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Supporting Information for

mRNA Enrichment Using Oligo(T) Click Nucleic Acids

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Experimental Section

CNA synthesis, polymerization, and characterization. Thymine CNA monomer was synthesized as previously described with a slight modification.[1] Specifically, the thymine imide was protected by an acid-labile tert-butoxide (BOC) group prior to backbone addition. Oligo(T) was synthesized as previously described via a light-initiated thiol-ene reaction.[1] The BOC protecting groups on the resulting oligomers were then deprotected by concentrated (37.2% w/w) hydrochloric acid. The product was then washed extensively with DI H_2O and Acetone to ensure complete removal of any salts. Finally, the average molecular weight and polydispersity index (PDI) was evaluated by gel permeation chromatography (TOSOH – HLC8320GPC) using an internal standard of short CNA oligomers.

Optimization of Cy-5 labelled RNA Pulldown. Oligo(T) was first dissolved in DMSO at prescribed concentrations while the RNA (A_{20}) was dissolved in the Binding buffer (B, see Table S1 for buffer compositions). The effect of DMSO concentration in the binding mixture was evaluated with four different concentrations, 5%, 10%, 20% and 50%. After mixing CNA solution with the RNA solution (final CNA concentration of 500 μM CNA, RNA concentration of 125 nM), the solutions were centrifuged at 6,000 g for 2 min to pellet the precipitate. To evaluate pulldown efficiency, Cy5 fluorescence of the supernatant was measured and compared to a negative control without oligo(T) CNA. To evaluate the effect of salt concentration on pulldown efficiency, pulldown was performed in solutions containing 5% DMSO and either no salt or 1 M LiCl. Pulldown efficiency was evaluated as described previously.

Using the optimized buffer and the procedure dictated above, the amount of CNA needed to effectively precipitate small RNA strands such as A_{20} was determined by a CNA titration. Pulldown efficiency was determined for a range of oligo(T) concentrations from 0.015 – 500 μM with a fixed RNA concentration of 125 nM. Samples were performed in triplicate. As a negative control, a non-complementary sequence (U_{20}) was used to confirm base-specific interactions. Release of the A_{20} RNA strand was achieved by reconstituting the pellet in release buffer R1 and heating to 75°C for 5 minutes. The solution was then spun at 15,000 g for 2 min to quickly pellet the unbound CNA, and the RNA concentration

in the supernatant was measured by Cy5 fluorescence. As a negative control, the resulting RNA concentrations of samples that received no heat were also measured.

Optimization of Cy-5 labelled mRNA pulldown. Using the optimized pulldown procedure described above, Cy5 labelled EGFP mRNA was used to evaluate oligo(T)'s ability to precipitate larger RNA strands. To evaluate the amount of CNA required for efficient pulldown, same titration experiment as described above was performed with the same fixed RNA concentration and the same concentration range of oligo(T). To demonstrate the range of mRNA concentrations that can be precipitated by this method, 250 μM of oligo(T) CNA was used to pull down mRNA in a concentration range of 2 – 64 μg/mL.

In-Vitro Translation. *In-vitro* translation was performed using Retic Lysate IVT™ Kit from Thermo Fisher Scientific following manufacturer's instructions. Samples of EGFP mRNA were subjected to the basic pulldown procedure as detailed above. To ensure the amount of mRNA used for IVT input remained in a workable range, an initial concentration of 62.5 ng/μL of EGFP mRNA was used for mRNA enrichment experiments. As negative controls, mRNA was incubated by itself or with oligo(A) CNA rather than oligo(T) CNA. After pulldown, samples were washed once with 0.67X SSC, 5% DMSO and then either kept at room temperature or heated at 75°C for 15 minutes in the same buffer before immediately centrifuging to remove any precipitated CNA and mRNA. Following this step, 5 μL of the supernatant was used as input for the IVT reactions. IVT was performed according to the manufacturer's protocols and a the resulting EGFP fluorescence was measured. A relative fluorescence factor was then calculated to determine how effective each condition was at concentrating EGFP mRNA. A factor of 1 corresponded to the fluorescence signal obtained from mRNA that was not pulled down. See Supporting Information for additional details.

Optimization of mRNA release. After observing poor mRNA release at low mRNA concentrations, washing and release steps were altered to gradually reduce the buffer's LiCl salt content. Specifically, CNA-mRNA pellets were washed once with buffer W1 (10 mM Tris pH 7.5, 150 mM LiCl, 1 mM EDTA) and once with buffer W2 (10 mM Tris pH 7.5, 5% DMSO). Release was accomplished by adding buffer R2 (10 mM Tris pH 7.5, 5% DMSO), mixing, and heating at 75°C for 5 minutes.

Pulldown specificity – rRNA vs. mRNA. To make sure oligo(T) CNA could selectively precipitate mRNA instead of other types of RNA, pulldown efficiency was compared between inputs of mRNA and rRNA. For the rRNA, the 100 ng/uL rRNA supplied with the Qubit™ RNA BR Assay was used and for mRNA, CleanCap® EGFP mRNA from TriLink Biotechnologies was used. For pulldown experiments, oligo(T) and RNA were supplied at 500 μM and 50 ng/μL respectively, and the procedure utilized the optimized binding buffer, B, detailed above. Pulldown efficiency was evaluated by measuring the RNA remaining in the supernatant using the Qubit™ RNA BR Assay according to manufacturer's protocols. The resulting fluorescence of each sample was used as an indicator for the amount of RNA not pulled out of solution by the CNA.

Pulldown and release from Total RNA. Total RNA extracts were obtained from MCF10a cells with TRIzol™ Reagent using the manufacturer's guidelines. The total RNA was then diluted to an initial concentration of 750 μg/mL. 10 μL of this concentration was added to individual tubes that were then subject to different pulldown conditions. Pulldown with CNA was accomplished by first mixing the total RNA with a 2x concentration of buffer B to a volume of 19 μL. After efficient mixing, 1 uL of 3 mM oligo(T) CNA was added to the total RNA solution. As a positive control, pulldown was performed with Dynabeads™ from an mRNA DIRECT™ Purification Kit. In this case, the total RNA was mixed with beads suspended in 10 μL of the supplied binding buffer. The samples were then incubated at room temperature for 5 minutes after which the CNA samples were centrifuged at 10,000 g for 2 minutes while the Dynabeads™ samples were placed on a magnet for 2 minutes. In both cases 15 uL of supernatant was taken and saved for RNA quantification. Both samples were then washed once with the wash buffer W1 and once with buffer W2. For mRNA release, pellets were reconstituted in buffer R2 and each sample was placed in a thermocycler and held at 75°C for 5 minutes. As quickly as possible, the CNA samples were spun at 6000 g for 2 min while the Dynabeads™ sample was placed on a magnet. The supernatant was then collected for quantification and downstream assays.

RT-PCR. Stocks of mRNA was obtained by the previously described pulldown conditions. As a negative control, RNA was degraded with Nucleoside Digestion Mix (New England Biolabs™).

Transcription to cDNA was performed with a high capacity reverse transcription kit (Applied Biosystems) using an oligo(dT)$_{12\text{-}18}$ primer (Invitrogen™). The oligo(dT) primer was used to ensure that only mRNA was transcribed to cDNA, which was necessary to control for the fact that the total RNA aliquots also contained rRNA and tRNA. Quantitative PCR (qPCR) was conducted with Fast SYBR Green Master Mix (Applied Biosystems) on a 7500 Fast Real-time PCR Machine. Relative expression (RE) is defined as the ratio of expression of a gene of interest (GOI) to a reference gene, *GAPDH*, through the following formula:

$$RE = \frac{\left(E_{ref}\right)^{C_{tref}}}{\left(E_{GOI}\right)^{C_{tGOI}}}$$

where E refers to the true efficiency of each primer pair and C_t is the number of cycles needed to reach a prescribed signal threshold.

Supplementary Figures and Tables

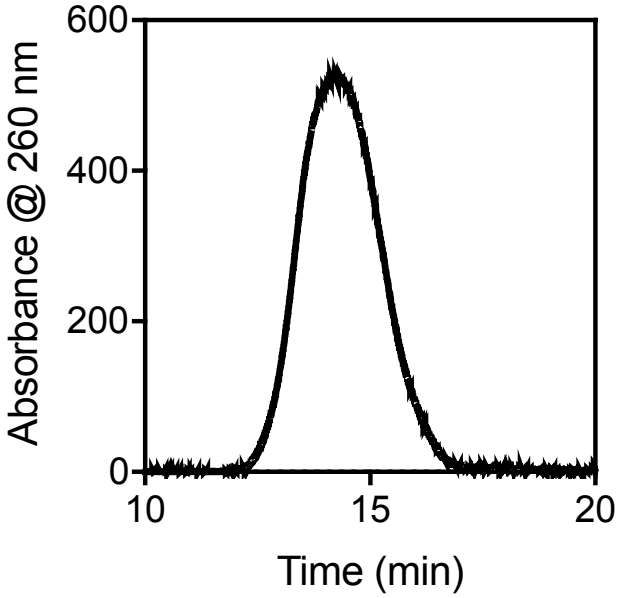

Figure S1 *– Gel Permeation Chromatography of oligo(T) CNA. The mean retention time corresponded to a degree of polymerization of 16 ± 3 repeat units and a PDI of 1.5 ± 0.2.*

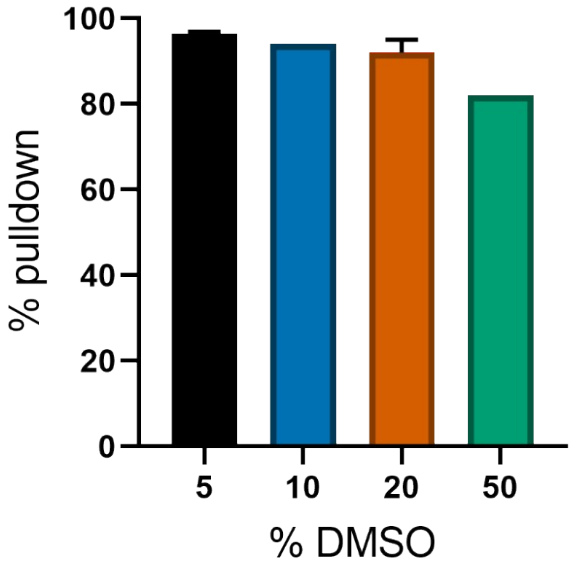

Figure S2 *– Pulldown efficiency of A_{20} increased as the % DMSO decreased with the highest percentage occurring at 5%. This was attributed to the fact that a larger aqueous phase better facilitated CNA precipitation. Results are represented as averages of at least 3 replicates and error bars as standard deviations.*

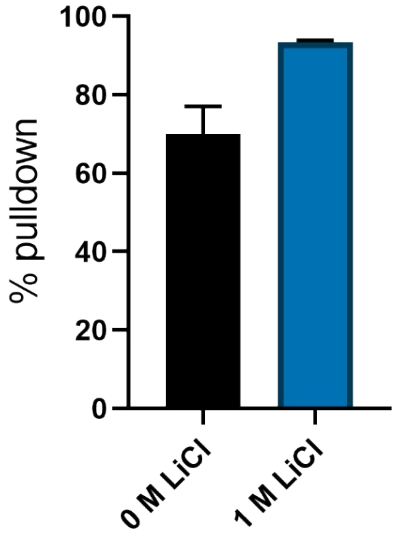

Figure S3 *– The addition of LiCl salt to the binding buffer resulted in an increase in pulldown efficiency. This was attributed to the ability of Li^+ ions to facilitate RNA precipitation.*

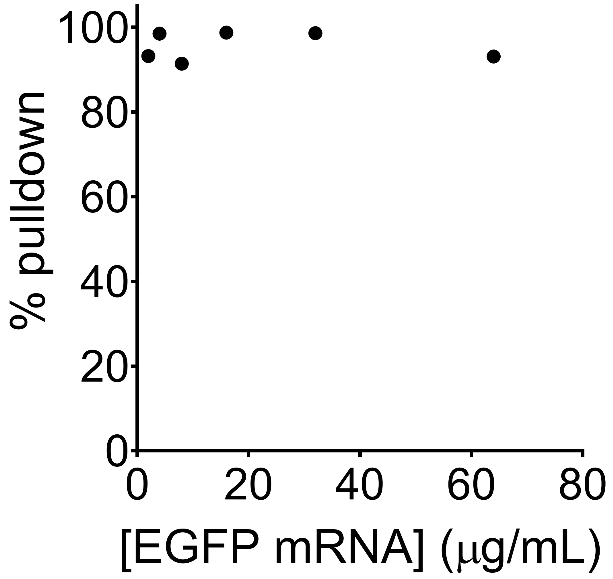

Figure S4 *– Pulldown efficiency remained >90% for concentrations up to 64 μg/mL, demonstrating the robustness of this technique.*

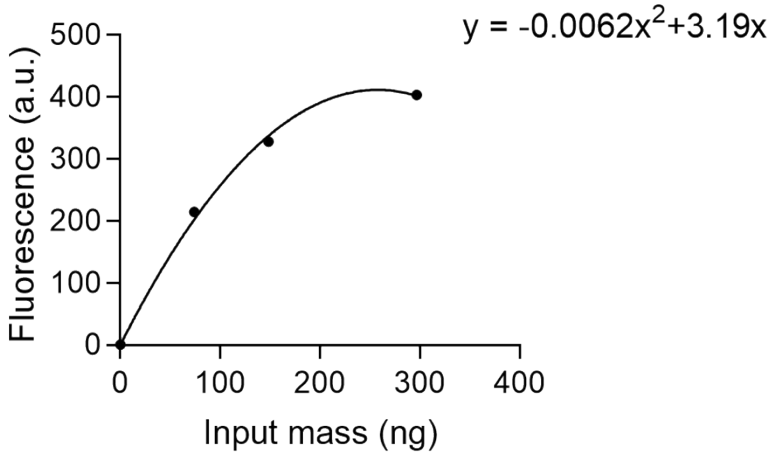

Figure S5 *– In order to correlate the EGFP fluorescence to input mRNA concentration, a standard curve was generated with known concentrations of EGFP mRNA that were not subjected to pulldown and release. From this curve, it was determined that the fluorescence of the translated protein was easily correlated to starting mRNA amounts in the range of 0-300 ng. For in vitro translation experiments, the mRNA yield was low (<100 ng).*

Design of In Vitro Translation Experiments

To ensure the amount of mRNA used for IVT input stayed below 300 ng, an initial concentration of 62.5 ng/μL of EGFP mRNA was used for mRNA enrichment experiments, as this concentration would correspond to 297 ng of mRNA in 5 uL of the supernatant after release if 100% of the mRNA was recovered. At the opposite end, if there was no specific or non-specific pulldown, the concentration of mRNA remaining in 5 uL of the supernatant after release would be just 6.25% of the original. This value was determined based on the total dilution (1/16) of the initial concentration over the course of the procedure (1/4 dilution for washing step and 1/4 dilution for release step). The fluorescence that resulted from the *in vitro* translation assay was taken as a signal of the concentration of functional mRNA. A relative fluorescence factor was then calculated to determine how effective each condition was at concentrating specifically functional EGFP mRNA, where:

$$Relative\ Fluorescence\ Factor = \frac{Fl.\ sample}{Fl.\ of\ sample\ assuming\ no\ enrichment}$$

A factor greater than 1 indicated the concentration of functional mRNA and a factor of 1 corresponded to a situation where no concentration (i.e. pulldown) occurred.

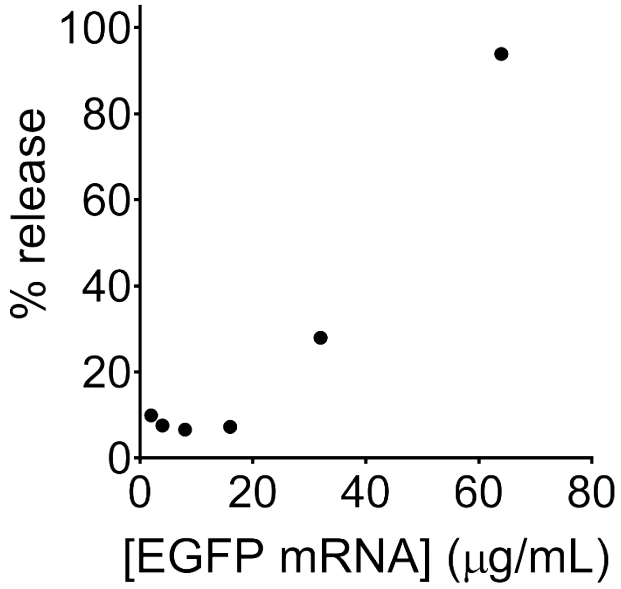

Figure S6 *– The % release of mRNA was found to be a function of mRNA concentrations using release buffer R1 which contains cationic salts that facilitates mRNA precipitation. It was observed that higher mRNA concentrations led to more efficient release.*

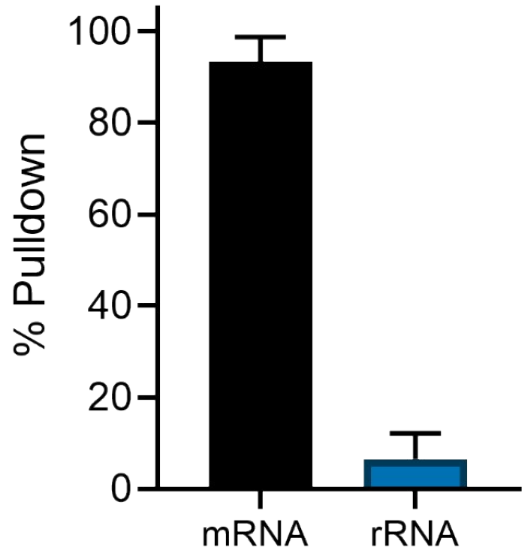

Figure S7 *– Oligo(T) mediated precipitation of mRNA compared to rRNA confirmed specificity for mRNA.*

Table S1 *– Summary of optimized buffer compositions for CNA-facilitated RNA enrichment*

Buffer Name	Composition
Binding (B)	10 mM Tris pH 7.5, 1 M LiCl, 1 mM EDTA
Wash 1 (W1)	10 mM Tris pH 7.5, 150 mM LiCl, 1 mM EDTA
Wash 2 (W2)	10 mM Tris pH 7.5, 5% DMSO
Release (R1)	0.67X SSC, 5% DMSO
Release (R2)	10 mM Tris pH 7.5, 5% DMSO

Table S2 *– List of qPCR Primer Sequences and Efficiencies*

Gene	Primer Sequence	Efficiency
GAPDH	F: 5' – GCAAGAGCACAAGAGGAAGAG – 3' R: 5' – AAGGGGTCTACATGGCAACT – 3'	107%
IL1B	F: 5' – TACCTGTCCTGCGTGTTGAA – 3' R: 5' – TCTTTGGGTAATTTTTGGGATCT – 3'	92%
MMP2	F: 5' – AGAAGGCTGTGTTCTTTGCAG – 3' R: 5' – AGGCTGGTCAGTGGCTTG – 3'	100%

References

(1) Han, X.; Domaille, D. W.; Fairbanks, B. D.; He, L.; Culver, H. R.; Zhang, X.; Cha, J. N.; Bowman, C. N. New Generation of Clickable Nucleic Acids: Synthesis and Active Hybridization with DNA. *Biomacromolecules* **2018**, *19* (10), 4139–4146. https://doi.org/10.1021/acs.biomac.8b01164.