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mRNA Display Using Covalent Coupling of mRNA to Translated Proteins

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Abstract

mRNA display is a powerful technique that allows for covalent coupling of a translated protein with its coding mRNA. The resulting conjugation between genotype and phenotype can be used for the efficient selection and identification of peptides or proteins with desired properties from an mRNA-displayed peptide or protein library with high diversity. This protocol outlines the principle of mRNA display and the detailed procedures for the synthesis of mRNA-protein fusions. Some special considerations for library construction, generation and purification are discussed.

Keywords

mRNA display; covalent coupling of mRNA and protein; genotype-phenotype conjugation; *in vitro* protein selection; high diversity

1. Introduction

The ability to rapidly identify and select proteins with desired properties from synthetic polypeptide or natural proteome libraries has become increasingly useful in biological and biomedical studies. A number of approaches such as phage-display, ribosome-display, and yeast two-hybrid have been developed to address the challenge (1–4). Phage display is a widely used method to isolate peptide sequences with desired functions, often from a short combinatorial peptide library (3). Yeast two-hybrid is often used to isolate interacting protein sequences of a target protein from natural cDNA libraries (4). Ribosomal display is another powerful genotype-phenotype conjugation method that allows the selection of polypeptide sequences with desired properties from a highly diversified polypeptide library displayed on the ribosome as described in the previous chapter (2).

mRNA display is an *in vitro* selection technique that allows the identification of polypeptide sequences with desired properties from both a natural proteome library and a synthetic combinatorial peptide library (5–9). The central feature of this method is that the polypeptide chain is covalently linked to the 3' end of its own mRNA. This is accomplished by synthesis and *in vitro* translation of an mRNA template with puromycin attached to its 3'

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end via a short oligo linker. During *in vitro* translation, when the ribosome reaches the RNA-Oligo junction and translation pauses, puromycin, an antibiotic that mimics the aminoacyl moiety of tRNA, enters the ribosome “A” site and accepts the nascent polypeptide by forming a peptide bond. This results in tethering the nascent polypeptide to its own mRNA (Figure 1). When the initial mRNAs are composed of many different sequences, the corresponding protein or proteome library will be generated. Since the genotype coding sequence and the phenotype polypeptide sequence are covalently combined within the same molecule, the selected protein can be revealed by DNA sequencing after reverse transcription and PCR amplification. Therefore, mRNA display provides a powerful means for reading and amplifying a peptide or protein sequence after it has been functionally isolated from a library with high diversity. Multiple rounds of selection and amplification can be performed, enabling enrichment of rare sequences with desired properties. Compared to prior peptide or protein selection methods, mRNA display has several major advantages. First, the genotype is covalently linked to and is always present with the phenotype. This stable linkage makes it possible to use any arbitrary and stringent conditions in the functional selection. Second, unlike cell-based systems such as yeast two-hybrid or phage display that are limited by the transformation efficiency, the complexity of the peptide or protein library that is allowed by using cell-free system can be close to that of the mRNA or cDNA pools. The reaction scale is tunable, typically from microliters to milliliters. Peptide or protein libraries containing as many as 10^{12} – 10^{14} unique sequences can be readily generated and selected, a few orders of magnitude higher than that can be achieved using phage display or other peptide/protein selection platforms. Therefore, both the likelihood of isolating rare sequences and the diversity of the sequences isolated in a given selection are significantly increased.

The generation of mRNA-protein fusion molecules using mRNA display consists of the following steps: library construction and amplification, *in vitro* transcription, DNase digestion, conjugation with puromycin oligo linker, *in vitro* translation/fusion formation, oligo(dT) mRNA purification, reverse transcription, and protein affinity purification. Specifically, a cDNA library is first *in vitro* transcribed to generate mRNAs using T7, T3, or SP6 RNA polymerase. The resulting mRNA templates are modified by covalently linking to a short oligo linker containing a puromycin at the 3' ends. Such a linkage can be achieved by photo-crosslinking, splint, or Y ligation (8, 10–12). Creation of the mRNA-protein fusion is accomplished by *in vitro* translation in a cell-free system using rabbit reticulocyte lysate that has low nuclease activity. Efficient mRNA-protein fusion formation can be accomplished through a post-translational incubation with high concentrations of Mg^{2+} and K^{+} (8). mRNA templates and mRNA-protein fusion molecules can be readily purified from the lysate by using an oligo(dT) column, taking advantage of the oligo(dA) residues in the puromycin-containing oligo linker. To remove the secondary structures of mRNAs that may interfere with the selection step, the fusion molecules are often converted to DNA/RNA hybrids by reverse transcription. The resulting mRNA-displayed protein library is then purified on the basis of the affinity tags engineered at the N and C termini and used for subsequent selection. We describe here the general procedures for the generation of mRNA-protein fusion molecules that can be used for the selection of peptide or protein sequences with

desired properties. The procedures for the functional selection using mRNA-displayed peptide or protein libraries are detailed in the next chapter.

2. Materials

2.1. Reagents

1. Expand long template PCR system (Roche)
2. T7 RNA polymerase (NEB)
3. 10× RNA polymerase buffer: 400 mM, Tris-HCl, pH7.9, 60 mM MgCl₂, 100 mM DTT, 20 mM spermidine
4. RNase-free DNase (Promega)
5. 10× DNase buffer: 400 mM Tris-HCl, pH8.0, 100 mM MgSO₄, 10 mM CaCl₂
6. Acidic phenol chloroform : isoamyl alcohol (Ambion)
7. 7.5 M LiCl RNA precipitation solution (Ambion)
8. Puromycin oligo linker: 5'-Psoralen-(TAGCCGGTG)₂-OMe-dA₁₅ C9C9dAdCdC-Puromycin-3'
9. Retic lysate IVT™ kit (Ambion)
10. [³⁵S]-L-methionine (Perkin Elmer)
11. Oligo(dT) cellulose (Ambion)
12. 1× Oligo(dT) binding buffer: 100 mM Tris-HCl, pH 8.0, 1 M NaCl, 10 mM EDTA, 0.2% Triton X-100
13. Oligo(dT) wash buffer: 20 mM Tris-HCl, pH 8.0, 300 mM KCl, 0.1% Tween-20
14. RNase-free 10 mL poly-prep chromatography column (Biorad)
15. SuperScript II RNase H⁻ reverse transcriptase (Invitrogen)
16. Reverse transcription primer: TTTTTTTTTTNNCCAGATCCAGACATTCCCAT
17. Anti-FLAG M2 affinity gel (Sigma)
18. FLAG peptide (Sigma)
19. 100 mM glycine buffer pH3.5
20. 1× TBST buffer: 20 mM Tris-HCl, pH8.0, 150 mM NaCl, 0.2 % Tween-20
21. 1× TE buffer: 10 mM Tris-HCl, pH8.0, 1 mM EDTA
22. NAP-5 column (GE Healthcare)
23. NAP-10 column (GE Healthcare)
24. QIAquick PCR purification kit (Qiagen)
25. QIAquick gel extraction kit (Qiagen)

2.2. Equipment

1. Thermal cycler
2. Nanodrop spectrophotometer
3. UV lamp (Black Ray Lamp 365 nm, 0.16 Amps)
4. Barnstead labquake shaker/rotator
5. Scintillation counter

3. Methods

3.1. General Design of the cDNA Library

Both natural cDNA and synthetic cDNA libraries can be used for mRNA display-based selections (13, 14). In general, the variable region is flanked with two consensus regions at the 5' and 3' ends, respectively. The 5' consensus region contains a transcription promoter and a short UTR that facilitate efficient *in vitro* transcription and translation, respectively. Depending on the choice of RNA polymerase used for *in vitro* transcription, T7, T3, or SP6 promoter can be used. A short 5'-UTR originated from TMV (tobacco mosaic virus) results in efficient *in vitro* translation when rabbit reticulocyte lysate is used. If T7 RNA polymerase is chosen, the 5'UTR should begin with GGG to facilitate transcription initiation. A general 5' consensus sequence upstream of the start codon has the following sequence: TTC TAA TAC GAC TCA CTA TAG GGA CAA TTA CTA TTT ACA ATT ACA; in which the T7 promoter is underlined and the TMV 5'-UTR is bolded. If necessary, a sequence encoding a 5-amino-acid recognition site (RRASV) by protein kinase A (PKA) can be incorporated for universal radiolabeling with ³²P. The right consensus region contains a short sequence for hybridizing and crosslinking with the puromycin-containing oligo linker (8, 10). In addition, various affinity tags such as His×6, E, HA, and FLAG tags can be incorporated at the N- and/or C-termini to facilitate affinity purification of mRNA-protein fusion molecules from *in vitro* translation reaction mixture.

Because mRNA is covalently linked with the C-terminus of the translated polypeptide, several flexible and hydrophilic amino acid residues such as Gly or Ser are often engineered at the very C-terminal region of the protein sequence to minimize the possible rigid structure and steric hindrance. Some special sequences that facilitate subsequent selections are optional, as discussed in the next chapter that focuses on functional selections from mRNA-displayed peptide or protein libraries.

3.2. Amplification of the Initial Library

With the availability of a high quality cDNA library that is compatible for mRNA display, the initial library should be amplified by PCR. One critical issue is to avoid over-amplification that could result in preferential enrichment of some sequences before the selection. The PCR conditions should be optimized prior to large scale amplification, particularly the annealing temperature, number of cycles, concentrations of cDNA template, primers, Mg²⁺, and dNTPs.

1. Set up reactions of less than 100 µL for optimization.

2. Run a hot-start PCR program with desired parameters. Save 5–10 μ L samples every 2–3 cycles.
3. Run 1~2% agarose gel to compare the quality and quantity of each PCR product.

The best PCR conditions should allow efficient amplification of the initial library with an amplification factor close to 2 each cycle. The number of PCR cycles should be chosen so that the amount of desired full-length products is maximal, whereas that of undesired shorter byproducts is minimal. The optimized PCR conditions are applied to the large scale amplification of the whole library. The amplified cDNA library should be cleaned up by using a PCR purification kit to remove PCR primers and salts. Further ethanol precipitation is recommended. The final cDNA library is dissolved in a buffer that contains 10 mM Tris-HCl, pH8.0 and 75 mM NaCl and stored at -20°C before use.

3.3. Generation of mRNAs by In Vitro Transcription

The next step for the generation of mRNA-protein fusion molecules is the synthesis of large quantities of mRNAs by *in vitro* transcription. The following procedures describe the synthesis and purification of mRNAs corresponding to the initial cDNA library using T7 RNA polymerase. Typically, the concentration of cDNA library used for *in vitro* transcription is around 200 nM. The scale of *in vitro* transcription is tunable from 0.25 mL to 10 mL, depending on the diversity of the initial library and the average copy number of each unique sequence. Typically, 0.5–1 mL of *in vitro* transcription is performed for the first round of selection from natural proteome libraries, whereas 3–10 mL reaction is required for the first round of selection from synthetic peptide libraries with very high diversities (14, 15).

1. Assemble an *in vitro* transcription reaction mixture in a nuclease-free eppendorf tube on ice that contains $1\times$ T7 RNA polymerase buffer, 25 mM MgCl_2 , 5 mM each rNTP, 200 nM initial cDNA library, and 3 U/ μ L T7 RNA polymerase. Nuclease-free DEPC water should be used to obtain the desired final volume (see Note 1).
2. Incubate the reaction mixture at 37°C for 6 to 12 hours. The reaction mixture will become opaque if *in vitro* transcription is successful, presumably due to the generation of magnesium pyrophosphate as byproduct.
3. Add EDTA to a final concentration of 30 mM (1.2 equivalent of $[\text{Mg}^{2+}]$) and incubate at room temperature for 5 minutes to dissolve the white precipitate. The solution should become clear after such treatment.
4. Extract the reaction mixture with one volume of acidic phenol/chloroform followed by one volume of chloroform.
5. Desalt the product using NAP-5 column and collect the product in 1 mL nuclease-free DEPC water. Concentrate the sample to 400 μ L in a Speedvac.

¹Nuclease inhibitors can be added but are usually not necessary if all the procedures are strictly performed under RNase-free conditions.

6. Load 1–5 μL of product onto a denaturing PAGE gel to check the quality of purified mRNAs (see Note 2).

3.4. Removal of cDNAs from mRNAs by DNase Digestion

Due to the amplification nature of mRNA display-based selection, the presence of even trace amount of cDNAs in the selected pool could result in enrichment of sequences that do not possess desired properties. Therefore, the cDNA templates in the transcription reaction mixture should be completely removed by DNase digestion using the following procedures.

1. Assemble a 500 μL reaction in a nuclease-free eppendorf tube that contains 1 $\times$ DNase Buffer, 400 μL desalted *in vitro* transcription product from step 3.3, and 0.05 U/ μL RNase-free DNase.
2. Incubate at 37 $^{\circ}\text{C}$ for 30 min to perform DNase digestion.
3. Add EDTA to a final concentration of 13.2 mM (1.2 equivalents of $[\text{Mg}^{2+}]$) to stop the reaction.
4. Extract the reaction mixture with one volume of acid phenol/chloroform followed by one volume of chloroform.
5. Desalt using NAP-5 column and collect the product in 1 ml nuclease-free DEPC water.
6. Add 0.5 volume of 7.5 M LiCl RNA precipitate solution and incubate at -80°C for 2 hours.
7. Spin at 14,000 rpm for 15 minutes at 4 $^{\circ}\text{C}$, and wash the pellet with 500 μL 75% ethanol twice.
8. Allow the pellet to air dry until no ethanol is detectable. Resuspend the purified mRNAs in 150 μL nuclease-free DEPC water.
9. Measure O.D. using Nanodrop, and calculate the molar concentration of mRNAs based on the (average) length of cDNA library. Load 1–5 μL of product onto a denaturing PAGE gel to check the quality of purified mRNAs (see Note 2).

3.5. Conjugation with Puromycin-containing Oligo Linker

The mRNA templates with puromycin at 3' ends can be generated by conjugating with an oligonucleotide containing a puromycin residue at the 3' end. Various conjugation methods can be used, including UV crosslinking and enzymatic ligation (8, 10, 16). In general, psoralen-mediated UV crosslinking is much simpler to perform (Figure 2), without need of purifying the products from the reaction mixture. The conjugation efficiency is typically in the range of 40% to 60%, and further purification is not necessary. The following

²The quality of the RNAs can be judged by running a denaturing PAGE gel. The bands on the gel should have the molecular weights as shown by the corresponding cDNA library. Short RNA bands usually suggest the presence of abortive transcription byproducts or RNA degradation. Because the mRNAs conjugated with the puromycin-containing oligo linker are larger in size than the unconjugated ones, they can be easily separated by using a 5–10% denaturing PAGE. There should be two bands for the crosslinking products. The lower band corresponds to the unconjugated mRNAs, while the higher band corresponds to the conjugation products. The ratio of these two bands can be used to estimate the conjugation efficiency, which is typically around 40% to 60%.

procedures describe the introduction of a puromycin-containing oligo linker to the 3' end of mRNAs by irradiation under ~365 nm UV light.

1. Assemble a reaction mixture that contains 20 mM HEPES, pH 7.4, 100 mM KCl, 5 μ M purified mRNAs from step 3.4, 12.5 μ M (2.5 equivalent of mRNAs) puromycin-containing oligo linker.
2. Mix well and aliquot 50 μ l sample into an RNase-free 8-strip PCR tube.
3. Anneal mRNA and puromycin-containing oligo linker on a thermal cycler under the following conditions: 85°C, 8 minutes; cool from 80°C to 25°C at 1°C/20 seconds; 25°C, 25 minutes.
4. Transfer strip tubes to an ice bath and irradiate under 365 nm UV light in darkness for 20 minutes at 4 °C.
5. Pool the samples into an RNase-free 1.5 ml eppendorf tube.
6. Add 0.5 volume of LiCl precipitate solution and incubate at –20°C for 4 hours to precipitate mRNA.
7. Wash pellet with 75% ethanol twice, air-dry the pellet, and resuspend mRNA product in 100 μ l DEPC water.
8. Measure O.D. using Nanodrop and calculate the molar concentration of mRNAs. If necessary, run a denaturing PAGE to check the conjugation efficiency and the quality of the resulting mRNAs (see Note 2).

3.6. In Vitro Translation and Fusion Formation

For the synthesis of protein sequences using *in vitro* translation, we recommend rabbit reticulocyte lysate from Ambion or Novagen. Wheat germ lysate can also be used, but bacterial lysate is not suitable due to the high levels of nucleases (9). It is critical to first optimize the translation conditions in a small scale, particularly the concentrations of Mg^{2+} , K^+ , and mRNA to get the highest yield. Radiolabeling of proteins can be achieved by adding [^{35}S]-methionine into the translation mixture in the absence of endogenous methionine. We strongly recommend radiolabeling that greatly facilitates the quantification of each step by liquid scintillation counting and/or autoradiography (see Note 3). After translation, addition of Mg^{2+} and K^+ to the optimized concentrations followed by incubation at –20°C overnight is critical to promote the formation of mRNA-protein fusions (8, 9). Depending on the lengths and sequences of the mRNA templates, the fusion efficiency varies, typically in the range of 5% to 40%, if high quality mRNAs and reticulocyte lysate are used for *in vitro* translation. The volume of *in vitro* translation is dependent on the diversity of the initial library. Typically, 1.0 mL (for natural proteome libraries) or 10 mL (for synthetic peptide libraries) of *in vitro* translation is performed for the first round of selection.

³Radiolabeling of the translation products is optional, but very helpful for monitoring the signals at every step. It is not necessary to radiolabel all the proteins in the reaction. Typically, two translation reactions, one hot and one cold, are performed in parallel. The hot reaction (10% of the reaction volume) includes [^{35}S]-methionine, while the cold reaction (90% of the reaction volume) uses normal methionine. After translation, the two reactions are mixed as one pot for all the subsequent procedures.

1. Assemble a reaction mixture that contains 1× translation mix without methionine, 50–120 mM KOAc or KCl, 0.3–1.0 mM Mg(OAc)₂, 200 nM puromycin-containing mRNAs, 0.5 μCi/μL [³⁵S]-L-methionine, and 40% (volume) reticulocyte lysate. It is critical that nuclease-free DEPC water should be used, and lysate should be as fresh as possible. Radiolabeling is optional but greatly facilitates signal monitoring at every step (see Note 3).
2. Incubate the reaction mixture at 30°C for 60–90 minutes.
3. Add MgCl₂ and KCl to a final concentration of 50 mM and 580 mM, respectively. Mix gently and incubate at room temperature for 30 min.
4. Incubate at –20°C overnight. The fusion mixture can be stored at –20°C for several days prior to purification. The translation and fusion efficiencies can be estimated by SDS-PAGE and autoradiography if a radioisotope is used to label the proteins or/and mRNAs (see Note 4).

3.7. Oligo(dT) Purification to Remove Free Proteins

After translation and fusion formation, the free mRNA templates and mRNA-protein fusions should be purified from lysate by using an oligo(dT) column, taking advantage of oligo(dA) residues in the puromycin-containing oligo linker. It is critical to dilute the translation reaction mixture at least 20 times using oligo(dT) binding buffer to reduce the possible degradation of small amount of mRNA-protein fusion molecules by proteases and nucleases that are present in the lysate. The manipulation should be performed as fast as possible in a cold room. The base-pairing between oligo(dT) on cellulose and poly(dA) linker at the 3' end of mRNAs are optimal under high salt conditions, but can be readily disrupted by using a buffer that contains low concentration of salt.

1. For a 500 μl translation reaction, weigh 60 mg of oligo(dT) cellulose into a 1.5 ml nuclease-free eppendorf tube.
2. Wash cellulose beads 3 times with 1 ml nuclease-free DEPC water, and 2 times with 1 ml Oligo(dT) binding buffer.
3. Resuspend oligo(dT) cellulose in 1 ml binding buffer and transfer to a 15 ml tube containing 9 mL of oligo(dT) binding buffer and 1 mM DTT.
4. Add the post-translation reaction mixture from step 3.6 to the slurry, wrap the tube in aluminum foil and rotate for 2 hours at 4°C.
5. Load the slurry mixture into a 10 ml nuclease-free poly-prep chromatography column.
6. Collect flow-through and reload to resin bed. Retain the final flow-through for analysis.

⁴The puromycin-mediated fusion between the mRNA and its coding protein sequence can be monitored based on comparing migration of radiolabeled fusion molecules on an SDS-PAGE gel. The resulting fusion molecules usually migrate near the top of the gel while the free proteins migrate as expected based on the MWs.

7. Wash oligo(dT) cellulose on column twice each with 1 ml of oligo(dT) binding buffer, followed by 3 times each with 1 ml of Oligo(dT) wash buffer. Retain each wash fraction for analysis.
8. Elute mRNA and mRNA-protein fusion molecules 4 times each with 600 μ l of DEPC plus 1 mM DTT.
9. If [35 S]-methionine is used to radiolabel the protein, take 1/100 of each fraction and 1/10 of the beads to quantify the radioactivity using a liquid scintillation counter. The fusion efficiency can be estimated by SDS-PAGE and autoradiography (see Note 5).

3.8. Reverse Transcription to Remove Secondary Structure of mRNAs

The single-stranded mRNAs on mRNA-protein fusions could adopt complicated structures and therefore likely interfere with the subsequent functional selection. One effective way to address the problem is by converting the fusion molecules into rigid DNA/RNA hybrids by reverse transcription. Additional advantages of this step include the protection of mRNAs from degradation and direct amplification of the selected library by PCR for iterative rounds of selection. However, the reverse transcriptase should not have RNase H activity to keep the mRNA/DNA hybrids from degradation.

Since reverse transcriptase is sensitive to the salt concentration, the best time to perform this reaction is after oligo(dT) purification when the eluted fusion molecules are in a buffer with relatively low salt concentrations. In general, the reverse transcription is efficient and can be performed at 37 °C if necessary.

1. Add reverse transcription primer (final concentration 2 μ M, see Note 6) to the mRNA-protein fusions purified from step 3.7. Mix and incubate at room temperature for 15 minutes before addition of the first strand synthesis buffer (1 $\times$), dNTPs (0.5 mM each), and DTT (10 mM). The mixture is incubated at 37 °C or 42 °C for 2 minutes.
2. Initiate the reverse transcription by adding an RNaseH⁻ reverse transcriptase (*e.g.* Superscript II, Invitrogen) to a final concentration of 2 U/ μ l and the reaction mixture is incubated at 37 °C or 42 °C for 50 minutes.
3. Terminate the reaction by adding EDTA to a final concentration of 3.6 mM (1.2 $\times$ [Mg²⁺]).
4. Change to TBST buffer by applying to an NAP-10 column equilibrated with 1 $\times$ TBST Buffer.

⁵Purification of mRNA-protein fusion molecules using oligo(dT) cellulose can be readily monitored by quantification of the radioactive counts present in 1% of the sample volume. Graphing the CPM data for the flow-through, washes, elution, and beads samples should illustrate an elution profile showing radioactive counts returning to background for the wash samples followed by a large increase in counts for the elution samples. The presence of a large excess of unincorporated [35 S]-methionine will always impart high counts to the flow-through sample.

⁶To make the reverse transcription more efficient, a unique primer with the following sequence is used: TTTTTTTTTTNNCCAGATCCAGACATTCCCAT, in which oligo(dT) is used to hybridize with the oligo(dA)-containing oligo linker at the 3' end of mRNAs, NN used to stride over the mRNA-oligo junction, and underlined sequence used to hybridize with the very 3' end of the mRNAs.

5. Collect the reverse transcribed product in 1.35 ml TBST buffer for anti-FLAG purification.

3.9. Anti-FLAG Purification to Remove Free mRNAs

While purification using an oligo(dT) column effectively removes free proteins that are not fused with their own mRNAs, the free mRNAs that are not fused with their encoded proteins are isolated together with mRNA-protein fusions. In some cases, such mixture can be directly used for selection, assuming the free mRNA/DNA hybrids do not possess secondary structures and are less likely to interfere with the selection. However, the presence of a trace amount of free mRNA/DNA hybrids in the selected pool increases the background when the selected molecules are PCR amplified for next round of selection. To remove free mRNA/DNA hybrids, the reverse-transcribed fusion molecules can be further purified if an affinity tag is engineered at the N- or C-terminus of the protein sequences. We found that FLAG and E tags work well for this purpose. The following procedure describes the purification of mRNA-protein fusion molecules from free mRNAs using an anti-FLAG column.

1. Quickly wash 600 μ l of anti-FLAG M2 affinity resin in a 10 ml poly-prep chromatography column 5 times each with 1 ml of 100 mM glycine buffer at pH 3.5.
2. Wash the resin 3 times each with 5 ml of 1 $\times$ TBST buffer.
3. Cap the bottom of the column and load the reverse transcribed product from step 3.8.
4. Cap the top of the column with lid, and rotate at 4 $^{\circ}$ C for 2 hours at an angle so that the resin is mixed well but attachment of solution to the top and side of the chromatography column is minimized.
5. Stand column upright and allow the resin to settle for 2 minutes. Remove top and bottom caps and collect and retain flow-through.
6. Wash the resin 5 times each with 1 ml of 1 $\times$ TBST, collect and retain each wash fraction.
7. Cap bottom of the column and add 600 μ l of 1 $\times$ TBST plus 30 μ l of 5 mg/ml FLAG peptide to slurry and rotate at 4 $^{\circ}$ C for 30 minutes to elute the captured fusion molecules.
8. Collect the elution fraction and repeat Step 7 three times.
9. If [35 S]-methionine is used to label the protein, take 1/100 of flow-through, wash, and elution fractions and 1/10 of the beads to quantify the amount of fusion molecules recovered using a liquid scintillation counter (see Note 7).
10. Change to a desired buffer using a NAP-10 column for the selection step.

⁷Anti-FLAG affinity purification of fusion molecules will show a similar elution profile to that of the oligo(dT) purification step. Radioactive counts should return to baseline during the wash steps and there should be a significant increase in counts in the elution fractions.

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Figure 1.

The formation of mRNA-protein fusion. Without puromycin-containing oligo linker (the black line), mRNA (grey line) and newly-synthesized polypeptide (dotted chain) will be separated from each other (top). Puromycin (**P**), which mimics the aminoacyl-tRNA, can enter the ribosome “A” site and be incorporated into the nascent polypeptide chain as the last amino acid, mediating the covalent conjugation between mRNA and its coded polypeptide (bottom).

Figure 2. Psoralen(PSO)-mediated photo-crosslinking between mRNA (grey line) and puromycin (P)-containing oligo linker (black line). The base-pairing between the 3' constant region of mRNA and the 5' portion of oligo linker is necessary for site-specific crosslinking.