cm of the gel, and allowed to polymerize to form a plug at the open bottom end of the glass plates. The rest of the gel was then poured and left to polymerize for several hours with the comb in place. A minor alteration was made to the casting procedure through the use of a 1.0mm rubber string available at many craft stores. The rubber string was greased and stretched until it could be fitted between the glass plates and wrapped around the outside of the Teflon spacers and along the open bottom end of the glass plates to create a U-shaped seal. Gels cast in this manner can be poured in a single step, omitting the polyacrylamide plug to seal the open bottom, and allowed to polymerize as before. The rubber string is gently pulled out to expose the bottom edge of the gel to the lower buffer chamber and the comb is removed to expose the wells to the upper buffer chamber. The apparatus was then placed in a cold case and electrophoreses is conducted at 10ºC ambient temperature. The acidic gel running buffer (100mM NaOAc pH 5.2) was prepared and the gels were pre-run at 9-10W for several hours. Once the samples were loaded, the gels were run at a constant 9-10W (approximately 10V/cm) until the bromophenol blue tracking dye was near the bottom edge (approx. 12 and 18 hours, respectively for 29cm and 37cm gels). The bands were visualized by soaking the gel in 1X TAE for 10 minutes to neutralize the acidic gel, followed by soaking in 1X TAE containing either 0.75μg/mL EtBr or 1X sybr gold for 30-45minutes. The gel was photographed and the negative of the image is used to enhance visualization of the tRNA bands. The assays are quantitatively analyzed by line densitometry as described in the Results.
## Appendix B: Buffers for Re-use of 5 Prime and Purelink Plasmid Purification Columns

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<tr>
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<td>EDTA</td>
<td>372.24</td>
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<tr>
<td></td>
<td>RNAse A*</td>
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<td>0.5mg/mL - 1mg/mL</td>
</tr>
<tr>
<td>Notes:</td>
<td>Add Tris and EDTA, adjust pH, autoclave, store at RT, *add RNAse immediately before use</td>
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<tr>
<td>PL2</td>
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<td>PEB</td>
<td>Elution buffer pH 8.5</td>
<td>Tris</td>
<td>121.1</td>
<td>10mM</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>372.24</td>
<td>1mM</td>
<td></td>
</tr>
<tr>
<td>Notes:</td>
<td>Add Tris, EDTA, adjust pH, autoclave</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1M HCl</td>
<td>HCl</td>
<td></td>
<td>1M</td>
<td></td>
</tr>
<tr>
<td>Notes:</td>
<td>Dilute HCl to 1M with autoclaved de-ionized water</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 6: Directions for preparation of 5 Prime kit replacement buffers (42).

<table>
<thead>
<tr>
<th>Kit</th>
<th>Buffer</th>
<th>Material</th>
<th>MW (g/mol)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3</td>
<td>Resuspension buffer pH 8</td>
<td>Tris</td>
<td>121.1</td>
<td>50mM</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>372.24</td>
<td>10mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RNAse A*</td>
<td></td>
<td></td>
<td>0.5mg/mL - 1mg/mL</td>
</tr>
<tr>
<td>Notes:</td>
<td>Add Tris and EDTA, adjust pH, autoclave, store at RT, *add RNAse immediately before use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L7</td>
<td>Lysis buffer</td>
<td>NaOH</td>
<td>40</td>
<td>200mM</td>
</tr>
<tr>
<td></td>
<td>SDS</td>
<td></td>
<td></td>
<td>1% (wt/vol)</td>
</tr>
<tr>
<td>Notes:</td>
<td>Make fresh (10X stocks of NaOH and SDS are convenient) DO NOT autoclave SDS solutions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N3</td>
<td>Precip buffer pH 5.5</td>
<td>Potassium Acetate</td>
<td>98.15</td>
<td>3100mM</td>
</tr>
<tr>
<td>Notes:</td>
<td>Add potassium acetate, adjust pH, autoclave</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EQ1</td>
<td>Equilibration buffer pH 5</td>
<td>Sodium Acetate</td>
<td>82.03</td>
<td>100mM</td>
</tr>
<tr>
<td></td>
<td>Sodium Chloride</td>
<td>58.44</td>
<td>600mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Triton X-100</td>
<td></td>
<td></td>
<td>0.15% (vol/vol)</td>
</tr>
<tr>
<td>Notes:</td>
<td>Add sodium acetate, sodium chloride, triton X-100, adjust pH, autoclave</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W8</td>
<td>Wash buffer pH 5</td>
<td>Sodium Acetate</td>
<td>82.03</td>
<td>100mM</td>
</tr>
<tr>
<td></td>
<td>Sodium Chloride</td>
<td>58.44</td>
<td>825mM</td>
<td></td>
</tr>
<tr>
<td>Notes:</td>
<td>Add sodium acetate, sodium chloride, adjust pH, autoclave</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td>Elution buffer pH 8.5</td>
<td>Tris</td>
<td>121.1</td>
<td>100mM</td>
</tr>
<tr>
<td></td>
<td>Sodium Chloride</td>
<td>58.44</td>
<td>1250mM</td>
<td></td>
</tr>
<tr>
<td>Notes:</td>
<td>Add Tris, sodium chloride, adjust pH, autoclave</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regenerate/Renew Buffer pH 8.5</td>
<td>Sodium Chloride</td>
<td>58.44</td>
<td>2500mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Triton X-100</td>
<td></td>
<td></td>
<td>0.15% (vol/vol)</td>
</tr>
<tr>
<td>Notes:</td>
<td>Add sodium chloride, triton X-100, adjust pH, autoclave</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 7: Directions for preparation of Purelink kit replacement buffers (43).
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