Video Article

Protein-tRNA Agarose Gel Retardation Assays for the Analysis of the N^6-threonylcarbamoyladenosine TcdA Function

Francisco J. Fernández[1,2,3], Sara Gómez[1], Sergio Navas-Yuste[1], Miguel López-Estepa[1], M. Cristina Vega[1]

[1]Department of Physical Chemical Biology, Center for Biological Research (CIB-CSIC), Spanish National Research Council (CSIC)

[2]Department of Immunology, Complutense University School of Medicine

[3]Abvance Biotech srl

Correspondence to: M. Cristina Vega at cvega@cib.csic.es

URL: https://www.jove.com/video/55638
DOI: doi:10.3791/55638

Keywords: Biochemistry, Issue 124, CsdL, cyclic t^6A (ct^6A), Cysteine Sulfinate Desulfinase (CSD), gel retardation assay, N^6-threonylcarbamoyladenosine (t^6A), TcdA, Transfer RNA (tRNA), tRNA hypermodification

Date Published: 6/21/2017

Citation: Fernández, F.J., Gómez, S., Navas-Yuste, S., López-Estepa, M., Vega, M.C. Protein-tRNA Agarose Gel Retardation Assays for the Analysis of the N^6-threonylcarbamoyladenosine TcdA Function. *J. Vis. Exp.* (124), e55638, doi:10.3791/55638 (2017).

Abstract

We demonstrate methods for the expression and purification of tRNA(UUU) in *Escherichia coli* and the analysis by gel retardation assays of the binding of tRNA(UUU) to TcdA, an N^6-threonylcarbamoyladenosine (t^6A) dehydratase, which cyclizes the threonylcarbamoyl side chain attached to A37 in the anticodon stem loop (ASL) of tRNAs to cyclic t^6A (ct^6A). Transcription of the synthetic gene encoding tRNA(UUU) is induced in *E. coli* with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cells containing tRNA are harvested 24 h post-induction. The RNA fraction is purified using the acid phenol extraction method. Pure tRNA is obtained by a gel filtration chromatography that efficiently separates the small-sized tRNA molecules from larger intact or fragmented nucleic acids. To analyze TcdA binding to tRNA(UUU), TcdA is mixed with tRNA(UUU) and separated on a native agarose gel at 4 °C. The free tRNA(UUU) migrates faster, while the TcdA-tRNA(UUU) complexes undergo a mobility retardation that can be observed upon staining of the gel. We demonstrate that TcdA is a tRNA(UUU)-binding enzyme. This gel retardation assay can be used to study TcdA mutants and the effects of additives and other proteins on binding.

Video Link

The video component of this article can be found at https://www.jove.com/video/55638/

Introduction

The gel retardation assay[1] (also known as electrophoretic mobility shift assay, EMSA) is an electrophoretic method designed to study and characterize protein-nucleic acid interactions. It allows the analysis of protein-DNA as well as protein-RNA interactions and in particular, interactions between tRNA and tRNA-binding proteins, using either purified components or complex mixtures of proteins (*e.g.*, cell lysates) or nucleic acids (*e.g.*, tRNA pools). We have applied gel retardation assays to the study of the interaction between purified tRNA(UUU) and TcdA, an N^6-threonylcarbamoyladenosine (t^6A) dehydratase, which cyclizes the threonylcarbamoyl side chain attached to A37 in the anticodon stem loop (ASL) of tRNAs to cyclic t^6 (ct^6A)[2,3]. TcdA is also known as CsdL[3,4]. The *tcdA*/*csdL* gene forms a cluster with the *csdA* and *csdE* genes[5], which encode the cysteine desulfurase and the sulfur acceptor of the cysteine sulfinase desulfinate (CSD) system and is required to sustain TcdA function *in vivo*[3].

The rationale behind the gel retardation assays is that, while free nucleic acid molecules migrate rapidly to the front of acrylamide or agarose gels by virtue of their large negative charge, the electrophoretic mobility of protein complexes of the same nucleic acids is dramatically reduced. The reduction in mobility is visualized as a "shift" in the nucleic acid-protein complexes, which resolve as discrete bands. Positively charged and larger nucleic acid-protein complex show a greater shift or reduction in mobility on a gel.

Since charge neutralization of the complex is a universal phenomenon for protein-nucleic acid complexes, the gel retardation assay can be applied to a wide range of complexes and nucleic acid types. The method is simple, inexpensive, and can be conducted in laboratories with minimum equipment. It requires only small amounts of proteins and tRNA. This is an advantage over alternative biophysical techniques, which usually consume larger quantities of sample.

The gel retardation assay has been widely used for the study of transcription factors. The assay has been used to analyze binding kinetics, strength, and specificity of transcription factors for many different DNA sequences. It was indeed in this field that the EMSA was first developed[6,7]. Gel retardation assays with RNA[8,9,10,11] and tRNA molecules have also been used in ribosome research and to analyze, as we do here, the enzymes that modify tRNA nucleosides post-transcriptionally.

Many different parameters affect the result of a gel retardation assay such as temperature, quality of the protein and tRNA sample, and the strength of the binding. Careful planning and execution of the experiment is crucial for the interpretation of the results of the free nucleic acid

and protein-bound species. Here, we used the synthetic gene encoding tRNA(UUU) cloned into an expression vector whose promoter lacks the Shine-Dalgarno ribosome binding site, leading to the synthesis of large amounts of the tRNA[2]. This detailed protocol is intended to provide a clear guide for performing tRNA gel retardation experiments while avoiding common mistakes.

Protocol

Caution: Consult all relevant material safety data sheets (MSDS) before use. Several of the chemicals used in this protocol are toxic and corrosive. Use appropriate practices when performing the extraction of tRNA using phenol, including the use of a fume hood and personal protective equipment (safety glasses, gloves, lab coat, full-length pants, closed-toe shoes, *etc.*). This protocol uses a non-toxic nucleic acid stain. The use of alternative stains may require additional precautions and specialized disposal of the staining agent if toxic and/or carcinogenic (*e.g.*, ethidium bromide).

1. Preparation of the Agarose Gel

1. Weigh 2 g of agarose and add it to a glass bottle to prepare a 2% w/v agarose gel.
2. Add 100 mL of 1x tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) running buffer to the agarose-containing bottle. Melt by heating in a microwave. At 30 s intervals, swirl the contents to mix well and repeat this step until the agarose is completely dissolved. Allow the agarose solution cool down to approximately 50-60 °C.
3. Tape the open edges of a gel tray to create a mold, place an appropriate comb to create the wells and cast the molten agarose into it. Avoid bubble formation and let it solidify at room temperature.
4. Once solidified, place the gel tray in an electrophoresis unit and fill it with TBE buffer until the gel is fully covered.

2. Preparation of the tRNA

1. **Express tRNA(UUU) in *E. coli***
    1. In the morning, use a vial of frozen *E. coli* BL21(DE3) transformed with the expression vector pET23d-EcUUU[2] (harbors the gene encoding *E. coli* tRNA(UUU)), to streak a low-salt Luria Broth (LB) plate supplemented with 100 µg/mL ampicillin. Invert the plate and place it into a 37 °C incubator until colonies appear (late afternoon).
    2. Inoculate one well-grown, single colony in 5 mL LB plus 100 µg/mL ampicillin and grow overnight at 220 rpm at 37 °C.
    3. The next morning, pellet the cells (6,500 x g for 10 min at 4 °C), replace medium with 5 mL fresh LB plus 100 µg/mL ampicillin and resuspend. Use this suspension to inoculate 200 mL LB plus 100 µg/mL ampicillin. Grow for 5 h at 220 rpm at 37 °C.
    4. When the optical density of the culture at 590 nm (OD_{590}) has reached 0.6 - 0.8, add 1 mM IPTG to the culture to trigger the expression of the tRNA(UUU) gene. Incubate at 37 °C for 3 h.
    5. Harvest the cells by centrifuging the culture at 6,500 x g for 10 min at 4 °C.

2. **Lysis and extraction**
    1. Resuspend the cell pellet in 5 mL lysis buffer (0.3 M sodium acetate, 10 mM EDTA). From this point onward, used autoclaved buffers and keep all the tRNA-containing samples at 4 °C to prevent tRNA degradation.
    2. Add 1 volume of acid phenol (pH 4.3) to the resuspended cell pellet in the fume hood. Mix for 1 min by inversion to lyse the cells and centrifuge at 10,000 x g (10 min, 4 °C).
    3. Collect the upper aqueous phase containing the soluble tRNA(UUU) (and smaller concentrations of the other tRNA pools) using a pipette taking care not to aspirate the organic (bottom) phase. Discard the bottom layer and the pellet, which consists of the organic phase and cell debris, into an adequate container.
    4. Precipitate the tRNA by thoroughly mixing the soluble (aqueous) phase with 2.5 volume 100% v/v ethanol by inversion for 1 h at 4 °C. Centrifuge the mixture at 15,000 x g (30 min, 4 °C).
    5. Decant the supernatant carefully and resuspend the tRNA-rich pellet in 4 mL gel filtration buffer (20 mM sodium phosphate or potassium phosphate, pH 7.4, 0.1 mM EDTA). Run 5 µL of the resuspended tRNA pool on a 2% w/v agarose gel.
    NOTE: Good quality tRNA appears as a single band (molecular size between 50-100 bp).

3. **Purification of tRNA(UUU)**
    1. Following standard chromatographic practice[12], load the resuspended tRNA(UUU) onto a high-resolution preparative gel filtration column (dimensions: 16 x 600 mm, bed volume: 120 mL, resolution range: 1,000-70,000 Da) previously equilibrated in gel filtration buffer (mobile phase). Set the flow rate to 1 mL/min to elute the samples.
    NOTE: The tRNA(UUU) peak elutes approximately at 75.5 mL. Typically, a yield of 0.5-1.0 mg/L tRNA of culture can be obtained.
    2. Analyze the samples at representative elution volumes by electrophoresing 5 - 10 µL of each sample on a 2% w/v agarose gel.

3. Sample Preparation

1. Express and purify TcdA according to López-Estepa *et al.*[2].
NOTE: Up to 250 µM TcdA can be used for the study.
2. Pipette the corresponding amount of TcdA as per **Table 1** into properly labeled 1.5 mL centrifuge tubes. Add 1.6 mM *tris*(2-carboxyethyl)phosphine (TCEP) (final concentration) and gently mix using a pipette. Close the lid and incubate the mixture at room temperature for 5 min.
3. Add 10 µM of adenosine triphosphate (ATP) and 50 mM $MgCl_2$ (final concentrations). Mix with a pipette and incubate the mixture for 5 min at room temperature.
4. Add purified tRNA(UUU) (section 2.3) as per **Table 1**, mix with a pipette and incubate at room temperature for 5 min.
5. Add phosphate buffered saline (PBS) up to 100 µL final volume. Mix properly and add glycerol to a final concentration of 10% v/v.

6. Vortex the sample and briefly spin down the contents of the tube (10,000 x g, 1 min, 4 °C).

4. Gel Loading and Electrophoresis Development

1. Place electrophoresis unit into a tray filled with crushed ice to maintain the chamber at 4 °C during the electrophoresis run.
2. Carefully, pipette 5 µL of each sample into the corresponding well. Add a suitable DNA size marker.
3. Plug the electrodes into the corresponding slots of the power supply unit. Turn on the power supply unit and set the voltage to 100 V and run the gel for 90 min.

5. Gel Staining to Observe tRNA-Protein Interaction

1. Prepare the staining solution by mixing 50 mL of TBE with 1x of a suitable DNA stain. Avoid exposing the staining solution to sunlight by covering the staining container with aluminum foil.
   NOTE: DNA stains are usually stored as 10,000 or 20,000x concentrated stocks.
2. After 90 min of electrophoresis, carefully remove the gel from the tray and place it in a container with staining solution. Place it on a rocker at low rocking speed at room temperature for 30 min.
3. Remove the agarose gel from the staining container, tap it carefully on a piece of cellulose tissue or filter paper to remove excess liquid, and place it directly on a transilluminator. Switch on the UV light to visualize bands containing tRNA. Take a photograph of the gel.
4. **Protein staining (optional).**
   1. Discard the DNA staining solution safely (according to safety procedures) and substitute it with 50 mL Coomassie Brilliant Blue (CBB) solution. Place the tray on a rocker at room temperature and stain overnight (>12 h). Discard the CBB staining solution and replace it with 50 mL PBS to remove excess dye. Destain for 1 h on a rocker.

5. Visualize protein-containing tRNA complexes under a light transilluminator and take photographs of the gel to compare with the tRNA UV gel picture.

Representative Results

Large amounts of tRNA(UUU) can be obtained by expressing the tRNA gene in *E. coli* under the control of a strong inducible T7 promoter. The expressed tRNA(UUU) accumulates in the cytoplasm and is enriched over the pool of naturally abundant tRNAs. A two-step purification process consisting of a capture/extraction step and a gel filtration/polishing step was utilized to obtain EMSA-grade tRNA. The capture/extraction step uses pH 4.3 phenol to achieve the simultaneous precipitation of protein, cell debris, DNA, and the extraction of tRNA (and other RNA molecules). At this step, the amount of total tRNA pool can be assessed by electrophoresis on a 2% w/v agarose gel. The second step incorporates gel filtration chromatography that separates RNA species according to their molecular size. The UV trace at 254 nm is used to monitor the separation and identify the main elution peaks (**Figure 1 top**). Representative aliquots of peak fractions were run on a 2% w/v agarose gel for analysis (**Figure 1 bottom**). The majority of the tRNA molecules co-elute in a single peak (the middle) of the chromatography run (here, 75.5 mL in a 120 mL bed-size column). When separating pools containing a single overexpressed species, like tRNA(UUU), more than 90% of the tRNA molecules in the pool belong to that specific tRNA, while the remaining molecules correspond to the naturally abundant tRNAs. Secondary peaks are observed both before and after the tRNA-containing central peak. The peak(s) before contain partially-degraded larger RNA molecules (fractions 26 and 32; bars in **Figure 1**), and the peak(s) at the end of the purification run contain very small RNAs and contaminants (fractions 45 and 51; bars in **Figure 1**). After pooling the fractions under the central peak (fractions 39-43; bars in **Figure 1**), the amount of purified tRNA can be quantitated by UV spectroscopy and/or by comparison with molecular size standards on a 2% w/v agarose gel.

The TcdA and tRNA(UUU) samples can be mixed at different molar ratios from 1.0 to 10.0 using a semi-logarithmic sampling scheme (**Table 1**) and separated on 2% w/v agarose gels (**Figure 2**). Depending on the stability of the protein-tRNA complexes and the stoichiometry of binding, one or several bands can be visualized on the gel. For TcdA-tRNA(UUU), a stoichiometric complex is formed that runs as an isolated band containing both protein and tRNA. Typically, free tRNA migrates faster and can be observed at the bottom of the gel, while protein-tRNA complexes, which are less negatively charged and are larger in size, tend to migrate slower. **Figure 2** shows a representative 2% w/v agarose gel of TcdA-tRNA(UUU) complexes. In **Figure 2**, the TcdA-only and tRNA-only bands serve as negative controls. As the TcdA:tRNA(UUU) molar ratio increases, the amount of TcdA-tRNA(UUU) complexes formed at the expense of the free tRNA band, increases. Note that the intensity of free tRNA diminishes as the amount of TcdA increases. The first complex to form is a 2:1 stoichiometric complex containing one tRNA molecule per TcdA dimer (visible in the lanes with 10, 20 or 30 µM TcdA). At TcdA saturation, a larger molecular-weight TcdA-tRNA(UUU) complex develops that contains a 2:2 stoichiometric complex, where both the TcdA subunits are bound to one tRNA(UUU) molecule each (visible in lanes with 30, 50 or 75 µM). The two last lanes with TcdA (50 or 75 µM) have bound all the tRNA in the reaction and lack a free tRNA band.

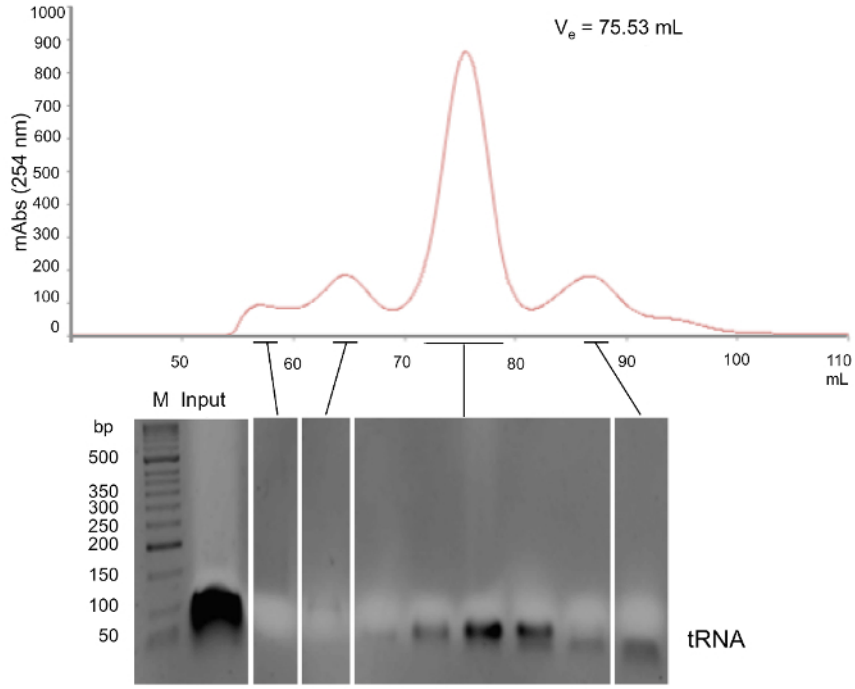

Figure 1: Purification of tRNA(UUU) by Gel Filtration. Chromatogram (top) of the gel filtration of tRNA(UUU) over a high-resolution preparative gel filtration column recorded at 254 nm. The highest peak, containing the tRNA pool enriched in tRNA(UUU) is eluted at ~75.5 mL. A 2% w/v agarose gel (bottom) was run with aliquots from the various tRNA elution fractions. M, molecular size ladder. Please click here to view a larger version of this figure.

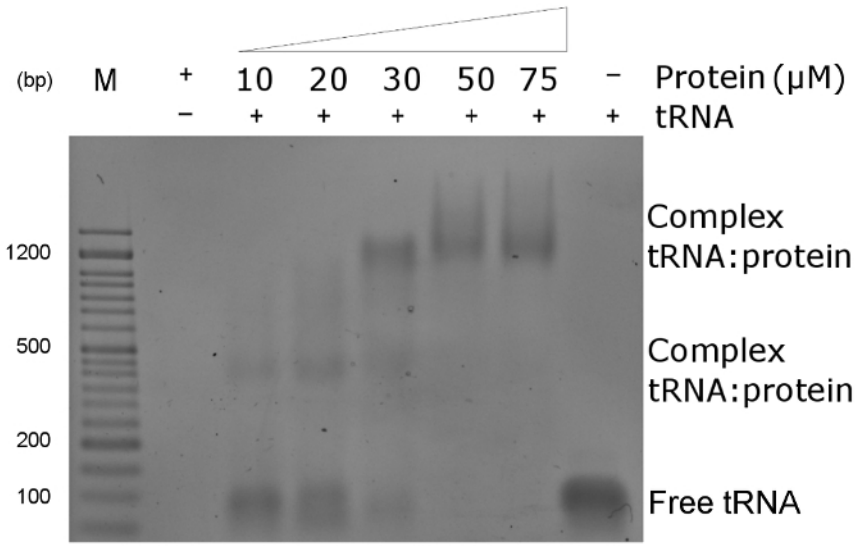

Figure 2: Gel Retardation Assay with TcdA and tRNA(UUU). EMSA provides evidence for the physical binding of tRNA to TcdA. Samples were run on a 2% w/v agarose gel at 4 °C for 90 min. A fixed amount of tRNA(UUU), 7.5 μM, was mixed with variable amounts of TcdA (see **Table 1**), and protein-tRNA complexes were separated during the electrophoresis run. The gel was stained and visualized on a UV transilluminator. Each lane contains 5 μL of sample. M, molecular size ladder. Please click here to view a larger version of this figure.

Discussion

The protein-tRNA agarose gel retardation assay described herein can be modified in a number of ways. First, the percentage of agarose in the gel can be reduced to allow the separation and visualization of protein-tRNA complexes significantly larger than the one analyzed here (120 kDa). Second, if the target protein is thermolabile, extra precautions must be taken to ensure that the temperature is kept below the maximum acceptable temperature by moving the electrophoresis apparatus to a cold room or by using ice packs inside the electrophoresis chamber to immediately dissipate the heat produced during the run. Other buffer compositions (*e.g.*, 0.5X TB, tris-borate) and running parameters (> 20 V/

cm) can be modified to shorten the run time if necessary or acceptable for the samples[11]. The suggested $MgCl_2$ concentration can be reduced if RNA degradation is observed during the sample preparation step, since divalent cations can potentially catalyze the cleavage of nucleic acids.

The major limitation of the technique is that the protein-tRNA bands are typically slightly more diffuse than those seen in acrylamide-based gel retardation methods, which tend to be sharper and clearer. Another limitation is that wider, longer gels may be necessary for the simultaneous analysis of multiple samples and if protein-tRNA complexes of various sizes and charge are to be resolved.

In comparison to existing methods, the protein-tRNA agarose gel retardation assay affords a significant reduction in labor and time from sample preparation to analysis. Acrylamide gels, contain harmful chemicals, are laborious to prepare and take longer to polymerize. In contrast, agarose gels are simple, fast to cast and set, and do not involve toxic chemicals. The experiment can be completed in 2 h. This includes 30 min for the gel to set, 15 min for sample preparation (which can be done while cooling the agarose), 90 min for the electrophoresis and visualization.

Future applications of the method may refine the detection methods used in this protocol and apply it to the detection of less abundant, more unstable RNA species, or to protein complexes that may be expressed only in very small amounts. Labeling the RNA or the protein component with highly sensitive fluorophores would allow the detection of smaller amounts of protein-RNA complexes. Furthermore, using fluorophores with non-overlapping emission/excitation peaks could allow for multiplexing experiments where different protein and/or RNA species could be visualized simultaneously in a single experiment.

There are three critical steps in this protocol. The first and most obvious critical step is the expression and purification of tRNA(UUU). If the quantity of purified tRNA(UUU) is below a certain minimum detection limit (between 25-200 ng tRNA per band), or degradation bands become apparent as lower molecular size contaminants, the tRNA should be discarded and re-purified. Using acid phenol (pH 4.3) during tRNA extraction is essential since it selectively precipitates DNA while keeping RNA soluble. The second critical step concerns the preparation of the protein-tRNA samples for analysis. Sufficient amounts of protein and tRNA must be used to allow for the robust detection of all complexes formed. The protein-tRNA molar ratios must be adjusted to displace the equilibrium toward complex formation. The third critical step is the electrophoresis. The TBE buffer must be used fresh (not reused) and it is necessary to keep the agarose gels at temperatures where the protein-tRNA complexes are stable and do not denature (typically, 4-10 °C). Care must be exercised to keep the agarose gel cold during the entire electrophoresis, for example, by surrounding the electrophoresis chamber with ice and, whenever possible, running the experiment in a cold room at 4 °C.

We have demonstrated a straightforward method for analyzing, characterizing protein-tRNA complexes using TcdA-tRNA(UUU) as a model system, and using agarose gel electrophoresis instead of acrylamide electrophoresis. The latter takes longer to complete and is considerably more laborious. Using this method, the electrophoretic separation on 2% w/v agarose gels (8 cm gel formats) can be accomplished in less than 90 min. This is a convenient tool for the analysis of protein-tRNA interactions and allows for the simultaneous analysis of multiple samples. Variants of tRNA and/or protein mutants can be screened for binding and stability using this method.

Disclosures

The authors have nothing to disclose.

Acknowledgements

MCV is member of the CIB Intramural Program “Molecular Machines for Better Life” (MACBET). The research leading to these results has received funding from the Spanish Instituto de Salud Carlos III (PI12/01667 to MCV), the Spanish Ministerio de Economía y Competitividad (CTQ2015-66206-C2-2-R and SAF2015-72961-EXP to MCV), the Regional Government of Madrid (S2010/BD-2316 to MCV), and the European Commission (Framework Programme 7 (FP7)) project ComplexINC (Contract No. 279039 to MCV). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Scott, V., Clark, A. R., & Docherty, K. The gel retardation assay. *Methods Mol Biol.* **31**, 339-347 (1994).
2. López-Estepa, M., *et al.* The crystal structure and small-angle X-ray analysis of CsdL/TcdA reveal a new tRNA binding motif in the MoeB/E1 superfamily. *Plos One.* **10** (4), e0118606 (2015).
3. Miyauchi, K., Kimura, S., & Suzuki, T. A cyclic form of N6-threonylcarbamoyladenosine as a widely distributed tRNA hypermodification. *Nat Chem Biol.* **9** (2), 105-111 (2013).
4. Bolstad, H. M., Botelho, D. J., & Wood, M. J. Proteomic analysis of protein-protein interactions within the Cysteine Sulfinate Desulfinase Fe-S cluster biogenesis system. *J Proteome Res.* **9** (10), 5358-5369 (2010).
5. Loiseau, L., Ollagnier-de Choudens, S., Lascoux, D., Forest, E., Fontecave, M., & Barras, F. Analysis of the heteromeric CsdA-CsdE cysteine desulfurase, assisting Fe-S cluster biogenesis in Escherichia coli. *J Biol Chem.* **280** (29), 26760-26769 (2005).
6. Fried, M. G., & Crothers, D. M. Equilibrium studies of the cyclic AMP receptor protein-DNA interaction. *J Mol Biol.* **172** (3), 241-262 (1984).
7. Garner, M. M., & Revzin, A. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. *Nucleic Acids Res.* **9** (13), 3047-3060 (1981).
8. Rio, D. C. Electrophoretic mobility shift assays for RNA-protein complexes. *Cold Spring Harb Protoc.* **2014** (4), 435-440 (2014).
9. Ryder, S. P., Recht, M. I., & Williamson, J. R. Quantitative analysis of protein-RNA interactions by gel mobility shift. *Methods Mol Biol.* **488**, 99-115 (2008).
10. Yakhnin, A. V., Yakhnin, H., & Babitzke, P. Gel mobility shift assays to detect protein-RNA interactions. *Methods Mol Biol.* **905**, 201-211 (2012).
11. Ream, J. A., Lewis, L. K., & Lewis, K. A. Rapid agarose gel electrophoretic mobility shift assay for quantitating protein: RNA interactions. *Anal Biochem.* **511**, 36-41 (2016).

12. Hagel, L. Gel-filtration chromatography. *Curr Protoc Mol Biol.* **Chapter 10**, Unit 10.9 (2001).