

Journal of Visualized Experiments

Antibody-free assay for RNA methyltransferase activity analysis

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59856R3
Full Title:	Antibody-free assay for RNA methyltransferase activity analysis
Keywords:	RNA methylation; in vitro RNA methyltransferase assay; in vitro transcription; gel purification; S-Adenosyl-Methionine; scintillation counts; urea denaturing gel; epigenetics; epitranscriptome.
Corresponding Author:	Blerta Xhemalce University of Texas at Austin Austin, TX UNITED STATES
Corresponding Author's Institution:	University of Texas at Austin
Corresponding Author E-Mail:	b.xhemalce@austin.utexas.edu
Order of Authors:	Samantha B Shelton Blerta Xhemalce
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.	Austin, Texas, USA

TITLE

Antibody-Free Assay for RNA Methyltransferase Activity Analysis

AUTHORS AND AFFILIATIONS:

Samantha B. Shelton¹, Blerta Xhemalçe¹

¹Department of Molecular Biosciences, University of Texas at Austin, Austin, TX, USA

Email address of authors:

Samantha B. Shelton (samanthashelton@utexas.edu)

Corresponding author:

Blerta Xhemalçe (b.xhemalce@austin.utexas.edu)

KEYWORDS:

RNA methylation, in vitro RNA methyltransferase assay, in vitro transcription, gel purification, S-adenosyl-methionine, scintillation counts, urea denaturing gel, epigenetics, epitranscriptome

SUMMARY:

Here, an antibody-free in vitro assay for direct analysis of methyltransferase activity on synthetic or in vitro transcribed RNA is described.

ABSTRACT:

There are more than 100 chemically distinct modifications of RNA, two thirds of which consist of methylations. Interest in RNA modifications, and especially methylations, has re-emerged due to the important roles played by the enzymes that write and erase them in biological processes relevant to disease and cancer. Here, a sensitive in vitro assay for accurate analysis of RNA methylation writer activity on synthetic or in vitro transcribed RNAs is provided. This assay uses a tritiated form of S-adenosyl-methionine, resulting in direct labeling of methylated RNA with tritium. The low energy of tritium radiation makes the method safe, and pre-existing methods of tritium signal amplification, make it possible to quantify and to visualize the methylated RNA without the use of antibodies, which are commonly prone to artifacts. While this method is written for RNA methylation, few tweaks make it applicable to the study of other RNA modifications that can be radioactively labeled, such as RNA acetylation with ¹⁴C acetyl coenzyme A. Overall, this assay allows to quickly assess RNA methylation conditions, inhibition with small molecule inhibitors, or the effect of RNA or enzyme mutants, and provides a powerful tool to validate and expand results obtained in cells.

INTRODUCTION:

DNA, RNA and proteins are subject to modifications that tightly regulate gene expression¹. Among these modifications, methylations occur on all three biopolymers. DNA and protein methylations have been very well studied during the last three decades. In contrast, interest in RNA methylation has been only recently reignited in light of the important roles that proteins that write, erase or bind RNA methylations play in development and disease². In addition to better known functions in the abundant ribosomal and transfer RNAs, RNA methylation pathways

regulate specific messenger RNA stability^{3,4}, splicing⁵ and translation^{6,7}, miRNA processing^{8,9} and transcriptional pausing and release^{10,11}.

Here, a simple and robust method for in vitro verification of RNA methyltransferase activity in the setting of a molecular biology lab is reported (summarized in **Figure 1**). Many studies assess the activity of an RNA methyltransferase through dot-blot with an antibody against the RNA modification of interest. However, dot-blot does not verify the integrity of the RNA upon incubation with the RNA methyltransferase. This is important because even minor contaminations of recombinant proteins with nucleases can lead to partial RNA degradation and confounding results. Moreover, even highly specific RNA modification antibodies can recognize unmodified RNAs with specific sequences or structures. The in vitro RNA methyltransferase assay reported here takes advantage of the fact that the S-adenosyl methionine can be tritiated on the methyl group donor (**Figure 1**), allowing for the methylated RNA to be accurately detected without the use of antibodies. Instructions are provided for in vitro transcription and purification of a transcript of interest and testing of the methylation of said transcript by the enzyme of interest. This method is flexible and robust, and can be adjusted according to the needs of any given project. For example, in vitro transcribed and purified RNAs, chemically synthesized RNAs, but also cellular RNAs can be used. This assay provides quantitative information in the form of scintillation counts, as well as qualitative information by showing where exactly the methylated RNA runs on a gel. This can provide unique insight into the function of an RNA methyltransferase, particularly when using cellular RNAs as a substrate, as it provides a method to directly observe the size of the RNA or RNAs that are targeted for methylation.

PROTOCOL:

1. In vitro transcription and gel purification of the target RNA

1.1. Clone the sequence of interest into plasmids containing T7 and/or SP6 promoters using established molecular cloning techniques¹² or kits.

1.2. Linearizing the DNA template for in vitro transcription

1.2.1. Amplify the sequence of interest by PCR of the plasmid with primers designed to include the T7 promoter region through the insert, +20-30 bp upstream and downstream as previously described¹³.

1.2.2. Alternatively, digest 5 µg of the plasmid using a restriction enzyme (RE) cut site available downstream the insert according to the instructions provided by the supplier of the RE.

NOTE: Remember to double-check that the insert is not cut by the selected RE. Selection of the RE used in this step can dramatically affect the yield of the transcript. Preferentially, linearize with a blunt or 5'-overhanging end producing RE to avoid template switching of the T7 RNA polymerase on the opposite DNA strand¹⁴. Try different RE if initial yields are insufficient.

1.3. Purify the resulting DNA using the kit for DNA agarose gel extraction and clean-up. Elute the DNA with 30 µL of water.

1.4. Mix well by vortexing, pipet 1.5 µL and measure DNA concentration with a microvolume spectrophotometer.

1.5. Use 250 ng of DNA to check the quality and size of the purified DNA on a 1% agarose gel electrophoresis in 1x TBE buffer migrated for 1 h at 4 V/cm.

1.6. Thaw the frozen reagents of the in vitro transcription kit. Place the T7 RNA Polymerase Mix on ice, and the other reagents on a nutator at room temperature. Immediately after complete thawing, quick spin the rNTP tubes for 5 s to collect solution at the bottom of the tubes and place on ice. Keep the 10x Reaction Buffer at room temperature to avoid precipitation.

1.7. Pipet into a 1.5 mL tube the following components of the 20 µL transcription reaction at room temperature in the indicated order: 6 µL of water, 2 µL (0.1 - 1 µg) of linear DNA template, 2 µL of 75 mM ATP solution, 2 µL of 75 mM GTP solution, 2 µL of 75 mM CTP solution, 2 µL of 75 mM UTP solution, 2 µL of 10x Reaction Buffer and 2 µL of T7 RNA Polymerase Mix.

NOTE: For DNA template generated by PCR, use 100 – 200 ng DNA; for DNA template generated by restriction enzyme digest of a plasmid, use ~1 µg. Active recombinant His₆-tagged T7 RNA Polymerase can be purified in *E. coli*¹⁵.

1.8. Mix the tube thoroughly. Quick spin for 5 s to collect reaction solution at the bottom of the tube, then incubate at 37 °C for 2-4 h.

NOTE: Every transcript will behave differently depending on the DNA template, its length, its sequence or structure. Optimize in vitro transcription conditions by testing different incubation times up to 6 h; different concentrations of DNA template, T7 RNA Polymerase, or NTP; or addition of supplementary MgCl₂ to what is present in the T7 RNA Polymerase Mix.

1.9. At the end of the incubation period, add 1 µL of DNase I per 20 µL reaction and incubate at 37 °C for 15 min.

NOTE: The transcription reaction can be temporarily kept on ice until the polyacrylamide gel is ready.

1.10. Proceed with purification by polyacrylamide gel.

NOTE: Since RNA is sensitive to pH and RNase contamination, use RNA dedicated equipment and RNase-free reagents.

1.11. Determine the percentage of polyacrylamide for the gel depending on the size of the transcript of interest: 3.5% for 100-2000 nt (XC: 460 nt ; BB: 100 nt), 5% for 80-500 nt (XC: 260 nt

; BB: 65 nt), 8% for 60-400 nt (XC: 160 nt ; BB: 45 nt), 12% for 40-200 nt (XC: 70 nt ; BB: 20 nt), 15% for 25-150 nt (XC: 60 nt ; BB: 15 nt), and 20% for 6-100 nt (XC: 45 nt ; BB: 12 nt).

1.12. Prepare the urea denaturing polyacrylamide gel by combining the following reagents in a 50 mL conical tube: 9.6 g of molecular grade urea, 2 mL of 10x TBE, x mL of 40% acrylamide:bis-acrylamide mix (29:1) and water up to the 20 mL mark on the tube, where $x = 20 \text{ mL} / (40\% / \text{gel percentage } \%)$.

CAUTION: Polyacrylamide gels often contain un-polymerized acrylamide which is a toxic material that can produce a hazard when introduced to the environment. Dispose of polyacrylamide gels through the institution's chemical waste program.

1.13. Microwave for 15 s at 30% power. Place on the nutator for 10 min at room temperature or until urea has completely dissolved.

1.14. While the gel mixture is rotating, retrieve a gel cassette (18-well, 1 mm thick; 13.3 x 8.7 cm (W x L)), remove the comb and position the cassette for gel casting.

1.15. Quick spin the 50 mL tube for 5 s to collect gel solution at the bottom of the tube. Add 125 μL of 10% ammonium persulphate solution (APS). Place on the nutator for ~ 1 min. Quick spin again for 5 s.

1.16. Add 25 μL of tetramethylethylenediamine (TEMED) and carefully mix by pipetting up and down 5 times with a 25 mL pipette avoiding bubbles.

1.17. Pipette into the gel cast and carefully insert the gel comb avoiding bubbles.

1.18. Tighten the seal by adding a large binder clip over the top of the cassette.

1.19. Allow the gel to polymerize for 1 h.

1.20. Once the gel has solidified, remove the binder clip. Insert the cassette into the electrophoresis box. Add 1x TBE buffer into the top and bottom reservoirs.

1.21. Retrieve transcription reaction. Add water up to 100 μL , then add 100 μL of 2x Gel Loading Buffer. Prepare the ladder by mixing the recommended amount with water to 10 μL and 10 μL of 2x Gel Loading Buffer to a separate 1.5 mL tube.

1.22. Incubate both the ladder and the in vitro transcription reaction in the thermomixer at 70 $^{\circ}\text{C}$ for 15 min.

1.23. While the samples are denaturing at 70 $^{\circ}\text{C}$, carefully remove the comb, clean the wells by pipetting up and down each well with a P1000 pipette, and immediately pre-run for 10 min at 100 V.

1.24. Once the samples have completed their 15 min denaturation at 70 °C, remove the tubes from the thermomixer and immediately place on ice.

1.25. Clean each well of the gel again with the P1000 pipette. Load 20 µL of the ladder on the first well to the left, 20 µL of the RNA sample on 10 separate wells, and 20 µL of 1x Gel Loading Buffer on the unutilized wells.

1.26. Run the gel at 100 V for 60 -240 min, depending on the % polyacrylamide of the gel.

NOTE: The dyes in the Gel Loading Buffer will separate into two bands as they traverse the gel, a slow migrating Bromophenol Blue (BB) blue band, and a fast migrating Xylene Cyanol (XC) cyan band. The approximate migration of these bands in different % polyacrylamide gels is well known (see step 1.11) and can be used to estimate the migration of RNA in the gel.

1.27. Once the gel has been stopped, carefully remove the gel from the cassette.

1.28. Place the gel in a clean box containing a solution of 50 mL of 1x TBE buffer with 50 µL of nucleic acid gel stain and incubate for 5 min on a rocker to stain the RNA.

1.29. Take a before and after band excision picture of the gel on the gel imaging system, preferably using blue light instead of UV transillumination.

1.30. Excise each band of interest using a nuclease-free disposable gel cutting tip. After each well, transfer the tip containing the gel slice to a 1.5 mL tube and briefly spin to collect the gel slice. Repeat until all the bands have been collected in the same 1.5 mL tube.

1.31. Once all the gel slices have been collected, add 100 µL of nuclease-free water or TE buffer to the 1.5 mL tube. Store at 4 °C for ~48 h. This allows the RNA to exit the gel slices into the solution.

1.32. After 48 h, pipet the water or TE buffer to a fresh 1.5 mL tube. Dispose of the remaining gel slices. Purify the RNA via clean-up kit as follows.

1.32.1. Equilibrate the spin column at room temperature for at least 30 min.

1.32.2. To the 100 µL of RNA solution in the new 1.5 mL tube from step 1.32, add 350 µL of RLT buffer and mix well for 2 min on a nutator. Spin for 1 s at 200 x *g* to collect solution at the bottom of the tube.

1.32.3. Add 675 µL of 100% EtOH and mix well for 2 min on the nutator. Spin for 1 s at 200 x *g* and immediately proceed to the next step.

1.32.4. Transfer 565 μL of the mixture onto the spin column and spin for 1 min at 15,000 $\times g$. Empty the collection tube by aspiration.

1.32.5. Repeat previous step with the second half of the sample.

1.32.6. Add 500 μL of RPE buffer to the column and spin for 1 min at 15,000 $\times g$. Empty the collection tube by aspiration.

1.32.7. Add 750 μL of 80% ethanol to the column and spin for 1 min at 15,000 $\times g$. Empty the collection tube by aspiration.

1.32.8. Place the column in a new 2 mL collection tube with the lid open and spin at 15,000 $\times g$ for 5 min.

1.32.9. Transfer the column to a new 1.5 mL tube.

1.32.10. Add 17 μL of water on the center of the column and spin at 15,000 $\times g$ for 1 min to elute. Elute again using another 17 μL of water. The total recovered volume should be 32 μL .

1.33. Mix well by vortexing, pipet 1.5 μL and measure the concentration of the RNA using a microvolume spectrophotometer.

1.34. Check the quality of the RNA purification by urea denaturing polyacrylamide gel as in steps 1.11-1.29.

2. In vitro RNA methyltransferase assay

2.1. Set up the 100 μL RNA methyltransferase assay in a 1.5 mL tube on ice as follows: 23 μL of water, 10 μL of 10x TBS (500 mM Tris-HCl, pH 7.5; 1.5 M NaCl), 2 μL of 0.05 M EDTA, 5 μL of 100 mM DTT, 40 μL of 50% glycerol, 4 μL of 58 μM ^3H -SAM, 5 μL of 20x Protease Inhibitor Cocktail, 1 μL of RNaseOUT (optional), 5 μL of RNA and 5 μL of Methyltransferase.

CAUTION: Radioactive tritiated material is hazardous and should only be handled while wearing gloves, a lab coat and any other necessary PPE. All pipette tips and tubes in contact with radioactive material are considered solid radioactive waste. Dispose of all solid and liquid radioactive waste according to the lab's approved radioactive waste protocol.

NOTE: Include control samples without the methyltransferase and without the RNA. The reagent concentrations may require optimization and/or inclusion of divalent cation salts, such as MgCl_2 , or unlabeled SAM. The optimal range of final RNA concentration is from 50 nM to 1 μM , while the methyltransferase concentration is from 25 nM to 300 nM.

2.2. Mix thoroughly by gently vortexing the tube. Spin 5 s at 200 $\times g$ to collect the solution at the bottom of the tube. Incubate the tube(s) at 37 $^\circ\text{C}$ for 2 h.

264
265 2.3. Clean up the reaction using column purification as in step 1.32.

266
267 CAUTION: Be very careful to properly dispose of any radioactive materials, particularly during the
268 column washes (pipette out instead of aspirating waste from collection tubes).

269
270 2.4. Perform liquid scintillation count

271
272 2.4.1. Set up the scintillation count rack with one vial per sample, one vial for background
273 measurement and one vial for the swipe test. Fill the vials with 5 mL of scintillation counting
274 solution.

275
276 2.4.2. Add 10 μ L of each eluted radioactive RNA sample into 1 vial, and tighten the lid and mix
277 gently.

278
279 2.4.3. Prepare the swipe test vials. Thoroughly rub cotton swabs on all surfaces and equipment
280 used during the protocol. Add swabs to the vials filled with 5 mL of scintillation solution and
281 tighten the lid.

282
283 2.4.4. Run the samples on the scintillation counter as follows. Open the counter hood, insert the
284 rack into the machine and close the hood. Select **Count Single Rack**. Select **Select User Program**.
285 Select or create a program that measures tritium (^3H) for 60 s. Hit **Count Rack**. Repeat the
286 scintillation count three times.

287
288 NOTE: The equipment will measure the scintillation count of each sample and output to both the
289 screen and a printout. Protocol can be paused here if desired. Remaining RNA samples from step
290 2.3 should be frozen at $-80\text{ }^{\circ}\text{C}$ for later use.

291
292 2.5. Proceed with the autoradiogram

293
294 2.5.1. Prepare and pre-run urea denaturing polyacrylamide gel as in steps 1.11-1.20.

295
296 2.5.2. Pipette 20 μ L of radioactive RNA material into a new 1.5 mL tube containing 20 μ L of 2x
297 Gel Loading Buffer. Mix well. Prepare the ladder as in step 1.21. Incubate the samples at $70\text{ }^{\circ}\text{C}$
298 for 15 min.

299
300 2.5.3. Wash the wells of the gel once more immediately before sample loading. Load 20 μ L of the
301 prepared ladder, 20 μ L of the samples, and 20 μ L of 1x Gel Loading Buffer on remaining lanes.
302 Run the gel at 100 V for 60-180 min, depending on polyacrylamide percentage.

303
304 2.5.4. Once the gel finishes running, remove the gel from the cassette and place it in a box
305 containing 50 mL of 1x TBE buffer with 5 μ L of ultrasensitive nucleic acid gel stain.

306
307 2.5.5. Incubate for 5 min on the rocker to stain the RNA.

2.5.6. Carefully take the gel out of the box and place it on the UV transilluminator of the gel imaging system with the wells up and the ladder on the left.

2.5.7. Focus the camera on the gel, turn on the UV light and then take an image of the gel by exposing from 50 ms to 1 s depending on signal intensity.

2.5.8. Turn off UV exposure, and save image as Tiff file.

2.5.9. Place the gel back into the box. Remove TBE. Fix the gel with 50 mL of fixing solution (50% methanol, 10% acetic acid, 40% ultra-pure water) for 30 min at room temperature on a rocker.

2.5.10. Gently move the gel again to a fresh black box containing 25 mL of the autoradiography enhancing solution. In the absence of a black box, cover the box with aluminum foil to protect the solution from light.

2.5.11. Incubate for 30 min at room temperature on the rocker.

2.5.12. Gently lift the gel and place it face-down on a sheet of plastic wrap with the wells up and the ladder on the right-hand side. Place two sheets of chromatography paper on the back of the gel. Gently flip the entire stack.

2.5.13. Pre-heat the gel dryer to 80 °C. Move back the plastic cover on the gel dryer. Insert wrap, gel and chromatography paper stack under the plastic cover and move the plastic cover back down to create a seal.

2.5.14. Dry for 1 h at 80 °C in the gel dryer.

2.5.15. Turn off the gel dryer and gently remove the stack. Remove the wrap and second chromatography paper. Tape remaining chromatography paper with the dried gel in an autoradiogram cassette.

2.5.16. Add 1 sheet of autoradiography film in the dark room.

2.5.17. Place the cassette at -80 °C and develop the film after 1 h to 4 weeks depending on signal intensity, which can be judged based on the previously measured scintillation counts: 1-4 weeks for 250-1,000 cpm, 24 h to 1 week for 1,000-10,000 cpm and 1 h to 24 h for >10,000 cpm.

2.5.19 Once the film is developed, place the film on top of the cassette and carefully mark with a lab marker the 4 edges of the gel, each well, and the position of the XC and BB dyes.

2.5.20 Scan the film at 300 or 600 pixels per inch resolution and save the image as Tiff file.

REPRESENTATIVE RESULTS

In vitro transcription reaction

Figure 2A represents a typical run from an in vitro transcription reaction with the T7 RNA polymerase of the 7SK snRNA, which is a relatively short (331 nt) and highly structured RNA. As shown on that raw image, there are multiple undesired bands, both shorter and longer than 7SK, probably resulting from random transcriptional initiation or termination events. Because of this, gel purification following the in vitro transcription reaction is important to obtain a clean RNA sample as shown in **Figure 2B**. At this point, the RNA of interest can be identified by its location relative to the ladder and purified by gel purification.

As mentioned previously, it may be necessary to optimize the length of the transcription reaction prior to the gel purification step. Transcription reactions that run for too long can result in extremely high amounts of RNA production, making downstream identification of the correct transcript difficult. It is also important to verify the identity of the purified transcript by Reverse Transcription and quantitative PCR (RTqPCR) using specific primers.

In vitro RNA methyltransferase assay

Figure 3 shows a representative result of an RNA methyltransferase assay described in the protocol using the lower limit of our recommended RNA and protein concentrations. This assay allows for both quantitative results from the scintillation counts, as well as qualitative results from the autoradiogram. Here, MePCE, an RNA methyltransferase known to methylate 7SK¹⁰, was shown to be able to also methylate U6. Moreover, as recently shown for 7SK¹⁶, binding of histone H4 to MePCE also inhibits U6 methylation. We were able to observe this in both the scintillation count (**Figure 3C**) and the autoradiogram (**Figure 3B**), showing the robustness of this protocol.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic representation of a typical RNA methyltransferase assay workflow and expected results. Sinefungin is a competitive methyltransferase inhibitor. L: ladder; WT: wild type; M: catalytic mutant; cpm: counts per min.

Figure 2. Representative experiment showing the product of in vitro transcription before (A) and after (B) purification on a denaturing urea-polyacrylamide 8% gel stained with nucleic acid stain. The arrows point to the 7SK transcript before and after gel purification. L: ladder.

Figure 3. In vitro RNA methyl-transferase assay performed with MePCE against U6. In vitro methyltransferase assay using recombinant GST-MePCE (25 nM), ³H-radioactive SAM as methyl group donor and in vitro transcribed U6 RNA (50 nM) as substrate. The autoradiogram was exposed for 2 weeks in order to detect the residual activity (250 cpm) in the +Histone H4 sample.

DISCUSSION:

Here, a simple and robust method for in vitro verification of RNA methyltransferase activity towards specific transcripts is reported. The assay takes advantage of the fact that S-adenosyl methionine can be tritiated on the methyl group donor (**Figure 1**), allowing for the methylated RNA to be accurately detected without the use of antibodies. However, it is important to note

that this assay cannot indicate which residue or chemical group is methylated by the enzyme. To identify or to verify that a specific residue is methylated, other methods such as mutational analysis, reverse transcription block or mass spectrometry analysis of the RNA substrate can be used in conjunction with the RNA methyltransferase assay.

The choice of RNAs and their mode of synthesis depends on the size of the RNA. Specific RNAs of sizes between 18 and 120 nt can be conveniently custom-synthesized from many reputed nucleic acid providers. However, most commonly, specific RNAs of various sizes are in vitro transcribed. The DNA templates for in vitro transcription can be generated in various ways, and require the sequence of interest to be located downstream of a T7 or SP6 promoter. When precise ends of RNAs are important, the pRZ plasmid is recommended¹⁷. The high sensitivity of the assay (**Figure 3**) also allows to substitute a purified RNA with a mixture of cellular RNA, either total or messenger RNAs for example. Indeed, the gel and autoradiogram provide a method to directly observe the size of the RNA(s) targeted for methylation.

The conditions reported here in step 2.1 of the protocol are optimal for the BIN3 family of methyltransferases, which has two homologs in humans, MePCE¹⁰ and BCDIN3D⁹. It is important to emphasize that the assay conditions need to be adjusted to the specific protein and RNA of interest. For example, it was shown that the presence of MgCl₂ decreases BCDIN3D activity¹⁸, however, MgCl₂ can be an important coordinator of RNA structure, and could thus constitutes an important component of the assay for other RNA methyltransferases.

The advantage of using an autoradiogram, which can be exposed for an extended period of time, is that it can allow to detect very weak activity that is not detected in the scintillation assay. Usually this indicates that the protein is a methyltransferase, but that one or more of the reaction conditions or reagents need to be optimized: enzyme; substrate, cofactor, buffer conditions, etc⁹. For example, the enzyme purification may need to be improved to remove or change the position of the tag. It may also be that the RNA used as a substrate needs to be folded properly or to interact with another protein or RNA factor to be methylated by the enzyme. Thus, pre-existing literature and/or own results in cells need to be carefully reviewed to set-up the assay conditions for the enzyme and RNA of interest.

The assay is also extremely flexible. For example, it can be a precursor step to another type of assay, such that the radioactively methylated RNA is used in quantitative and qualitative demethylase assays¹¹, or in RNA binding assays allowing to specifically visualize the behavior of the methylated RNA compared to the unmodified one. The assay can also be lightly adjusted to analyze the modification of RNAs that use coenzymes that can be radioactively labeled, such as acetyl coenzyme A for the study of RNA acetylation^{19,20}.

ACKNOWLEDGMENTS:

The authors would like to thank Dr. Turja Kanti Debnath for his help with ChemDraw. Research in the Xhemalçe lab is supported by the Department Of Defense – Congressionally Directed Medical Research Program – Breast Cancer Breakthrough Award (W81XWH-16-1-0352), NIH

Grant R01 GM127802 and start-up funds from the Institute of Cellular and Molecular Biology and the College of Natural Sciences at the University of Texas at Austin, USA.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

- 1 Xhemalce, B. From histones to RNA: role of methylation in cancer. *Briefings in Functional Genomics*. **12** (3), 244-253. doi: 210.1093/bfpg/els1064. Epub 2013 Jan 1011. (2013).
- 2 Shelton, S. B., Reinsborough, C. & Xhemalce, B. Who Watches the Watchmen: Roles of RNA Modifications in the RNA Interference Pathway. *PLoS Genetics*. **12** (7), e1006139. doi: 1006110.1001371/journal.pgen.1006139. eCollection 1002016 Jul. (2016).
- 3 Mauer, J. *et al.* Reversible methylation of m(6)Am in the 5' cap controls mRNA stability. *Nature*. **541** (7637), 371-375. doi: 310.1038/nature21022. Epub 22016 Dec 21021. (2017).
- 4 X, W. *et al.* - N6-methyladenosine-dependent regulation of messenger RNA stability. *Nature*. **505** (7481), 117-120 (2014).
- 5 Pendleton, K. E. *et al.* The U6 snRNA m(6)A Methyltransferase METTL16 Regulates SAM Synthetase Intron Retention. *Cell*. **169** (5), 824-835.e814. doi: 810.1016/j.cell.2017.1005.1003. (2017).
- 6 Meyer, K. D. *et al.* 5' UTR m(6)A Promotes Cap-Independent Translation. *Cell*. **163** (4), 999-1010. doi: 1010.1016/j.cell.2015.1010.1012. Epub 2015 Oct 1022. (2015).
- 7 Wang, X. *et al.* N(6)-methyladenosine Modulates Messenger RNA Translation Efficiency. *Cell*. **161** (6), 1388-1399. doi: 1310.1016/j.cell.2015.1305.1014. (2015).
- 8 Alarcon, C. R., Lee, H., Goodarzi, H., Halberg, N. & Tavazoie, S. F. N6-methyladenosine marks primary microRNAs for processing. *Nature*. **519** (7544), 482-485. doi: 410.1038/nature14281. Epub 12015 Mar 14218. (2015).
- 9 Xhemalce, B., Robson, S. C. & Kouzarides, T. Human RNA methyltransferase BCDIN3D regulates microRNA processing. *Cell*. **151** (2), 278-288. doi: 210.1016/j.cell.2012.1008.1041. (2012).
- 10 Jeronimo, C. *et al.* Systematic analysis of the protein interaction network for the human transcription machinery reveals the identity of the 7SK capping enzyme. *Molecular Cell*. **27** (2), 262-274. (2007).
- 11 Liu, W. *et al.* Brd4 and JMJD6-associated anti-pause enhancers in regulation of transcriptional pause release. *Cell*. **155** (7), 1581-1595. doi: 1510.1016/j.cell.2013.1510.1056. (2013).
- 12 JoVE Science Education Database. Basic Methods in Cellular and Molecular Biology. Molecular Cloning. *JoVE, Cambridge, MA*. (2019).
- 13 Lorenz, T. C. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *Journal of Visual Experimentation*. (63), e3998. doi: 3910.3791/3998. (2012).
- 14 Rong, M., Durbin, R. K. & McAllister, W. T. Template strand switching by T7 RNA polymerase. *Journal of Biological Chemistry*. **273** (17), 10253-10260. (1998).

- 15 Rio, D. C. Expression and purification of active recombinant T7 RNA polymerase from E. coli. *Cold Spring Harb Protocols*. **2013(11)**. (pii), 2013/2011/pdb.prot078527. doi: 078510.071101/pdb.prot078527. (2013).
- 16 Shelton, S. B. *et al.* Crosstalk between the RNA Methylation and Histone-Binding Activities of MePCE Regulates P-TEFb Activation on Chromatin. *Cell Reports*. **22** (6), 1374-1383. doi: 1310.1016/j.celrep.2018.1301.1028. (2018).
- 17 Walker, S. C., Avis, J. M. & Conn, G. L. General plasmids for producing RNA in vitro transcripts with homogeneous ends. *Nucleic Acids Research*. **31** (15), e82. (2003).
- 18 Blazer, L. L. *et al.* A Suite of Biochemical Assays for Screening RNA Methyltransferase BCDIN3D. *SLAS Discovery*. **22** (1), 32-39. doi: 10.1177/1087057116666276. Epub 1087057116662016 Sep 1087057116666227. (2017).
- 19 Arango, D. *et al.* Acetylation of Cytidine in mRNA Promotes Translation Efficiency. *Cell*. **175** (7), 1872-1886.e1824. doi: 1810.1016/j.cell.2018.1810.1030. Epub 2018 Nov 1815. (2018).
- 20 Ito, S. *et al.* Human NAT10 is an ATP-dependent RNA acetyltransferase responsible for N4-acetylcytidine formation in 18 S ribosomal RNA (rRNA). *Journal of Biological Chemistry*. **289** (52), 35724-35730. doi: 35710.31074/jbc.C35114.602698. Epub 602014 Nov 602619. (2014).

RNA:

- chemically synthesized, or
- in vitro transcribed/gel purified, or
- cellular RNA

Enzyme

- WT
- catalytic mutant (M)
- WT
- ± inhibitor

³H-SAM

RNA methyltransferase assay

methylated RNA

RNA:

methylated RNA

Stained Gel

Scintillation count

cpm

³H-SAM:

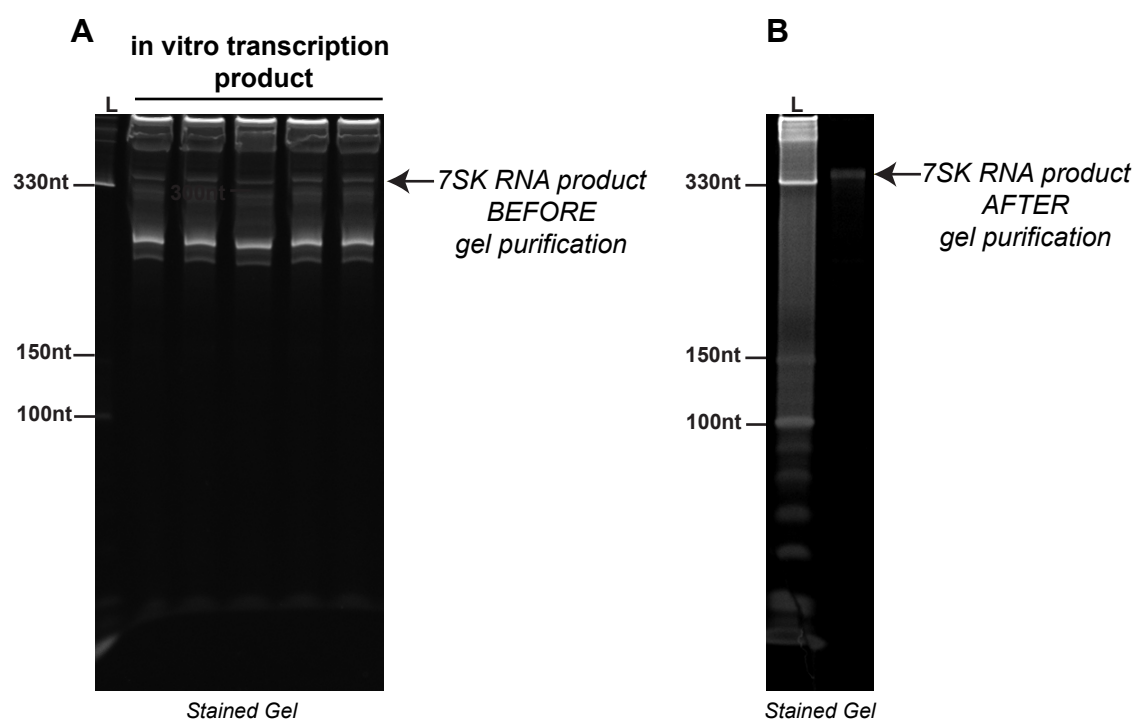
RNA:

Enzyme:

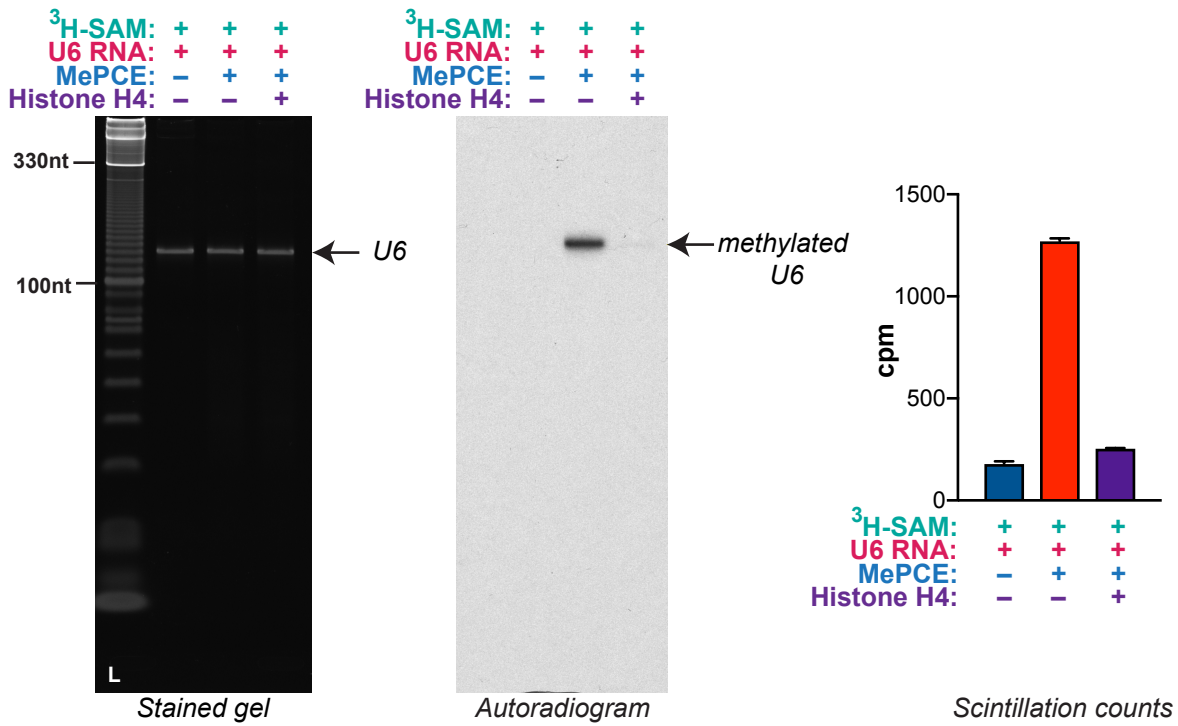
Inhibitor:

(Sinefungin)

Figure 2:



C



Name of Material/Equipment	Company	Catalog Number	Comments/Description
10 bp DNA Ladder	Invitrogen	10821-015	10 bp DNA Ladder kit. For urea denaturing polyacrylamide gel (For 10 mL, dissolve 1g in 8 mL of milliQ water; adjust volume to 10 mL with milliQ water; filter the solution using a 10 mL syringe equipped with a 0.45 µm filter).
10% Ammonium Persulfate (APS)	N/A	N/A	For urea denaturing polyacrylamide gel (For 1L, add 108 g of Tris Base, 55 g of Boric Acid to a cylinder with a stir bar; add 800 mL of distilled water and let dissolve; add 40mL of 0.5 M Na ₂ EDTA (pH 8.0); adjust volume to 1L with milliQ water; filter the solution using a 0.22µm filter).
10X TBE Buffer	N/A	N/A	For 1L, add 60.5 g of Tris Base, 87.6 g of NaCl to a cylinder with a stir bar; add 800 mL of distilled water and let dissolve; adjust pH to 7.5 with concentrated HCl; adjust volume to 1L with milliQ water; filter the solution using a 0.22 µm filter.
10X TBS	N/A	N/A	
Acrylamide: Bis-Acrylamide 29:1 (40% Solution/Electrophoresis), Fisher BioReagents	Fisher	BP1408-1	For urea denaturing polyacrylamide gel.
ADENOSYL-L-METHIONINE, S-[METHYL-3H]; (SAM[3H]) Amersham Hypercassette	Perkin Elmer	NET155V250UC	For <i>in vitro</i> methylation of RNA; Concentration = 1.0 mCi/mL; Specific activity = 17.1 Ci/mmol; Molarity= (1.0 Ci/L)/(83.2 Ci/mmol) = 0.0584 mmol/L = 58.4 µM. Upon receipt of the frozen 3H-SAM tube, thaw it at 4°C, make 20 µL aliquots, and freeze them at -30°C. Never refreeze and reuse a partially used aliquot.
Autoradiography Cassettes	GE Healthcare	RPN11649	For autoradiogram gel exposure.
Amersham Hyperfilm MP	GE Healthcare	28906846	For autoradiogram gel exposure.
Beckman Scintillation Counter	Beckman	LS6500	For liquid scintillation count.

Biorad Mini Horizontal Electrophoresis System	Biorad	1704466	Mini Horizontal Electrophoresis System.
cOMplete Mini EDTA-free Protease Inhibitor Cocktail Tablets	Roche Applied Science	4693159001	For a 20X solution, dissolve 1 tablet in 0.525 mL of nuclease free water.
Criterion Cell	Biorad	345-9902	RNase free empty cassette for polyacrylamide gel.
Criterion empty Cassettes	Biorad	1656001	Vertical midi-format electrophoresis cell.
DeNovix DS-11 Microvolume Spectrophotometer	DeNovix	DS-11-S	Microvolume Spectrophotometer for measuring DNA and RNA concentration.
Ecoscint Original	National Diagnostics	LS-271	For liquid scintillation count.
Fisherbrand 7mL HDPE Scintillation Vials	Fisher	03-337-1	For liquid scintillation count.
Fluoro-Hance-Quick Acting Autoradiography Enhancer	RPI CORP	112600	For autoradiogram gel pretreatment.
Gel dryer	Biorad	1651745	For drying gel.
Gel Loading Buffer II	Ambion	AM8547	For loading RNA in denaturing polyacrylamide urea gel (composition: 95% Formamide, 18 mM EDTA, and 0.025% SDS, Xylene Cyanol, and Bromophenol Blue).
GeneCatcher disposable gel excision tips	Gel Company	NC9431993	For removing bands from agarose and polyacrylamide gels.
Megascript Kit	Ambion	AM1333	For <i>in vitro</i> transcription with T7 RNA polymerase.
Perfectwestern Extralarge Container	Genhunter Corporation	NC9226382 (clear)/ NC9965364 (black)	Gel staining box.
pRZ	Addgene	#27663	Plasmid for producing <i>in vitro</i> transcripts with homogeneous ends
Qiagen RNeasy MinElute Cleanup	Qiagen	74204	For RNA clean-up, use modified protocol provided in the protocol.
QIAquick Gel Extraction Kit (50)	Qiagen	28704	Kit for gel extraction and clean up of dsDNA fragment used for <i>in vitro</i> transcription.
Saran Premium Plastic Wrap	Saran Wrap	Amazon	For drying gel.
SYBR Gold	Invitrogen	S11494	Ultra sensitive nucleic acid gel stain.
SYBR Safe	Invitrogen	S33102	Nucleic acid gel stain.
TE	Sigma	93283-100ML	10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0

TEMED	Fisher	110-18-9	For urea denaturing polyacrylamide gel.
Thermomixer with SMARTBLOCK		5382000023/536100003	
24X 1.5mL TUBES	eppendorf	8	For temperature controlled incubation of 1.5 mL tubes.
TOPO TA Cloning Kit	<i>life</i> technologies		Kits for fast cloning of Taq polymerase–amplified PCR products into vectors containing T7 and/or SP6 promoters for in vitro RNA transcription.
TURBO DNase (2 U/μL)	Ambion	AM2238	For DNA removal from <i>in vitro</i> transcription reactions.
Urea	Sigma	51456-500G	For urea denaturing polyacrylamide gel.
Whatman 3MM paper	GE Healthcare	3030-154	Chromatography paper for drying gel.

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Sensitive assay for accurate analysis of methyltransferase activity on RNA

Author(s):

Samantha B Shelton, Blerta Xhemalce

Item 1: The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via:

☒ Standard Access

☐ Open Access

Item 2: Please select one of the following items:

☒ 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.

ARTICLE AND VIDEO LICENSE AGREEMENT

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. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **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.

10. **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.

11. **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

ARTICLE AND VIDEO LICENSE AGREEMENT

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 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.

12. **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.

13. **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.

14. **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 be 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 is required per submission.

CORRESPONDING AUTHOR

Name:	Blerta Xhemalce	
Department:	Molecular Biosciences	
Institution:	University of Texas at Austin	
Title:	Dr.	
Signature:		Date: 02/16/2019

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

1. Upload an electronic version 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 02140

ANSWERS TO THE EDITORIAL COMMENTS:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

→ **Completed as requested.**

2. Please do not highlight notes/cautions for filming.

→ **We have now removed the highlight from all Cautions and Notes.**

3. Generic steps cannot be filmed. Please specify the volume of all reagents in protocol steps (Step 1.7, 2.1).

→ **We have now specified the volumes in protocol steps 1.7 and 2.1 as requested, as well as in step 1.12.**