bio-protocol www.bio-protocol.org/e2799 Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2799

Purification of RNA Mango Tagged Native RNA-protein Complexes from Cellular Extracts Using TO1-Desthiobiotin Fluorophore Ligand

Shanker Shyam Sundhar Panchapakesan[1], Sunny C. Y. Jeng[2] and Peter J. Unrau[2,*]

[1]Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT, USA; [2]Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, Canada

*For correspondence: punrau@sfu.ca

[Abstract] A native purification strategy using RNA Mango for RNA based purification of RNA-protein complexes is described. The RNA Mango aptamer is first genetically engineered into the RNA of interest. RNA Mango containing complexes obtained from cleared cellular native extracts are then immobilized onto TO1-Desthiobiotin saturated streptavidin agarose beads. The beads are washed to remove non-specific complexes and then the RNA Mango containing complexes are eluted by the addition of free biotin to the beads. Since the eluted complexes are native and fluorescent, a second purification step such as size exclusion chromatography can easily be added and the purified complexes tracked by monitoring fluorescence. The high purity native complexes resulting from this two-step purification strategy can be then used for further biochemical characterization.

Keywords: RNA Mango, TO1-Desthiobiotin, RNP complex purification, Fluorophore, Native purification, Non-coding RNAs, RNA-protein complexes

[Background] Current RNA tags suffer from limitations such as poor K_D, large size, potential biological interference or lack of intrinsic fluorescence (Panchapakesan *et al.*, 2015). RNA Mango is small, can be simply integrated into stem-loop structures, in particular, GNRA tetraloops, is biologically tolerated and above all has a high affinity for its thiazole orange-based (TO1) ligand, TO1-Desthiobiotin (TO1-Dtb). This allows Mango tagged complexes to be easily bound and washed on streptavidin beads (summarized in Figure 1). The Mango:TO1-Desthiobiotin complex is highly fluorescent and can be eluted from streptavidin beads by the addition of biotin. The fluorescence of Mango tagged native complexes allows additional purification steps to be used to obtain highly purified native complexes.

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2799

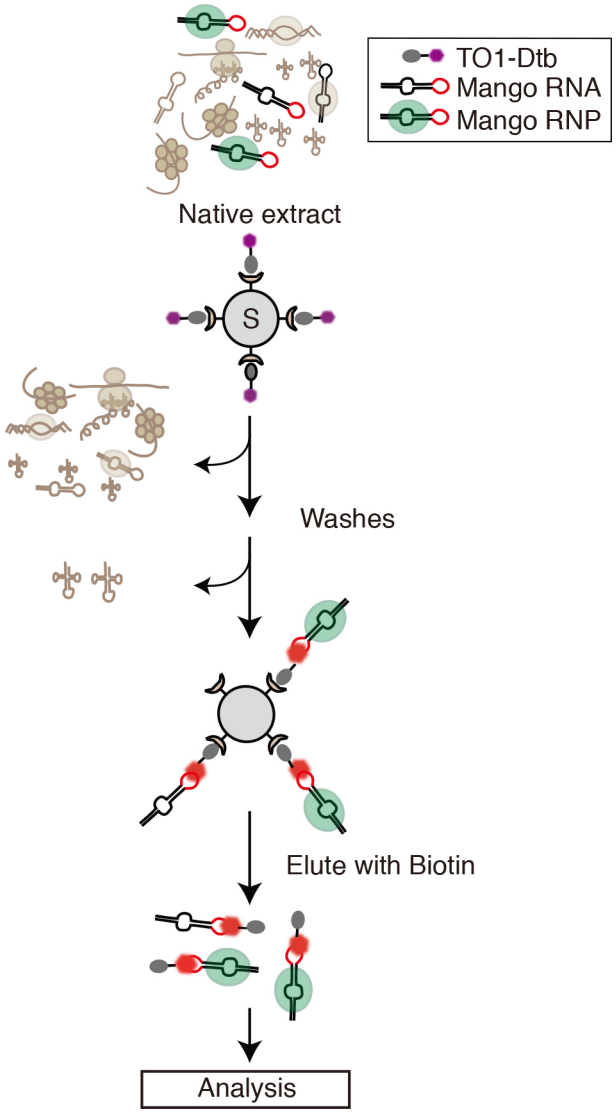

Figure 1. Purification of Mango tagged RNA-protein complexes out of native extract. RNA constructs are designed (Procedure A, Figure 2) so that the Mango tag is located in a biologically compatible location. After bacterial expression of this construct, a native extract containing the Mango tagged RNA (Mango tag shown in red highlight), is prepared (Procedure B). Mango tagged RNA and RNA complexes are then bound to streptavidin beads (Black circle containing S) that have been derivatized with TO1-Desthiobiotin (Dtb, Procedure C). The thiazole orange moiety of TO1-Dbt is shown in purple and becomes highly fluorescent once bound by Mango (bright red highlight). Bound complexes are then extensively washed in native conditions (Procedure D). After washing fluorescent Mango tagged complexes can be eluted in native conditions by addition of biotin (Procedure E). Downstream analysis including further purification steps can then be simply implemented (Procedures F and G).

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2799

Materials and Reagents

1. Low retention pipette tips (Fisher Scientific, catalog numbers: 02-717-134, 02-717-143, 02-707-511)
2. Microcentrifuge tubes, pre-lubricated (Corning, Costar®, catalog numbers: 3206, 3207)
3. 96-well black wall, clear bottom, non-binding, non-sterile, Greiner Bio-One microtiter plate (Greiner Bio One International, catalog number: 655906)
4. Tricorn XK 16/40 SEC column (GE Healthcare, catalog number: 28988938)
5. Bacterial cells (*E. coli*) with RNA Mango tagged RNA of interest
6. Liquid nitrogen
7. TO1-PEG3-Desthiobiotin (ABM, catalog number: G956)
8. High capacity streptavidin agarose beads (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 20357)
9. Superdex 200 resin (GE Healthcare, catalog number: 17104301)
10. SYBR Green II RNA Gel Stain (Thermo Fisher Scientific, Invitrogen™, catalog number: S7564)
11. 19:1 Acrylamide:Bis 40% (Thermo Fisher Scientific, catalog number: AM9022)
12. *N,N,N',N'*-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, catalog number: T9281)
13. Ammonium persulphate (APS) (Bio-Rad Laboratories, catalog number: 1610700)
14. Boric acid (ACP Chemicals, catalog number: B2940)
15. EDTA (ACP Chemicals, catalog number: E4320)
16. Tris base (Fisher Scientific, catalog number: BP152)
17. Tris-HCl (Sigma-Aldrich, Roche Diagnostics, catalog number: 10812846001)
18. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9541)
19. Magnesium chloride ($MgCl_2$) (Sigma-Aldrich, catalog number: M8266)
20. DTT (Sigma-Aldrich, Roche Diagnostics, catalog number: 10197777001)
21. Sodium hydroxide (NaOH) (VWR, BDH, catalog number: BDH9292)
22. Sodium chloride (NaCl) (ACP Chemicals, catalog number: S2830)
23. HEPES (Sigma-Aldrich, catalog number: H3375)
24. Heparin (Sigma-Aldrich, catalog number: H0777-25KU)
25. Biotin (Sigma-Aldrich, catalog number: B4501)
26. Yeast extract (Fisher Scientific, catalog number: BP1422)
27. Tryptone (Fisher Scientific, catalog number: BP1421)
28. Potassium hydroxide (KOH) (VWR, BDH, catalog number: BDH9262)
29. 10x Bacterial Native Extract (**BNE**) buffer (see Recipes)
30. 10x Buffer **A** (see Recipes)
31. 10x HEPES KCl (**HK**) buffer (see Recipes)
32. 10x **Binding** buffer (see Recipes)
33. 10x **Biotin elution** buffer (see Recipes)
34. **LB** media (see Recipes)

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2799

Equipment

1. Standard personal protective equipment
2. Pipettes
3. Peristaltic pump
4. Fraction collector and appropriate collection tubes
5. SpectraMax M5 fluorescent plate reader (Molecular Devices, model: SpectraMax M5)
6. Temperature controlled room (To be maintained at 4 °C)
7. Preparative centrifuge, microcentrifuge and benchtop Picofuge
8. Tube rotator for 1.5 ml tubes
9. Polyacrylamide gel running equipment
10. French press
11. Sorvall RC6+ centrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: Sorvall™ RC 6 Plus)
12. Sorvall SLA-1500 rotor (Thermo Fisher Scientific, Thermo Scientific™, catalog number: SLA-1500)
13. NanoDrop
14. Autoclave

Procedure

A. Construct design

1. A single copy of RNA Mango is usually sufficient for the pull down analysis and can be inserted into pre-existing stem loops that do not interfere with the function of the RNA of interest (**Figure 2** (Panchapakesan *et al.*, 2017; Trachman III *et al.*, 2017). If inserting into a pre-existing stem loop structure, the RNA Mango quadruplex core and GAAA tetraloop-like isolation domain then the Mango aptamer can be inserted within the stem structure using the following sequence: 5'-...NNN NNN *GAA* **GGG ACG GUG CGG AGA GGA G***A*N' N'N'N' N'N'.., where the left arm of the arbitrary RNA helix is indicated by N residues. The right arm (being the reverse complement of the left) is indicated by N' symbols. The GAAA tetraloop-like isolation motif (shown in italics) together with the TO1-Desthiobiotin binding quadruplex core (in bold) being inserted within the arbitrary helix. While GNRA tetraloops are common in nature, RNA Mango can also be inserted into the 3' or 5' end of an unstructured RNA of interest. In this case, RNA Mango (including the GAAA tetraloop-like motif) together with an arbitrary stem should be inserted. New Mango tags (Mango II, III and IV) can be inserted in a similar fashion (Autour *et al.*, 2018).

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2799

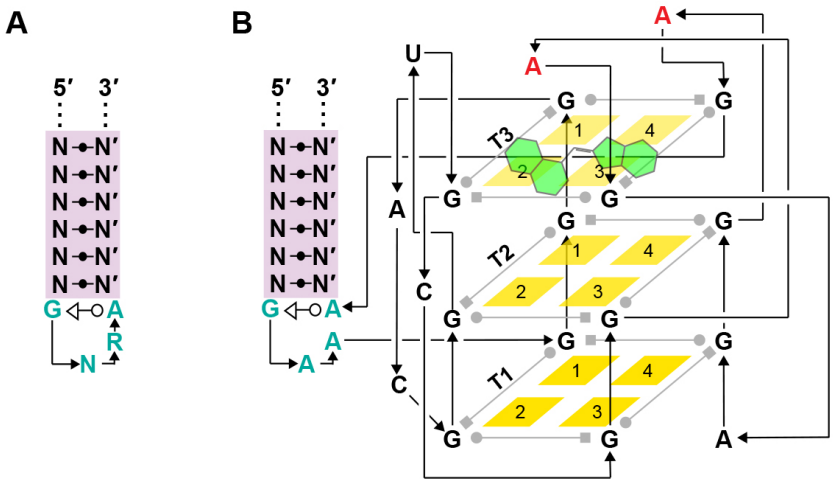

Figure 2. Design of biologically compatible Mango-I tags. A. A general GNRA tetraloop structure is shown, depicted using Leontis-Westhof nomenclature (Leontis and Westhof, 2001). B. Structure of Mango I (figure modified from Autour *et al.* (2018)). Mango can be incorporated into naturally occurring stem-loop structures by replacing the N-N' stem highlighted in purple with the stem sequence of the RNA of interest. In Mango I, a GAAA tetraloop-like motif (blue) encloses the core G-quadruplex (3 tiers: T1, T2, T3) by flanking it such that the Mango I core fits into GAA/A, where '/' is the point of the Mango core insertion. Thus, Mango I can be inserted into a pre-existing GNRA tetraloop structure in a similar manner, so long as Mango does not interfere with the tetraloop's biological function.

2. The Mango tagged RNA should be tested for the functionalities of the Mango tag as well as the RNA of interest. The functionality of the Mango tag can be tested by measuring the fluorescence of the Mango tagged RNA of interest and comparing it to *in vitro* transcribed RNA Mango (Panchapakesan *et al.*, 2017). Typically, a 1 µM solution of RNA is made along with two-fold excess of TO1-Dtb ligand in 1x **Binding** buffer. After incubating at RT for 5 min, fluorescence assays can be performed using a fluorometer. For a SpectraMax M5 fluorometer, the following excitation and emission setup is optimal for analyzing RNA Mango mediated fluorescence: Excitation at 495 nm; Emission 535 nm PMT: Medium, Cut-off 515 nm–Bottom read. Using a SpectraMax M5 under the aforementioned settings, RNA Mango concentrations as low as 5 nM can be reliably detected. The user should optimize the excitation and emission wavelengths to minimize interference from the excitation light source of the specific fluorometer being used.

B. Native extract preparation

1. Bacterial cells (*E. coli*) with RNA Mango tagged RNA of interest should be grown in appropriate growth conditions to maximize the formation of the RNP complex desired. We typically grew cells overnight in 1 L **LB** media.
2. Cells are then pelleted at 3,800 *x g* (RCF) for 10 min in a refrigerated Sorvall RC6+ centrifuge, using a Sorvall SLA-1500 rotor. The resulting cell pellet is then re-suspended in 20 ml of pre-

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2799

chilled **BNE** buffer in ice. Cells are lysed by passaging through a 4 °C French press twice at 1,000 psi. If a French press is unavailable, then alternative cell disruption techniques such as sonication or enzymatic methods such as lysozyme digestion can be used. This crude lysate is then cleared by centrifugation at 13,200 *x g* (RCF) for 10 min at 4 °C. The cleared lysate is flash frozen in liquid nitrogen and stored at -80 °C until ready for use. For other organisms, including yeast, appropriate native extract preparation protocols should be used.

3. Ensure the presence of KCl (90 to 150 mM), depending upon the stability of RNP complex of interest, as binding to TO1-Desthiobiotin requires KCl (Dolgosheina *et al*., 2014; Jeng *et al*., 2016; Panchapakesan *et al*., 2017). Handle all material in Steps B2 and B3 PROMPTLY on ice.

C. TO1-Desthiobiotin streptavidin agarose bead preparation

Prepare an appropriate amount of streptavidin agarose bead slurry. Using 7 ml of bacterial extract and 700 µl of streptavidin agarose bead slurry, derivatized with 42 nanomoles of TO1-Desthiobiotin, we obtained high quality results when overexpressing the 6S regulatory RNA (Dolgosheina *et al*., 2014; Panchapakesan *et al*., 2017). Scale the protocol below to suit experimental conditions.

1. Carefully re-suspend streptavidin agarose bead slurry by gentle agitation.
2. Take up 700 µl streptavidin agarose beads in a 1.5 ml Eppendorf tube.
3. Wash twice at room temperature (RT) with 1 ml Buffer **A**, 2 min each, using a standard laboratory rotator. The tubes can be centrifuged briefly in a Picofuge to remove excess liquid from the beads.
4. Wash beads three times using 1 ml **HK** buffer for 2 min each wash, again using a rotator.
5. For every 100 µl streptavidin agarose beads incubate with 6 nanomoles of TO1-Desthiobiotin in 100 µl of **HK** buffer. Incubate at RT for 15 min on a rotator. Note the OD_{500} (ε_{500} = 63,000 M^{-1} cm^{-1}) of the fluorophore solution before and after binding to the beads to estimate the amount of fluorophore bound using a NanoDrop. More than 90% of TO1-Desthiobiotin should be bound. The color of the beads will now turn orange because of the fluorophore binding. Different beads may have distinct binding capacities, which should be empirically determined if needed.
6. Wash with 1 ml of **HK** buffer to remove residual unbound fluorophore.

D. Binding of extract to prepared agarose beads

Since the final goal of this procedure is to purify RNP complexes in native conditions, care should be taken not to denature the complex in every step of the purification viz., bead binding, washing and elution. Since RNA Mango requires KCl for proper folding and binding to the TO1-Desthiobiotin ligand (Dolgosheina *et al*., 2014; Jeng *et al*., 2016; Trachman III *et al*., 2017), KCl should be included in all binding, washing and elution steps. Other components of the buffer can be modified according to experimental needs.

1. Bind 7 ml of bacterial extract to beads at RT. Incubate at RT for 15 min on a rotator (in the case of yeast extract (Panchapakesan *et al*., 2017), binding is performed at 4 °C, to ensure

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2799

stabilization of the RNP complex). The extract can be diluted, if required, by the addition of **BNE** or **Binding** buffer to the extract.

2. Wash twice with 1 ml **Binding** buffer for 2 min each on a rotator. The number of washes selected depends on the anticipated stability of the RNP complex. Likewise, the temperature (4-37 °C) and time (2-15 min) of each wash can be significantly adjusted if optimization is required.

E. Biotin-mediated native elution

Elute using 20 mM **Biotin elution** buffer in 500 µl volume at 37 °C (Panchapakesan *et al*., 2017) for 20 min on a rotator as biotin effectively displaces desthiobiotin from streptavidin (Hirsch *et al*., 2002). It is desirable to perform downstream processing immediately following biotin-mediated native elution. If not, the sample should be aliquoted and immediately flash frozen in liquid nitrogen for future use.

F. Downstream processing: Microtiter plate fluorescence assay and gel staining assays

1. Load 200 µl of biotin eluate into a 96-well black wall, clear bottom microtiter plate and measure fluorescence emission.
2. To estimate the purity, expression level and integrity of the RNA fraction of the Mango pulldown, phenol chloroform extract 20 µl of the biotin eluate, together with equivalent volumes of input, flow through and wash. Perform denaturing 5% PAGE with TBE as described elsewhere. If the RNA is well expressed, SYBR staining can be used to visualize both the RNA of interest and judge levels of RNA contamination. If the RNA expression levels are low, a Northern blot can be used to judge RNA integrity and expression levels.

G. Size Exclusion Chromatography (SEC) of streptavidin-purified Mango complex

As the biotin-eluted RNP complex is highly fluorescent (while it is bound to TO1-Desthiobiotin), it can be further purified with secondary purification techniques such as SEC (Panchapakesan *et al*., 2017). For SEC purification, load onto a size exclusion column containing SEC resin appropriate for the expected molecular size of the RNP complex being purified. To purify the Mango-tagged 6S RNA in complex with bacterial RNAP, we used a Superdex 200 column (24 ml, 16 mm diameter) using **HK** buffer at 0.1 ml/min flow rate. In this example, 380 µl of the biotin extract was loaded onto the column. The column was run at 4 °C and 500 µl fractions were collected in silanized plastic tubes.

1. Fraction analysis: 200 µl from each fraction was placed into a 96-well black wall, clear-bottom microtiter plate, and fluorescence measured using a SpectraMax M5 fluorescence plate reader (settings as previously described). Note that RNA Mango fluorescence is temperature sensitive, maximum fluorescence being obtained at lower temperatures (Jeng *et al*., 2016). Hence, if the signal to noise ratio is lower at RT, the signal can in principle be improved by measuring fluorescence at 4 °C provided that condensation can be avoided.

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2799

2. Pool the fluorescent fractions of interest. The purified RNP complexes can be analyzed using a variety of techniques such as Mass Spec analysis, Western blot *etc*. We have used the University of British Columbia Proteomics Core Facility, where samples were analyzed using LC-MS/MS after purification from SDS PAGE gel with success.

Data analysis

For more data analysis such as denaturing gel (Step F2), SEC trace of the purified RNP complex (Step G1), the readers may refer to (Dolgosheina *et al*., 2014; Panchapakesan *et al*., 2017).

Notes

1. KCl is essential for the RNA Mango aptamer to bind TO1-Desthiobiotin, but this binding is only modestly dependent on KCl concentration (Dolgosheina *et al.*, 2014), which can be adjusted as needed (90-150 mM final concentration). However, subsequent generations of Mango (Mango II, III and IV) are much more flexible in optimal KCl concentration range (5-150 mM, Mango III functions optimally at 20 mM KCl) and are functional up to at least 256 mM $MgCl_2$ (Autour *et al.*, 2018).
2. We have had success performing purification in the absence of RNase and protease inhibitors, but consider adding these to both the **BNE** and **Binding** buffers as needed.
3. Although this protocol extensively deals with purification from bacterial cells, it can also be adopted to work with eukaryotic systems such as yeast cells. If using yeast cells, steps such as native extract preparation (Step B1), binding of extract to beads (Procedure D) and elution (Procedure E) should be changed accordingly.

Recipes

Note: Buffers (prepare as 10x sterile filtered stocks, store at -20 °C or lower).

1. 10x Bacterial Native Extract (**BNE**) buffer
   200 mM Tris pH 8.0
   900 mM KCl
   10 mM $MgCl_2$
   Prepare a 100 mM DTT stock and make up 1x **BNE** buffer to include 1 mM fresh DTT (final) before use
2. 10x Buffer **A**
   1,000 mM NaOH
   500 mM NaCl

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2799

3. 10x HEPES KCl (**HK**) buffer
   150 mM HEPES pH 7.5
   900 mM KCl
4. 10x **Binding** buffer
   150 mM HEPES pH 7.5
   900 mM KCl
   750 µg/ml heparin*
   Prepare 1x **Binding** buffer from **HK** buffer by adding 1 mM fresh DTT (final) immediately before use. As many RNA binding proteins are positively charged, heparin (a negatively charged sulfated carbohydrate) may be added as a cheap competitor to inhibit nonspecific RNP complex formation
5. 10x **Biotin elution** buffer
   200 mM Biotin in 10x **Binding** buffer
6. **LB** media
   10 g tryptone
   5 g yeast extract
   10 g NaCl
   Autoclave prior to use

Acknowledgments

PJU acknowledges an NSERC Discovery operating grant. The authors declare no conflicts of interest or competing interests.

References

1. Autour, A., S, C. Y. J., A, D. C., Abdolahzadeh, A., Galli, A., Panchapakesan, S. S. S., Rueda, D., Ryckelynck, M. and Unrau, P. J. (2018). Fluorogenic RNA Mango aptamers for imaging small non-coding RNAs in mammalian cells. *Nat Commun* 9(1): 656.
2. Dolgosheina, E. V., Jeng, S. C., Panchapakesan, S. S., Cojocaru, R., Chen, P. S., Wilson, P. D., Hawkins, N., Wiggins, P. A. and Unrau, P. J. (2014). RNA mango aptamer-fluorophore: a bright, high-affinity complex for RNA labeling and tracking. *ACS Chem Biol* 9(10): 2412-2420.
3. Hirsch, J. D., Eslamizar, L., Filanoski, B. J., Malekzadeh, N., Haugland, R. P., Beechem, J. M. and Haugland, R. P. (2002). Easily reversible desthiobiotin binding to streptavidin, avidin, and other biotin-binding proteins: uses for protein labeling, detection, and isolation. *Anal Biochem* 308(2): 343-357.
4. Jeng, S. C., Chan, H. H., Booy, E. P., McKenna, S. A. and Unrau, P. J. (2016). Fluorophore ligand binding and complex stabilization of the RNA Mango and RNA Spinach aptamers. *RNA* 22(12): 1884-1892.

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2799

5. Leontis, N. B. and Westhof, E. (2001). Geometric nomenclature and classification of RNA base pairs. *RNA* 7(4): 499-512.
6. Panchapakesan, S. S. S., Ferguson, M. L., Hayden, E. J., Chen, X., Hoskins, A. A. and Unrau, P. J. (2017). Ribonucleoprotein purification and characterization using RNA mango. *RNA* 23(10): 1592-1599.
7. Panchapakesan, S. S., Jeng, S. C. and Unrau, P. J. (2015). RNA complex purification using high-affinity fluorescent RNA aptamer tags. *Ann N Y Acad Sci* 1341: 149-155.
8. Trachman, R. J., 3rd, Demeshkina, N. A., Lau, M. W. L., Panchapakesan, S. S. S., Jeng, S. C. Y., Unrau, P. J. and Ferre-D'Amare, A. R. (2017). Structural basis for high-affinity fluorophore binding and activation by RNA mango. *Nat Chem Biol* 13(7): 807-813.