Journal of Visualized Experiments

Fluorescent visualization of Mango-tagged RNA in polyacrylamide gels: A post-staining method

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59112R2
Full Title:	Fluorescent visualization of Mango-tagged RNA in polyacrylamide gels: A post-staining method
Keywords:	RNA Mango, Fluorescence, Denaturing Gels, Native Gels, Gel staining, Detection Method, PAGE
Corresponding Author:	Peter J Unrau Burnaby, British Columbia CANADA
Corresponding Author's Institution:	
Corresponding Author E-Mail:	punrau@sfu.ca
Order of Authors:	Peter J Unrau
	Iqra M. Yaseen
	Quiana R. Ang
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Burnaby, BC, Canada

TITLE:

Fluorescent Visualization of Mango-tagged RNA in Polyacrylamide Gels via a Poststaining Method

AUTHORS & AFFILIATIONS:

Iqra M. Yaseen[1,*], Quiana R. Ang[1,*], Peter J. Unrau[1]

[1]Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada

*These authors contributed equally.

Corresponding Author:

Peter J. Unrau (punrau@sfu.ca)
Tel: (778) 782-3448

Email Addresses of Co-authors:

Quiana R. Ang (qang@sfu.ca)
Iqra M. Yaseen (iyasin@sfu.ca)

KEYWORDS:

RNA Mango, fluorescence, denaturing gels, native gels, gel staining, detection method, PAGE

SUMMARY:

Here we present a sensitive, rapid, and discriminating post-gel staining method to image RNAs tagged with RNA Mango aptamers I, II, III, or IV, using either native or denaturing polyacrylamide gel electrophoresis (PAGE) gels. After running standard PAGE gels, Mango-tagged RNA can be easily stained with TO1-Biotin and then analyzed using commonly available fluorescence readers.

ABSTRACT:

Native and denaturing polyacrylamide gels are routinely used to characterize ribonucleoprotein (RNP) complex mobility and to measure RNA size, respectively. As many gel-imaging techniques use nonspecific stains or expensive fluorophore probes, sensitive, discriminating, and economical gel-imaging methodologies are highly desirable. RNA Mango core sequences are small (19–22 nt) sequence motifs that, when closed by an arbitrary RNA stem, can be simply and inexpensively appended to an RNA of interest. These Mango tags bind with high affinity and specificity to a thiazole-orange fluorophore ligand called TO1-Biotin, which becomes thousands of times more fluorescent upon binding. Here we show that Mango I, II, III, and IV can be used to specifically image RNA in gels with high sensitivity. As little as 62.5 fmol of RNA in native gels and 125 fmol of RNA in denaturing gels can be detected by soaking gels in an imaging buffer containing potassium and 20 nM TO1-Biotin for 30 min. We demonstrate the specificity of the Mango-tagged system by imaging a Mango-tagged 6S bacterial RNA in the context of a complex mixture of total bacterial RNA.

INTRODUCTION:

Mango is an RNA tagging system consisting of a set of four small fluorescent RNA aptamers that bind tightly (nanomolar binding) to simple derivatives of the thiazole-orange (TO1-Biotin, **Figure 1A**)[1,2,3]. Upon binding, the fluorescence of this ligand is increased 1,000- to 4,000-fold depending on the specific aptamer. The high brightness of the Mango system, which for Mango III exceeds that of enhanced green fluorescent protein (eGFP), combined with the nanomolar binding affinity of the RNA Mango aptamers, allows it to be used both in the imaging and the purification of RNA complexes[2,4].

The X-ray structures of Mango I[5], II[6], and III[7] have been determined to high resolution, and all three aptamers utilize an RNA quadruplex to bind TO1-Biotin (**Figure 1B–D**). The compact cores of all three aptamers are isolated from the external RNA sequence via compact adaptor motifs. Mango I and II both utilize a flexible GNRA-like loop adaptor to connect their Mango cores to an arbitrary RNA duplex (**Figure 1B,C**). In contrast, Mango III uses a rigid triplex motif to connect its core to an arbitrary RNA helix (**Figure 1D**, purple residues), while the structure of Mango IV is not currently known. As the ligand-binding core of each of these aptamers is separated from the external RNA sequence by these helical adaptors, it appears likely that they can all be simply incorporated into a variety of RNAs. The bacterial 6S regulatory RNA (Mango I), components of the yeast spliceosome (Mango I), and the human 5S RNA, U6 RNA, and a C/D scaRNA (Mango II and IV) have all been successfully tagged in this fashion[2,8], suggesting that many biological RNAs can be tagged using the RNA Mango aptamer system.

Denaturing and native gels are commonly used to study RNAs. Denaturing gels are often used to judge RNA size or RNA processing, but typically, in the case of a northern blot, for example, require several slow and sequential steps in order to generate an image. While other RNA fluorogenic aptamers, such as RNA Spinach and Broccoli, have been used successfully for gel imaging[9], no fluorogenic aptamer system to date possesses the high brightness and affinity of the Mango system, making it of considerable interest to investigate Mango's gel-imaging abilities. In this study, we wondered if the RNA Mango system could be simply extended to gel imaging, as the excitation and emission wavelengths of TO1-Biotin (510 nm and 535 nm, respectively) are appropriate for imaging in the eGFP channel common to most fluorescent gel-scanning instrumentation.

The post-gel staining protocol presented here provides a rapid way to specifically detect Mango-tagged RNA molecules in native and denaturing polyacrylamide gel electrophoresis (PAGE) gels. This staining method involves soaking gels in a buffer containing potassium and TO1-Biotin. RNA Mango aptamers are G-quadruplex based and potassium is required to stabilize such structures. Using RNA transcribed from minimal Mango-encoding DNA templates (see the protocol section), we can simply detect as little as little as 62.5 fmol of RNA in native gels and 125 fmol of RNA in denaturing gels, using a straightforward staining protocol. In contrast to common nonspecific nucleic acid stains (see **Table of Materials**, referred to SG from hereon), we can clearly identify Mango-tagged RNA even when high concentrations of total untagged RNA are present in the sample.

PROTOCOL:

1. Preparation of the reagents

1.1. TO1-Biotin poststaining solution (gel staining solution)

1.1.1. Make 1 L of 1 M phosphate buffer at pH 7.2 at 25 °C by adding 342 mL of 1 M of Na_2HPO_4 and 158 mL of 1 M NaH_2PO_4. Adjust pH to 7.2 at 50 mM by adding the appropriate phosphate solution. Sterile-filter using a 0.2 µm filter and store the solution in plasticware at room temperature.

1.1.2. Prepare 5x gel staining solution (without TO1-Biotin) as follows. Make up a 1 L solution by mixing 247.5 mL of ddH_2O, 700 mL of 1 M KCl, 50 mL of 1 M phosphate buffer (pH 7.2), and 2.5 mL of Tween 20. Sterile-filter using a 0.2 µm filter and store the solution in plasticware. It can be stored at room temperature.

NOTE: $MgCl_2$ can be added to this solution if required as it can potentially stabilize RNA complexes. This will have a modest impact on the fluorescent signal[2].

1.1.3. Make up a 1x gel staining solution using the 5x gel staining solution from step 1.1.2 and, immediately prior to use, supplement it with TO1-Biotin fluorophore (see **Table of Materials**) to a final concentration of 20 nM. For example, add 20 mL of 5x gel staining solution to 80 mL of deionized water to make 100 mL of 1x gel staining solution, and add 2 µL of a 1 mM TO1-Biotin stock (prepared in dimethylformamide).

NOTE: The extinction coefficient for the TO1-Biotin dye at 500 nm is 63,000 $M^{-1} \cdot cm^{-1}$, measured in staining solution[1].

1.2. Denaturing PAGE (2x denaturing gel loading solution and solutions A, B, C, ammonium persulfate, and tetramethylethylenediamine)

1.2.1. Prepare 50 mL of 2x denaturing gel loading solution by mixing 40 mL of formamide, 0.5 mL of 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 8.0), and 9.5 mL ddH_2O. The solution can be stored at room temperature. Loading dyes are not added to this solution as it may obscure fluorescent imaging.

1.2.2. Prepare 2x denaturing gel loading dye as follows. When purifying RNA and DNA oligonucleotides, add 0.5 mL of 2.5% (w/v) bromophenol blue (BB) and 0.5 mL of 2.5% (w/v) xylene cyanol (XC) to the solution described in step 1.2.1, and add 8.5 mL of ddH_2O instead of 9.5 mL. The solution can be stored at room temperature.

1.2.3. Prepare 100 mL of solution A by weighing out 200.2 g of urea (formula weight: 60.06 g/mol) and adding it to 312.5 mL of 40% 19:1 acrylamide:N,N'-methylenebisacrylamide. Stir it with a magnetic stir bar at maximum speed until dissolved (about 3 h) and make up the solution to 500

mL using ddH_2O. Note that the final concentrations are 6.667 M urea and 25% 19:1 acrylamide:N,N'-methylenebisacrylamide. Store at 4 °C in clean plasticware.

1.2.4. Prepare 1 L of solution B by weighing out 400.4 g of urea and make up the solution to 1 L with ddH_2O; stir until the urea has dissolved. Solution B can be kept at room temperature.

1.2.5. Prepare solution C (10x Tris-borate-EDTA [TBE]) as follows. Prepare 4 L of 10x TBE by weighing out 432 g of Tris base and 220 g of boric acid, adding 160 mL of 0.5 M EDTA with a pH of 8 (filtered), and making up the solution to 4 L with ddH_2O. A large stock of this buffer is made as it is also used as gel running buffer.

1.2.6. Prepare 10% ammonium persulfate (APS) by dissolving 1 g of APS into a total volume of 10 mL of ddH_2O. Store at 4 °C.

1.2.7. Keep tetramethylethylenediamine (TEMED) handy. Store it at 4 °C together with the APS solution.

1.3. Native PAGE (2x native gel loading solution)

1.3.1. Prepare 50 mL of 2x native gel loading solution by mixing 25 mL of 100% glycerol, 10 mL of 5x gel staining solution, and 15 mL of ddH_2O to make up the solution to 50 mL.

NOTE: As in the denaturing gel section, loading dyes can be added to this stock, but their addition can potentially obscure the fluorescent signal in the gel and, thus, should be avoided.

2. Preparation and loading of denaturing gels

2.1. Prepare a denaturing gel with an appropriate percentage by following **Table 1**. Consider the specific gel casting system; 30 mL gels are commonly used. The appropriate gel percentage can be estimated by using **Table 2**: select the polyacrylamide percentage where the BB and XC dyes have motilities faster and slower, respectively, than the RNA of interest, to ensure high-band separation in the relevant size range.

2.2. Mix solutions A, B, and C according to **Table 1** and add APS and TEMED immediately prior to pouring the gel. Mix the solutions well in clean plasticware.

2.3. Pour the gel solution into an appropriate gel casting apparatus, after ensuring that all components are scrupulously clean. Lift one side of the apparatus so that the gel is slightly tilted when pouring, to avoid any air bubbles becoming trapped within the gel itself.

2.4. Insert the desired comb and leave it to polymerize (approximately 30 min). Observe the polymerization by looking closely for a change in the index of refraction around the gel wells. Make up the gel tank by using 1x solution C (1x TBE) and carefully remove the comb. Using a syringe, aspirate out the wells immediately prior to sample loading.

2.5. Prepare denaturing samples as follows. Add 2x denaturing gel loading solution to the RNA samples of interest to make the denaturing gel loading solution 1x and heat-denature at 95 °C for 5 min by using a thermocycler or water bath.

2.6. Prior to loading the samples, air-cool them several minutes until they are cool to the touch. Load the samples by layering them on the bottom of each well, using gel-loading tips.

NOTE: Round tips can be used for gels of 1 mm thickness or more; use flat tips for thinner gels.

2.7. Run the denaturing gels at room temperature. Ensure that the wattage is sufficiently low so that the glass plates of the gel system do not crack.

NOTE: In our laboratory, 28 W for 20 cm x 16 cm plates was sufficient for this purpose.

3. Preparation and loading of native gels

3.1. Prepare a native gel with an appropriate percentage by referring to **Table 3**. Consider the specific gel casting system, but keep in mind that 30 mL gels are commonly used. Mix the solutions of 1x TBE, 40% 29:1 acrylamide:N,N'-methylenebisacrylamide, and glycerol according to **Table 3**, and add APS and TEMED immediately prior to pouring the gel. Proceed as described in steps 2.3 and 2.4.

3.2. Prepare native samples by adding 2x native gel loading solution to the RNA samples of interest to make the solution 1x and allow it to incubate at room temperature for 100 min prior to running the gel to ensure complete RNA folding.

3.3. Run the native gel in a 4 °C cold room, ensuring that the wattage is sufficiently low to not heat the gel.

NOTE: In our laboratory, 14 W for 20 cm x 16 cm plates was sufficient for this purpose.

4. RNA Preparation by run-off T7 transcription

NOTE: DNA sequences used for the run-off transcription[10] of RNA Mango constructs were ordered commercially. In this method, DNA oligonucleotides containing the reverse complement (RC) of both the sequence to be transcribed and the T7 promoter are hybridized to a T7 promoter top strand sequence and then transcribed in vitro. Below, for each oligonucleotide, the RC of the Mango core sequence is shown in bold and the RC of the T7 promoter region is shown in italics. Residues in regular font correspond to otherwise arbitrary complementary helical regions required to allow the Mango core to properly fold.

Mango I: GCA CGT AC**T CTC CTC TCC GCA CCG TCC CTT C**GT ACG TGC C*TA TAG TGA GTC GTA TTA AAG*

Mango II: GCA CGT AC**T CTC CTC TTC CTC TCC TCT CCT C**GT ACG TGC C*TA TAG TGA GTC GTA TTA AAG*

Mango III: GGC ACG TAC GAA **TAT ACC ACA TAC CAA TCC TTC C**TT CGT ACG TGC C*TA TAG TGA GTC GTA TTA AAG*

Mango IV: GCA CGT AC**T CGC CTC ATC CTC ACC ACT CCC TCG** GTA CGT GCC *TAT AGT GAG TCG TAT TAA AG*

T7 Top Strand: CTT TAA TAC GAC TCA CTA TAG G

4.1. Preparative gel purification of DNA oligonucleotides

4.1.1. For a 0.2 µmol-scale DNA synthesis, resuspend the deprotected DNA oligonucleotide in 100 µL of ddH_2O and 100 µL of 2x denaturing loading dye (from step 1.2.2). In this case, including gel loading dyes in the loading solution at the same concentration as in step 1.2.2 is preferred.

4.1.2. Purify the DNA oligonucleotide using a 50 mL preparative scale denaturing gel of the appropriate polyacrylamide percentage; use **Table 2** to choose the gel percentage. For example, use an 8% gel for a 50 nt sequence. Ideally, bromophenol blue should run faster than the oligonucleotide, and xylene cyanol should run slower.

NOTE: In our laboratory, such gels were cast using casting spacers that were 1.5 mm thick and a gel-loading comb with wells that were 2–2.5 cm wide.

4.1.3. Load 100 µL of the DNA oligonucleotide solutions prepared in step 4.1.2 per preparative gel well as described in steps 2.4–2.7.

4.1.4. Carefully dry the outside of the gel, remove the glass plates, and cover both sides of the gel in plastic wrap. Place it onto a fluorescent imaging screen (aluminum-backed thin-layer chromatography plates impregnated with fluorophore are an economical solution) and use a short-wavelength UV hand-held lamp to visualize the DNA bands by UV shadowing.

4.1.5. A crisp, well-defined shadow should be observed if the DNA synthesis is of high quality. Mark the bands on the plastic wrap, using a permanent marker.

NOTE: Protect skin and eyes from UV light and keep exposures short to avoid damaging the nucleic acid sample.

4.1.6. Placing the gel on a clean glass plate, carefully cut out the marked bands and place each gel fragment in 400 µL of 300 mM NaCl. Elute the DNA overnight, using a rotator at room temperature.

4.1.7. Recover the eluent in a clean centrifuge tube and add 2.5 equivalents of ethanol to precipitate the DNA. Vortex well and place the tube at -20 °C for 30 min.

4.1.8. Pellet the sample in a bench top centrifuge at 156,000 x *g* for 30 min at 4 °C. Carefully remove the supernatant and resuspend the pellet in ddH_2O. Use a spectrophotometer to accurately determine the DNA oligonucleotide concentration. Store the sample as a 10 µM stock for convenience at -20 °C.

4.2. Run-off transcription and gel purification of RNA samples

4.2.1. Prepare 5x nucleoside triphosphate (NTP) stock by mixing liquid NTP stocks made up of a final concentration of 40 mM guanosine triphosphate (GTP, 11,400 $M^{-1}{\cdot}cm^{-1}$), 25 mM cytidine triphosphate (CTP, 7,600 $M^{-1}{\cdot}cm^{-1}$), 25 mM adenosine triphosphate (ATP, 5,000 $M^{-1}{\cdot}cm^{-1}$), and 10 mM uridine triphosphate (UTP, 10,000 $M^{-1}{\cdot}cm^{-1}$); all extinction coefficients at 260 nm.

NOTE: Liquid or powdered stocks can be obtained commercially. If preparing primary stocks from powder, carefully adjust the pH to a final pH of 7.9, using 1 M NaOH. Aliquot the stock in 1.5 mL microcentrifuge tubes and store it at -20 °C.

4.2.2. Prepare 100 mL of 10x T7 transcription buffer stock by mixing 25.6 mL of 1 M Tris-HCl, 14.4 mL of 1 M Tris base, 26 mL of 1 M $MgCl_2$, 10 mL of 10% Triton X-100, and 0.637 g of spermidine. Make up the stock to a final volume of 100 mL by adding ddH_2O.

NOTE: A final pH of 7.9 of the 1x solution should be confirmed using a calibrated pH meter. The 10x should be sterile-filtered and stored at -20 °C in suitably sized aliquots.

4.2.3. Perform transcription as follows.

4.2.3.1. Add the following reagents to make up a final concentration of 1x T7 transcription buffer using the 10x T7 transcription buffer stock and ddH_2O (from step 4.2.2), 1x NTPs, 10 mM of dithiothreitol (DTT), 1 µM T7 top strand sequence (see section 4 Note for the sequence), 1 µM gel-purified Mango DNA sequence (step 2.1), and T7 RNA polymerase enzyme (1 U/µL).

4.2.3.2. Vortex, spin down, and incubate the solution at 37 °C for 2 h or until it becomes cloudy and a white precipitate forms at the bottom of the tube. A 50 µL transcription should result in 50 µL of ~50 µM RNA after gel purification. Add an equal volume of 2x denaturing dye and store it at -20 °C until ready for gel purification.

4.2.4. Gel-purify the resulting RNA as described in the DNA oligonucleotides gel purification section by following steps 4.1.2–4.1.8, substituting the RNA sample for the DNA.

NOTE: RNA is extremely sensitive to RNase degradation, so ensure that in all steps, gloves and a clean lab coat are worn at all times. Ensure all samples are prepared and stored in single-use plasticware to protect against RNase contamination, and wash glass plates carefully with hot soap and water prior to use, rinse them thoroughly with ddH_2O, and dry the glassware with the assistance of ethanol applied from a squeeze bottle.

4.2.5. Use 1 µL of the final RNA sample and, using a droplet-based spectrophotometer, determine the absorbance at 260 nm. Using an extinction coefficient determined by the nearest neighbor method, calculate the RNA concentration and adjust the sample concentration to 10 µM with ddH_2O for convenience. Store the RNA samples at -20 °C.

4.3. *Escherichia coli* total crude nucleic acid extraction

NOTE: The following protocol is an example. This protocol uses endogenously expressed Mango I-tagged 6S RNA from a plasmid in *E. coli*, and induction from this plasmid is described elsewhere in detail[4].

4.3.1. For the purposes of this study, prepare 500 mL of induced cells for both the pEcoli-RNA Mango and the pEcoli-T1 plasmids (the cells are induced at an OD_{600nm} of 1). Pellet the cells and store them at -80 °C prior to use.

4.3.2. Perform RNA extraction as follows.

4.3.2.1. Take 0.5 mL of the induced pEcoli cell pellet and add 500 µL of equilibrated phenol. Then, vortex the sample; the solution will become milky white. Centrifuge at maximum speed for 2 min to separate the layers.

4.3.2.2. Extract the top layer, which will be slightly yellow, and repeat step 4.3.2.1 by adding an equal volume of phenol, centrifuging, and extracting for a total of 5x (or until the middle layer, which is opaque white, goes away and only two clear layers are left).

4.3.2.3. Add the phenol-extracted aqueous volume to an equal volume of chloroform, vortex, and then, centrifuge at maximum speed for 2 min.

4.3.2.4. Extract the aqueous layer and add NaCl to a final concentration of 300 mM. Precipitate the resulting nucleic acid by the addition of 2.5 equivalents of ethanol, vortex, spin down, and precipitate at -20 °C for at least 30 min.

4.3.2.5. Pellet in a bench top centrifuge at 15.6 x 1,000 x *g* at 4 °C for 30 min. Carefully remove the supernatant and resuspend the pellet in 100 µL of ddH_2O.

4.3.2.6. Add a DNase I digestion step to this procedure, following the referenced protocol[11], to obtain total RNA.

NOTE: Vortex vigorously till the pellet is completely dissolved in ddH_2O.

5. Post-gel staining

5.1. Prepare 1x gel staining solution as per the recipe in step 1.1.3 and add it to a clean glass container that is wide enough to comfortably fit the gel.

NOTE: Borosilicate glass containers with snap fit lids serve this purpose well.

5.2. Add enough 1x gel staining solution to the container so that the gel is completely covered with the solution and the liquid sloshes over the top of the gel when it is placed on the orbital rotator.

NOTE: The container must be big enough to fit the gel so that it can move around and have enough buffer in the container to fully cover the gel.

5.3. Once the native or denaturing gel has finished running (section 2 or 3, respectively), remove the gel from the apparatus and cut off its wells. It can be useful to remove a corner of the gel for orientation later in the analysis.

5.4. Carefully transfer the gel to the 1x gel staining solution prepared in step 5.2.

NOTE: The gels are fragile and prone to breaking so be careful when transferring the gel. Keep the lid on the staining container at all times so as not contaminate the gel.

5.5. Place the gel on an orbital rotator at a speed of 100 rpm for 30 min at room temperature.

NOTE: Make sure that the gel does not fold back onto itself; otherwise, the RNA may diffuse out of the gel and so label a different part of the gel.

6. Imaging Mango-tagged RNAs in gel

6.1. Carefully decant the imaging buffer. Rinse the gel quickly with water, ensuring enough liquid remains to keep the gel slightly mobile in the container.

6.2. Carefully transfer the gel onto the imager. Transfer by carefully picking up the sides of the gel and placing it onto the imager tray; alternatively, the gel can be slowly poured onto the tray. Make sure there are no bubbles underneath the gel and that there is no excess liquid under the gel. A pipette rolled over the gel can be useful to remove any excess liquid.

NOTE: Use a paper towel to soak up the excess fluid.

6.3. Take a gel image, observing fluorescence between excitation wavelength 510 nm and emission wavelength 535 nm (for example, green light at 520 nm wavelength fluorescence settings on the imager). Make sure the settings are fully optimized on the instrument and carefully follow the instructions for the instrument used.

REPRESENTATIVE RESULTS:

Short Mango-tagged RNAs were prepared as described in the protocol section. Assuming that fluorescence in denaturing conditions would be most difficult to observe owing to the presence

of urea in the gels, we first studied the resistance of the Mango aptamers to urea, which acts as a nucleic acid denaturant. We found that Mango aptamers are substantially resistant to denaturation up to a urea concentration of approximately 1 M (**Figure 2A**). Prior to adding gel staining solution to a denaturing gel, the final concentration of urea in the gel is 6 M. Adding sufficient staining solution to decrease this concentration to 1 M would, therefore, be optimal to ensure full Mango fluorescence for all four aptamers. In practice, the staining protocol achieved less than this fully optimal result, but this could simply be rectified if needed by using more staining solution or by the simple expedient of changing the solution once during staining.

Once the Mango-tagged RNA constructs were run into a denaturing gel, the staining time was optimized for maximum gel fluorescence by loading three different Mango III amounts into an 8% denaturing gel (**Figure 2B**). A time course revealed that after 5 min of soaking in gel staining solution, fluorescence was clearly visible. Maximum fluorescence of the Mango III construct was obtained after 20–40 min of staining, after which time the small RNAs used in this study started to diffuse out of the gel, resulting in a loss of fluorescent signal (**Figure 2B**). Consequently, both native and denaturing gels were stained for 30 min each. Longer RNA constructs could easily tolerate longer stained times as they would be much less likely to diffuse out of the gel.

Some of the RNA Mango aptamers folded more rapidly than others. Each of the Mango aptamers used in this study was incubated in a gel buffer supplemented with 1.5 M urea and 100 nM TO1-Biotin dye and analyzed using a fluorometer. Mango I, II, and III were fully folded after 10 min, whereas Mango IV became substantially folded only after 40 min (**Figure 3A**). In the absence of urea, folding was much more rapid as was expected (**Figure 3B**). To ensure that the native gel samples were fully folded, we preincubated samples for 100 min prior to running them into native gels. Practically, the data in **Figure 3** suggests this time could be substantially reduced depending on the Mango aptamer used.

Once the protocol was optimized to detect RNA Mango aptamer fluorescence, the sensitivity of the poststaining method was determined for each of the Mango variants in both native gels. Single bands corresponding to well-folded RNAs were observed for each of the four Mangos in native gels (**Figure 4A**). Upon serial dilution, as little as 62.5 fmol of Mango II could be observed, while as little as 125 fmol of Mango I, III, and IV were easily visualized. The quantification of native gels was log-linear over about 1.5 orders of magnitude, with Mango I, II, and IV behaving in a more linear fashion than Mango III (**Figure 4B**).

The results of the denaturing gels were slightly less sensitive than the native gels but were more linear. As little as 125 fmol of Mango II and III were easily detected (**Figure 4C**). Interestingly, quantification (**Figure 4D**) indicated that denaturing gels were log-linear or two orders of magnitude. We hypothesize that, in contrast to the native gels where RNA folds were perhaps subjected to partial denaturation during the gel running process, the presence of urea in the denaturing gel might provide a more homogenous way to fold the aptamers once they are placed in the TO1-Biotin staining solution.

As with all gel staining methodologies, if the gel is not carefully transferred into the container, or the rotating speed is too high during the staining period, the gel may fold back onto itself (**Figure 5A**). This can result in the Mango-tagged RNA sample diffusing from one place of the gel to another but can be easily avoided in practice. In native gels and, particularly, for Mango IV, we observed that incomplete folding can manifest in the appearance of multiple bands presumably corresponding to partially/misfolded RNA conformations (**Figure 5B**) resulting from shorter folding times. Folding issues in native gels can be avoided by preincubating RNA samples appropriately as previously described and by running native gels in a cold room. In denaturing gels, where RNA folds in situ within the gel, misfolding was not a significant problem. Finally, in the absence of RNA, very little background fluorescence was observed in either gel system.

Next, the specificity of the RNA Mango tag was studied by overexpressing the 6S regulatory RNA in bacteria. This RNA was previously tagged using Mango I (**Figure 1E**)[4]. Bacterial cells were transformed with either the pEcoli-RNA Mango plasmid (henceforth M plasmid) or the pEcoli-T1 plasmid as a negative control (henceforth E plasmid). Transformed cells were grown in liquid lysogeny broth medium till an OD_{600} of 1.0 was reached. The cultures were then induced with 50 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 40 min. The cells were harvested via centrifugation at 6,000 x *g* for 15 min. Total RNA was extracted using phenol-phenol chloroform extraction from cell pellets[12] as described in the protocol section. The total RNA samples were concentrated by ethanol precipitation and then treated with DNase I, following the protocol, to remove DNA[11]. Before using, the RNA was concentrated by ethanol precipitation.

Total bacterial RNA was run into 8% denaturing gels (**Figure 6**) and stained with either SG[13] or TO1-Biotin. For SG staining, 10 µL of 10,000x SG was added to 100 mL of gel buffer; otherwise, the staining protocol was identical to that used for TO1-Biotin. As expected, strong SG staining was observed for a multitude of RNAs, but most prominently for ribosomal (rRNA) and transfer RNAs (tRNA) (**Figure 6**, left panel). While the Mango-dependent staining (M lanes) could be seen in these SG-stained gels, they could not be uniquely identified given the complex staining pattern observed using this universal stain. In contrast, the TO1-Biotin-stained gels highlighted Mango-dependent bands as the most prominent bands. Only the ribosomal RNA bands are seen to be in competition with the 6S Mango-dependent bands. A number of nonspecifically stained bands could also be observed. Nevertheless, the Mango-dependent bands were again dominant, having only the rRNA and tRNA bands as weakly competing competitors (**Figure 6**, right panel).

FIGURE AND TABLE LEGENDS:

Figure 1: Mango aptamer system. (**A**) TO1-Biotin fluorophore. (**B**) Mango I. (**C**) Mango II. (**D**) Mango III. Panels **B–D** show the secondary structure of each aptamer. P1 is an arbitrary stem. The GNRA-like stem-loop (here GAAA) found in Mango I and II is shown in red, the triplex motif of Mango III is shown in purple. (**E**) The 6S regulatory RNA tagged with Mango I.

Figure 2: Effect of urea on Mango aptamer fluorescence and optimal staining times. (**A**) A urea titration using 50 nM RNA Mango I (orange circles), Mango II (green circles), Mango III (purple circles), and Mango IV (blue circles), together with 100 nM TO1-Biotin dye and at the indicated

urea concentrations. The samples were incubated for 40 min before fluorescence was read at an excitation wavelength of 510 nm and an emission wavelength of 535 nm. (**B**) Mango III RNA was loaded into an 8% denaturing gel and stained with a gel solution containing 20 nM final TO1-Biotin dye. For each indicated time point, 0.064, 0.32, and 1.6 pmol of Mango III RNA was used from left to right. The gel image was visualized with a fluorescence imager using a 520 nm laser and a 10 min exposure.

Figure 3: Folding times of the Mango aptamers in the presence and absence of urea. (**A**) In the presence of 1.5 M urea and 100 nM TO1-Biotin, fluorescence time courses were performed using 50 nM of each RNA Mango construct (RNA Mango I: orange dots, Mango II: green dots, Mango III: purple dots, and Mango IV: blue dots). (**B**) Identical to panel **A**, except in the absence of urea. All time courses were performed at room temperature.

Figure 4: Fluorescent images of native and denaturing PAGE with RNA Mango constructs. (**A**) An 8% native gel with serially diluted RNA Mango constructs. Lanes I, II, III, and IV each contain 8 pmol final quantities of RNA Mango I, II, III, and IV, respectively. The right panels are twofold serial dilutions containing 4 pmol, 2 pmol, 1 pmol, 0.5 pmol, 0.25 pmol, 0.125 pmol, and 0.0625 pmol of either Mango I, II, III, or IV, as indicated. Lane 12 contains no RNA. (**B**) Quantification of three replicates of the native gel (standard deviation of the mean shown for each). (**C**) An 8% denaturing gel with the same samples loaded as in panel **A**, except for the fact that denaturing gel loading solution was used instead of native gel loading solution. (**D**) Three quantified replicates of denaturing gel (standard deviation of the mean shown for each). All gel images were visualized a gel imager with a 520 nm laser and a 10 min exposure.

Figure 5: Suboptimal gels and incomplete folding of Mango IV in an 8% native gel. (**A**) A serial dilution of the sort shown in **Figure 4** for Mango II but showing the effect of gel folding during the staining protocol. (**B**) Mango IV native gel samples that were not allowed to fold for long enough in native buffer prior to gel loading exhibit double bands. Otherwise, these results are similar to the Mango IV results shown in **Figure 4A**. All gel images were visualized using a gel imager with a 520 nm laser and a 10 min exposure.

Figure 6: Mango-tagged RNAs can be detected in the presence of total RNA, using TO1-Biotin staining. 8% denaturing gels were loaded with 100 ng of total RNA and were run for 30 min. The left gel was stained with SG and the right panel with TO1-Biotin. For both panels, lanes labeled E were loaded with 100 ng of pEcoli-T1 (no Mango tag) and lanes labeled M were loaded with 100 ng of pEcoli-RNA Mango (6S RNA tagged with a Mango I tag). TO1-Biotin-stained gel images were visualized using an imager with a 520 nm laser and a 10 min exposure. SG-stained gel images were visualized using a gel imager using a 460 nm laser and a 10 min exposure.

Table 1: Denaturing PAGE gel casting table. A = Solution A, B = Solution B, C = Solution C.

Table 2: Approximate gel mobilities of bromophenol blue (BB) and xylene cyanol (XC) gel loading dyes in polyacrylamide denaturing gels.

Table 3: Native PAGE gel casting table.

DISCUSSION:

A significant advantage of the Mango fluorescent tag is that a single tag can be used in multiple ways. The high brightness and affinity of these aptamers make them useful not only for in cell visualization[2] but also for in vitro RNA or RNP purification[4]. Therefore, gel imaging extends the versatility of the Mango tag in a straightforward way. Mango gel imaging sensitivity is slightly less than that of a northern blot[14] but can easily detect 60–120 fmol of RNA sample, without needing lengthy and tedious membrane transfer and probing steps. This is comparable to the hybridization-based probing efficiency found previously for small RNAs in gel[15]. While other fluorogenic aptamer methodologies—particularly, RNA Spinach—have greater sensitivity and specificity[9], none currently have simultaneously the high brightness and affinity of the Mango aptamer system, which allows a single RNA tag to be used for cellular imaging, RNP purification, and now gel imaging.

There are a few critical steps in this gel staining protocol. When working with RNA solutions, the solutions should be sterile filtered, and single-use plasticware should be used. Catuion, running native gels as complexes or RNA structures can be easily denatured if the power levels for the gel are too high and result in gel heating. Ensure any used glassware is clean and not contaminated with RNases. Additionally, always be careful when transferring and picking up gels as they are fragile and can be prone to breakage.

The TO1-Biotin stain penetrates gels rapidly, but the data presented here also indicate that Mango IV folding, in particular, can be rate limiting (**Figure 2** and **Figure 3**). Using the conditions stated in the protocol section, we observed log-linear behavior for all four aptamers over two orders of magnitude in denaturing gels, making the method useful for quantification (**Figure 4C,D**). Since the small Mango aptamers used in this study easily diffused out of the gel matrix, we expect the quantitation to improve for longer RNA constructs.

The Mango tag gel imaging methodology demonstrated here is robust and is anticipated to be able to be simply extended in terms of sensitivity and specificity. Mango I, II, and III fold reliably, while Mango IV does not. While we have not explored destaining protocols, we anticipate that such an approach could also simply improve specificity. While beyond the scope of this work, the fluorescence and biotin tag conferred to Mango-tagged RNA when using TO1-Biotin fluorophore appears highly likely to further streamline gel analysis and purification. Commercially available secondary biotin-labeling techniques, for example, promise to further enhance the detection limits of this simple RNA Mango-tagging system. Likewise, it appears probable that native Mango-tagged RNA protein complexes can be eluted from a gel and recovered using streptavidin magnetic beads so as to capture the eluted RNA complex. This would further simplify the routine purification of biologically important RNAs and RNA complexes by the simple expedient of adding a Mango tag to the RNA of interest.

ACKNOWLEDGMENTS:

The authors thank Razvan Cojocaru and Amir Abdolahzadeh for their technical assistance and Lena Dolgosheina for proofreading the manuscript. Funding was provided for this project by a Canadian Natural Sciences and Engineering Research Council (NSERC) operating grant to P.J.U.

DISCLOSURES:

A patent is pending on the Mango fluorogenic system.

REFERENCES:

1. Dolgosheina, E. V. et al. RNA Mango Aptamer-Fluorophore: A Bright, High-Affinity Complex for RNA Labeling and Tracking. *ACS Chemical Biology* (2014).
2. Autour, A. et al. Fluorogenic RNA Mango aptamers for imaging small non-coding RNAs in mammalian cells. *Nature Communications.* **9**, 656 (2018).
3. Dolgosheina, E. V., Unrau, P. J. Fluorophore-binding RNA aptamers and their applications: Fluorophore-binding RNA aptamers. *Wiley Interdisciplinary Reviews: RNA* (2016).
4. Panchapakesan, S. S. et al. Ribonucleoprotein Purification and Characterization using RNA Mango. *RNA.* 1592–1599 (2017).
5. Trachman III, R. J. et al. Structural basis for high-affinity fluorophore binding and activation by RNA Mango. *Nature Chemical Biology.* **13**, 807 (2017).
6. Trachman, R. J. et al. Crystal Structures of the Mango-II RNA Aptamer Reveal Heterogeneous Fluorophore Binding and Guide Engineering of Variants with Improved Selectivity and Brightness. *Biochemistry.* **57**, 3544–3548 (2018).
7. Trachman, R. et al. Mango-III is a compact fluorogenic RNA aptamer of unusual structural complexity. *Nature Chemical Biology.* **in review**.
8. Panchapakesan, S. S. S., Jeng, S. C. Y., Unrau, P. J. RNA complex purification using high-affinity fluorescent RNA aptamer tags. *Annals of the New York Academy of Sciences* (2015).
9. Filonov, G. S., Kam, C. W., Song, W., Jaffrey, S. R. In-gel imaging of RNA processing using Broccoli reveals optimal aptamer expression strategies. *Chemistry & Biology.* **22**, 649–660 (2015).
10. Milligan, J. F., Groebe, D. R., Witherell, G. W., Uhlenbeck, O. C. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Research.* **15**, 8783–8798 (1987).
11. A Typical DNase I Reaction Protocol (M0303) | NEB. https://www.neb.com/protocols/1/01/01/a-typical-dnase-i-reaction-protocol-m0303.
12. Sambrook, J., Russell, D. W. Purification of Nucleic Acids by Extraction with Phenol:Chloroform. *Cold Spring Harbor Protocols.* **2006** (1), pdb.prot4455 (2006).
13. Tuma, R. S. et al. Characterization of SYBR Gold Nucleic Acid Gel Stain: A Dye Optimized for Use with 300-nm Ultraviolet Transilluminators. *Analytical Biochemistry.* **268**, 278–288 (1999).
14. Streit, S., Michalski, C. W., Erkan, M., Kleeff, J., Friess, H. Northern blot analysis for detection and quantification of RNA in pancreatic cancer cells and tissues. *Nature Protocols.* **4**, 37–43 (2009).
15. Ebhardt, H. A., Unrau, P. J. Characterizing multiple exogenous and endogenous small RNA populations in parallel with subfemtomolar sensitivity using a streptavidin gel-shift assay. *RNA.* **15**, 724–731 (2009).

Figure 1

Click here to access/download;Figure;Fig_1.pdf

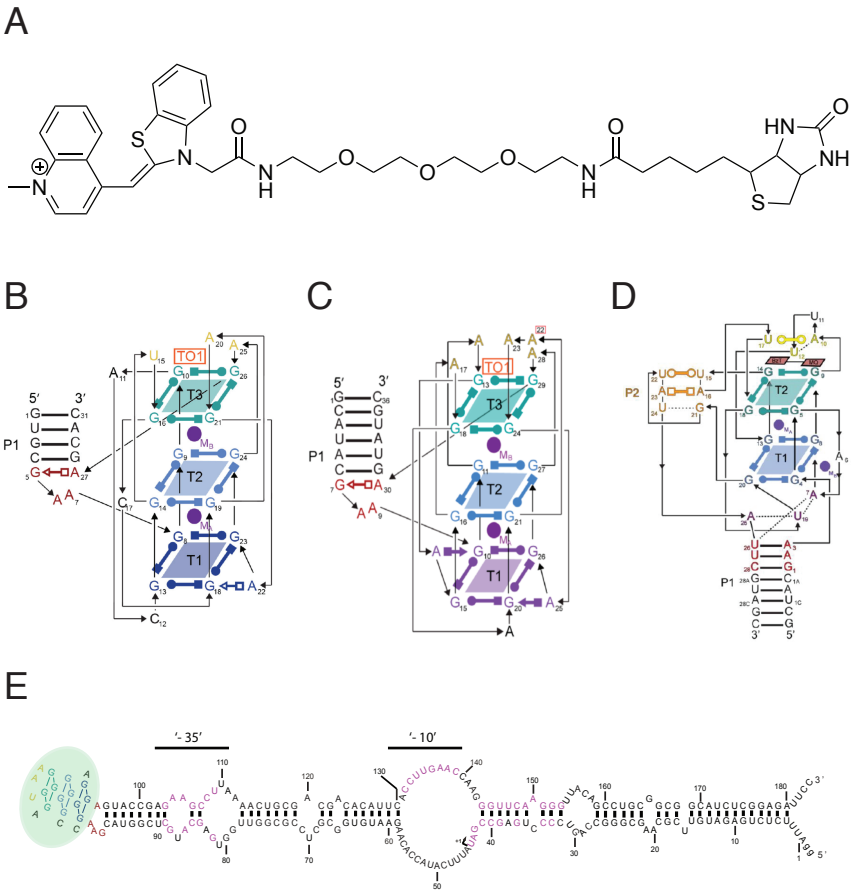
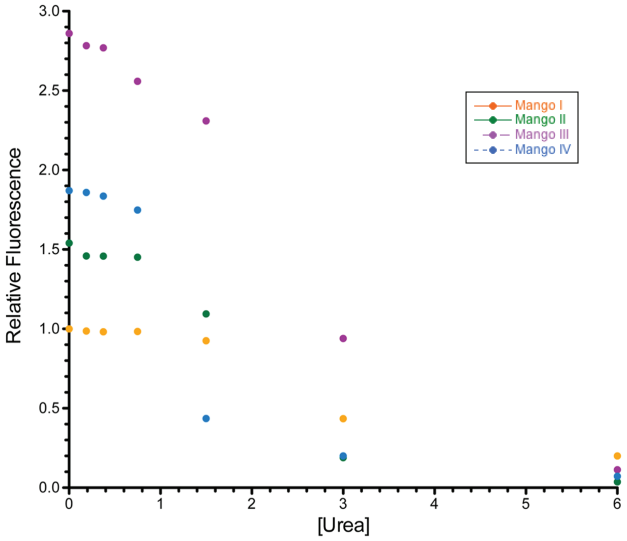


Figure 2

A



B

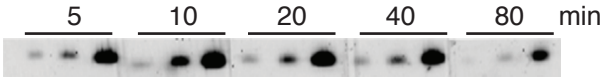
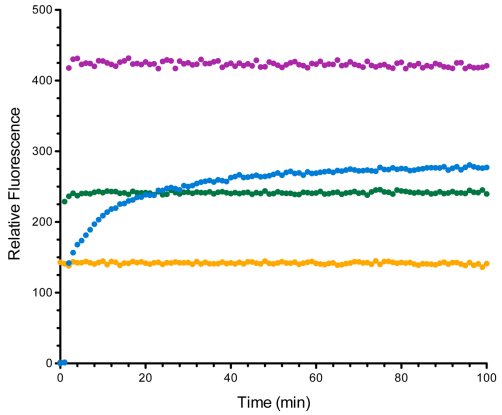
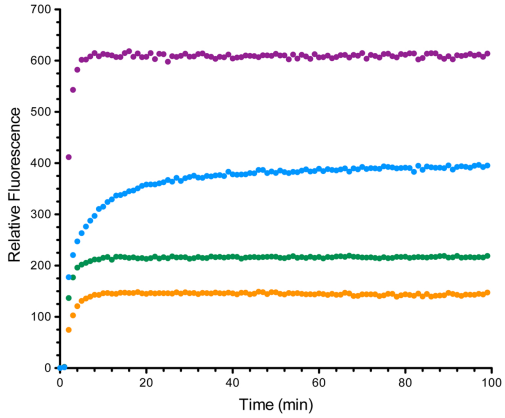


Figure 3

A



B



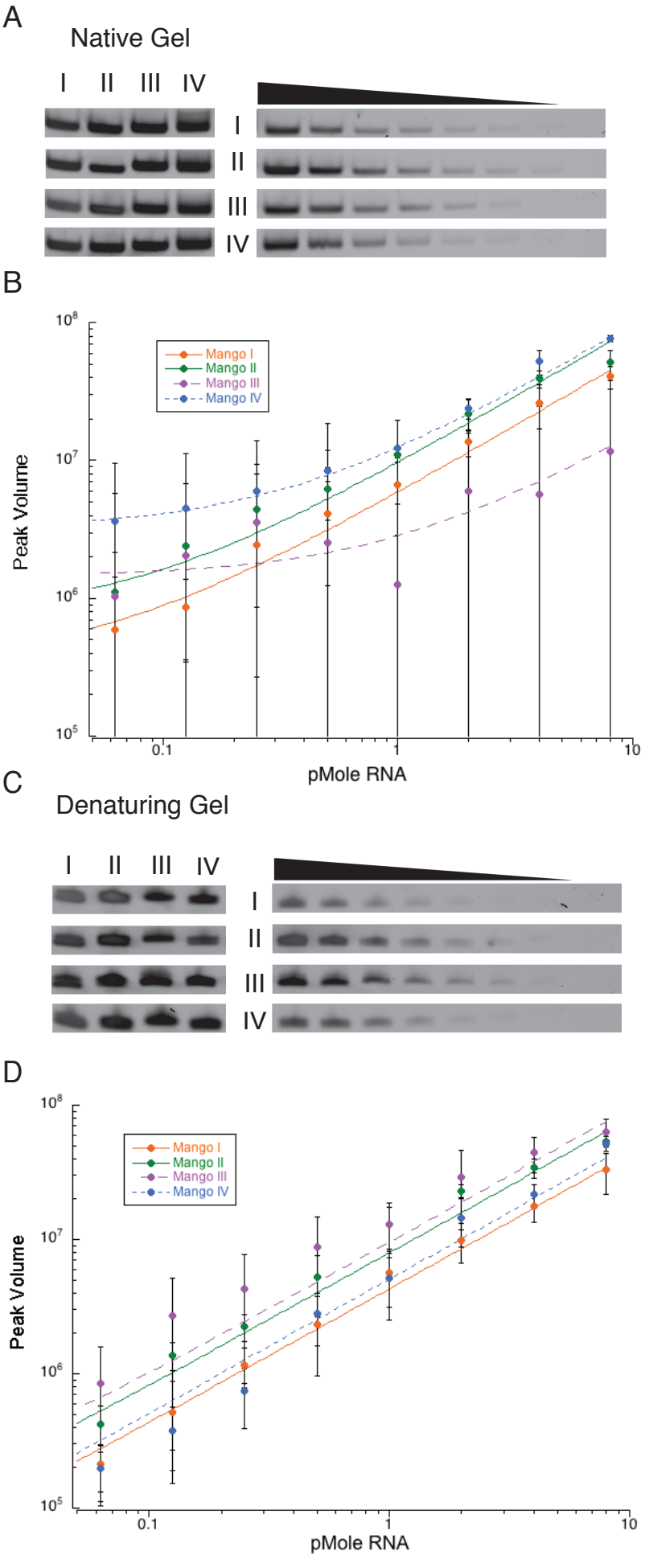


Figure 5

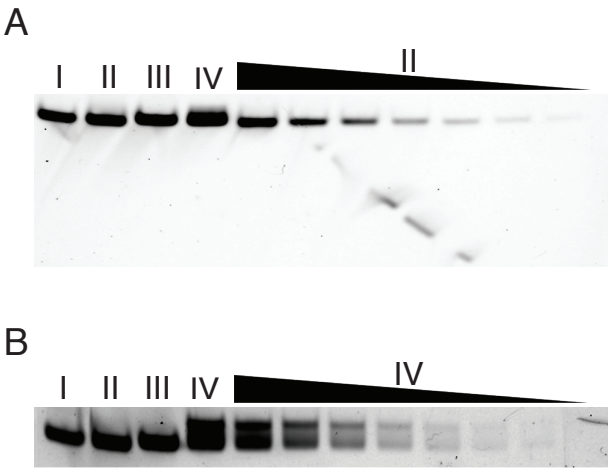


Figure 6

Click here to access/download;Figure;Fig_6.pdf

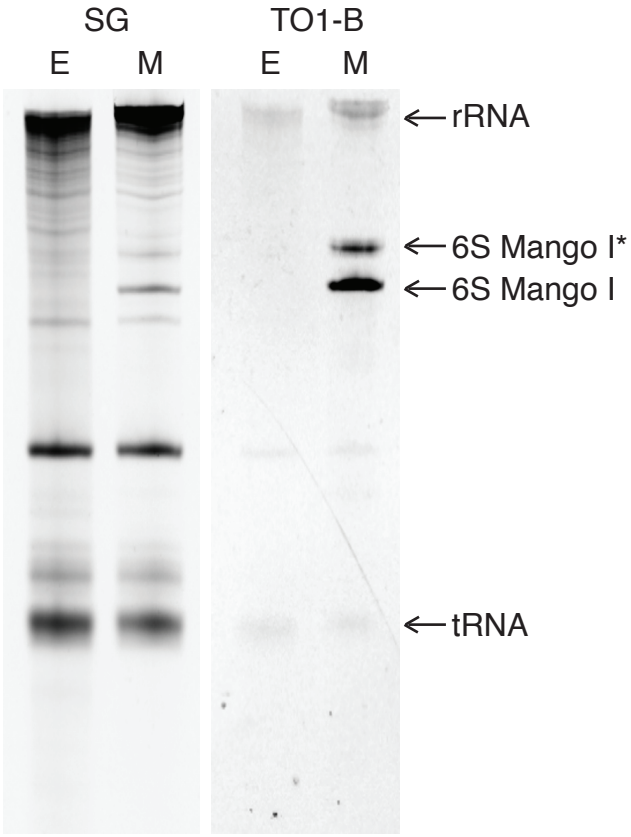


Table 1

PERCENTAGE		GEL VOLUME		
		20 mL	30 mL	50 mL
5%	A	4	6	10
	B	14	21	35
	C	2	3	5
6%	A	4.8	7.2	12
	B	13.2	19.8	33
	C	2	3	5
8%	A	6.4	9.6	16
	B	11.6	17.4	29
	C	2	3	5
10%	A	8	12	20
	B	10	15	25
	C	2	3	5
12%	A	9.6	14.4	24
	B	8.4	12.6	21
	C	2	3	5
15%	A	12	18	30
	B	6	9	15
	C	2	3	5
20%	A	16	24	40
	B	2	3	5
	C	2	3	5
APS (µL)		48	72	120
TEMED (µL)		20	30	50

Table 2

Denaturing Gel %	BB (~mobility nt)	XC (~mobility in nt)
5	35	130
6	26	106
8	19	70-80
10	12	55
20	8	28
23	5-6	

Table 3

PERCENTAGE		GEL VOLUME		
		20 mL	30 mL	50 mL
5%	1X TBE	16.5	24.75	41.25
	40% 29:1 acrylamide:N,N'-methylenebisacrylamide	2.5	3.75	6.25
	Glycerol	1	1.5	2.5
6%	1X TBE	16	24	40
	40% 29:1 acrylamide:N,N'-methylenebisacrylamide	3	4.5	7.5
	Glycerol	1	1.5	2.5
8%	1X TBE	15	22.5	37.5
	40% 29:1 acrylamide:N,N'-methylenebisacrylamide	4	6	10
	Glycerol	1	1.5	2.5
10%	1X TBE	14	21	35
	40% 29:1 acrylamide:N,N'-methylenebisacrylamide	5	7.5	12.5
	Glycerol	1	1.5	2.5
12%	1X TBE	13	19.5	32.5
	40% 29:1 acrylamide:N,N'-methylenebisacrylamide	6	9	15
	Glycerol	1	1.5	2.5
15%	1X TBE	11.5	17.25	28.75
	40% 29:1 acrylamide:N,N'-methylenebisacrylamide	7.5	11.25	18.75
	Glycerol	1	1.5	2.5
20%	1X TBE	9	13.5	22.5
	40% 29:1 acrylamide:N,N'-methylenebisacrylamide	10	15	25
	Glycerol	1	1.5	2.5
APS (µL)		48	72	120
TEMED (µL)		20	30	50

Table of Materials

Click here to access/download;Table of Materials;Table of Specific Materials_Equipment_2_1_19.xlsx

	Name of material/Equipment	Company	Catalog Number	Comment/Description
	0.8mm Thick Comb 14 Wells for 30 mL PAGE gels	LabRepCo	11956042	
	101-1000 µL tips	Fisher	02-707-511	
	20-200 µL low retention tips	Fisher Scientific	02-717-143	
	Acrylamide:N,N'-methylenebisacrylamide (40% 19:1)	Bioreagents	BP1406-1	Acute toxicity
	Acrylamide:N,N'-methylenebisacrylamide (40% 29:1)	Fisher	BP1408-1	Acute toxicity
	Agar	Anachemia	02116-380	
	Aluminium backed TLC plate	Sigma-Aldrich	1164840001	
	Amersham Imager 600	GE Healthcare Lifesciences	29083461	
	Ammonium Persulfate	Biorad	161-0700	Harmful
	BL21 cells	NEB	C2527H	
	Boric Acid	ACP	B-2940	
	Bromophenol Blue sodium salt	Sigma	B8026-25G	
	Chlololform	ACP	C3300	
	Dithiothreitol	Sigma Aldrich Alcohols	D0632-5G	
	DNase I	ThermoFisher	EN0525	
	EDTA Disodium Salt	ACP	E-4320	
	Ethanol	Commerial	P016EAAN	
	Flat Gel Loading tips	Costar	CS004854	
	Formamide 99%	Alfa Aesar	A11076	
	Gel apparatus set with spacers and combs	LabRepCo	41077017	
	Glass Dish with Plastic lid	Pyrex	1122963	Should be large enough to fit your gel piece
	Glycerol	Anachemia	43567-540	
	HCl	Anachemia	464140468	
	ImageQuanTL	GE Healthcare Lifesciences	29000605	
	IPTG	Invitrogen	15529-019	
	KCl	ACP	P-2940	
	MgCl2	Caledron	4903-01	
	MgSO4	Sigma-Aldrich	M3409	
	NaCl	ACP	S-2830	
	NaOH	BDH	BDH9292	
	Orbital Rotator	Lab-Line		
	Phenol	Invitrogen	15513-039	
	Round Gel Loading tips	Costar	CS004853	
	Sodium Phosphate dibasic	Caledron	8120-1	
	Sodium Phosphate monobasic	Caledron	8180-01	
	SYBRGold	ThermoFisher	S11494	
	T7 RNA Polymerase	ABM	E041	
	TEMED	Sigma-Aldrich	T7024-50 ml	
	TO1-3PEG-Biotin Fluorophore	ABM	G955	
	Tris Base	Fisher	BP152-500	
	Tryptone	Fisher	BP1421-500	
	Tween-20	Sigma	P9496-100	
	Urea	Fisher	U15-3	
	Xylene Cyanol	Sigma	X4126-10G	
	Yeast Extract	Bioshop	YEX401.500	

jove JOURNAL OF VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Fluorescent visualization of Mango-tagged RNA in polyacrylamide gels: A

Author(s): Iqra M. Yaseen*, Quiana R. Ang*, and Peter J. Unrau

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Peter Unrau

Department: MBB

Institution: SFU

Article Title: E. Visualization of Map-based tagged RNA in ...

Signature:

Date: 3/1/19

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Editor Comments

Please sign the article license agreement. (Line 2)

We edited for language, clarity and structure to meet our Journal's style. I have highlighted portions for filming (max 2.75 pages). Please check if appropriate. (Line 87)

We have revised the protocol to only include preparing the native and denaturing gels, running them and imaging them.

Gel staining solution? Please use consistent naming to avoid confusion.

Naming changed from 5X Gel buffer to 5X Gel Staining Solution to be consistent.

Bit confusing. Do you mean add 0.5+0.5 mL of BB and XC and 8.5 mL ddH2O? please clarify (Line 123)

Yes, this was changed to make it more clear. Both dyes are added into the loading dye solution.

Formula weight? (line 126)

This was changed to make it more clear. Previously said FW which is changed now to formula weight.

V:V? (Line 127)

What speed? (Line 127)

Maximum speed was used and has been clarified in text.

Please clarify, is solution C = 10X TBE? (Line 134)

This has been addressed this by adding 10X TBE in brackets next to solution C so as to imply that solution C is 10x TBE.

Which buffer exactly? The one from 1.1.2? Please be consistent with naming. (Line 148)

This has been addressed when all references to 5X Gel buffer were changed to 5X Gel staining in the third editor comments

Solution C? Please be consistent in terminology (Line 170)

Line 170 said 1X Buffer C which was changed to 1X Solution C to be consistent.

Aspirate? (Line 170)
The word “Blow” was changed to “aspirate” as per the editors request.

What ratio of samples to gel loading solution? (Line 173)

This was clarified to make the 2X denaturing gel loading solution to 1X.

Using a dry/water bath? (Line 174)

Both can be used so this was added.

Cool to what temperature? How long does cooling take? (Line 175)

Cool to touch. Should only be a few minutes. The samples can be cooled at room temperature

How much per well? (Line 175)

XXX This is highly dependent on the comb and the volume of the gel, not sure how to address this XXX

Unclear what is meant. Under which conditions do you skip to 2.1.1?? (Line 188)

Use of the word ‘otherwise’ here is confusing therefore I removed it

Do you mean 2.1.3? Mention exact step numbers, I think it should be 2.1.3-2.1.4 (Line 188)

Exact numbers are now mentioned. Changed from 2.1.1 to 2.1.3- 2.1.4, in order to increase clarity.

What ratio of samples to gel loading solution? (Line 191)

Make it 1X, this is the same as in step 2.1.5 which is addressed above.

Unclear which samples these are. Do you mean RNA samples?(Line 191)

‘Native gel samples” was changed to “RNA samples”

For how long?(Line 192)

Talking about the incubation time for the native gel samples which were incubated to 100 mins prior to running the gel therefore the time has been added.

When and how ate the above samples loaded?(Line 195)

XXX The samples are loaded using a pipette.. do we have to clarify this seems really obvious. XXX

Cannot be made into imperative voice so I have made this a note.(Line 202)

Add to the table of materials.(Line 203)

Reference?(Line 205)

Colors will not show up when the text is integrated on the jove website. Please use a different format or make this a figure instead. If you do so, reference this figure at the end of the last sentence (ending with “fold”) (Line 210)

XXX Should we make this into a figure or should we use different formatting?)

Commercially ordered from above note?(Line 222)

At the same concentration as in 1.2.2 correct? (Line 223)

Yes, added this for clarity.

Example %?(Line 227)

Addressed this by mentioning table 2 as well as giving an example.

Mention exact step numbers.(Line 231)

This was changed by adding the exact steps from just ‘step 2 to step 2.1.4-2.1.7’

When is the DNA loaded? (Line 233)

It was mentioned in the note above but in order to address this more clearly, this has been made into a step (4.1.3)

Add to the table of materials. (Line 234)

Fluorophore?(Line 235)

Change made.

What speed? (in g) (Line 245)

Eluent? (Line 248)

Elutant was changed to eluent, this was a spelling error.

Please double check, you are mixing NTP to CTP, ATP, amd GTP to produce 5X NTP?? Does not add up (Line 258)

XXX UTP is missing XXX

This is confusing. Do you simply mean mix 100 mM NTP, 40 mM GTP, 25 nM CTP etc? (Line 258)

Make a 100 mM NTP stock made up of the following GTP, CTP, ATP, and UTP.

T7 transcription buffer?(Line 274)

Yes, added into the step for additional clarification

What volume? (line 275)

Depends on the final volume that the person wants to make. It says 10X to 1X so dilute accordingly.

Volume? (Line 275)

Depends on the final volume that the person wants to make. It says 10X to 1X so dilute accordingly.

Volume? (line 275)

Depends on the final volume that the person wants to make. It says 10X to 1X so dilute accordingly.

From where? Unclear which one this is Line 276)

See section 4, this is added to step 4.2.3.1

Check and update (Line 277)

Volume? (Line 277)

Depends on the final volume that the person wants to make. It says 10X to 1X so dilute accordingly.

Add to the table of materials. (Line 277)

Aluminium backed TLC plates added to the table of specific materials

Reference (Line 294)

What cell density? (Line 302)

It is mentioned in the step that the protocol is described elsewhere in detail with a reference. However, I have added in brackets that the cells are induced at an OD_{600nm} of 1.

Induced-E. coli Cell pellet? (Line 307)

Previously mentioned just pellet, clarified to 'Induced-E. coli Cell pellet).
Concentration? (Line 307)

Not dependent on concentration. Is dependent on volume.

Speed and duration? (Line 308)

Max speed for 2 min.

You mean add phenol, vortex and then centrifuge? (Line 310)
Yes, fixed this to make it more clear.

Speed (in g) and duration? (Line 315)
Max speed for 2 min.

Centrifuge speed and duration?? (Line 318)
Max speed for 2 min.

Duration? (Line 319)

At least 30 min.
Add the kit to the table of materials. (Line 324)

XXX A kit was not used XXX the protocol is referenced

In which solution? (Line 327)

In ddH_2O, since a protocol was followed, it is mentioned in the protocol but was added to the note to make it clear.

Add to the table of materials (Line 352)

T7 RNA polymerase added to the table of materials

Please avoid use of commercial names. (line 435)

“NEB protocol” was corrected to just a protocol, there is a reference for clarity

Remove the commercial name. (Line 464)

Commercial name of the imager has been removed to just say ‘fluorescence imager’

Remove the commercial name. (Line 496)

Commercial name of the imager has been removed to just say ‘fluorescence imager’

Should “acrylamide:bis" be “acrylamide:N,N’-methylenebisacrylamide” in the table? (Line 504)

This correction has been made in Table 3 as well.