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Sebastian Vencken
Royal College of Surgeons in Ireland

Tidi Hassan
Royal College of Surgeons in Ireland

Noel G. McElvaney
Royal College of Surgeons in Ireland

Stephen GJ Smith
Trinity College Dublin

Catherine M. Greene
Royal College of Surgeons in Ireland, cmgreene@rcsi.ie

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miR-CATCH: microRNA Capture Affinity Technology

Sebastian Vencken[1], Tidi Hassan[1], Noel G. McElvaney[1], Stephen G. J. Smith[2], Catherine M. Greene[1$]

[1]Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin, and [2]Department of Clinical Microbiology, School of Medicine, Trinity College Dublin.

[$]Corresponding Author: Catherine M. Greene, Respiratory Research Division, Dept. Medicine, Royal College of Surgeons in Ireland, Education & Research Centre, Beaumont Hospital, Beaumont Road, Dublin 9, Republic of Ireland

E-mail: cmgreene@rcsi.ie. Contact No. +353-1-8093712; Fax No. +353-1-8093808

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Summary

Several experimental methods exist to explore the microRNA (miRNA) regulome. These methods almost exclusively focus on multiple targets bound to a single, or perhaps a few miRNAs of interest. Here, we describe a microRNA capture affinity technology (miR-CATCH) which uses an affinity capture oligonucleotide to co-purify a single target messenger RNA (mRNA) together with all its endogenously bound miRNAs. This bench-top method is similar to RNA immunoprecipitation (RIP) and provides an experimental alternative to computational miRNA target prediction.

Key words: MicroRNA, Affinity capture, MicroRNA target analysis, Oligonucleotide hybridisation, miR-CATCH

1. Introduction

Various computational and experimental methods exist to explore the microRNA (miRNA) regulome. Among the latter, a number of exploratory experiments exist based on first principles to determine miRNA targets *in vivo*. Several RNA immunoprecipitation (RIP)-based methods allow for the isolation of miRNA-target hybrids with the use of antibodies specific to the *Ago* proteins, the principal miRNA-carrying RNA binding proteins (RBPs) of the RNA-induced silencing complex (miRISC). Among these methods are tandem affinity purification of miRNA:mRNA (TAP-Tar) ***(1)***, high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP) ***(2)*** and photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) ***(3)***.

Besides immunopurification, a number of nucleic acid-based affinity capture methods exist. MiRNA target RNA affinity purification (miR-TRAP) reversibly cross-links a transfected biotinylated miRNA to its targets which are subsequently captured by streptavidin-coated beads ***(4)***. Aptamer-linked mRNA isolation, which involves the introduction of a vector containing an aptamer sequence and an RNA sequence of interest, can 'fish' for miRNAs and RBPs which bind to this RNA sequence ***(5)***. These can subsequently be co-purified with aptamer-binding proteins.

Almost all of these methods take an approach that centres on an miRNA of interest, rather than using an mRNA target-focused approach. The exception is aptamer-linked mRNA, which still requires exogenous manipulation of live samples.

To provide an *in situ* bench-top method to study all miRNAs endogenously bound to an mRNA target of interest and to overcome the limitations of current target-focused methods,

we developed an oligonucleotide affinity purification-based technique, we call miR-CATCH, to capture a target mRNA with all its attached miRNAs ***(6)***. Similar to RIP, miRISCs are reversibly cross-linked to their targets. Instead of immunopurification, biotinylated oligonucleotides complementary to predicted single-strand regions of the target mRNA capture that target with all its endogenously bound miRNAs through the use of streptavidin-coated magnetic beads. The reversal of the cross-links releases all individual components of the complex for further expression analysis.

This method yields an RNA sample enriched with the miRNAs bound to a desired target mRNA and is of a quality and purity comparable to RIP-based methods. Subsequent analyses can be performed such as mRNA quantification, miRNA expression analysis and high-throughput profiling.

2. Materials

RNase-free conditions are critical. All consumables should be RNase-free and filter pipette tips should be used. Prepare all salt solutions with analytical grade chemicals and treat with Diethylpyrocarbonate (DEPC) prior to the addition of Tris-EDTA (TE) buffer. RNase-free TE buffer should be prepared from a 100X stock solution. All buffers should be stored at 4°C and brought to room temperature prior to use. The protocol can be adapted to alternative equipment of similar purpose, although optimisation is recommended.

2.1. Buffers

1. FA Lysis Buffer: 140 mM NaCl, 50 mM HEPES (adjust to pH 7.5 with KOH), 1 mM EDTA, 1% v/v Triton-X 100, 0.1% w/v sodium deoxycholate. Always add PMSF, protease inhibitor cocktail and RNasin fresh before use.
2. 2X B&W Buffer: 1X TE buffer (pH 7.5), 2 M NaCl.
3. 1X B&W Buffer: 2X B&W Buffer, 50% v/v RNase-free water.
4. Solution A: 0.1 M NaOH, 0.05 M NaCl.
5. Solution B: 0.1 M NaCl.
6. Hybridisation Buffer: 2X TE buffer (pH 7.5), 1 M LiCl.
7. Wash Buffer A: 1X TE buffer (pH 7.5), 0.15 mM LiCl, 0.5% sodium dodecyl sulphate (SDS).
8. Wash Buffer B: 1X TE buffer (pH 7.5), 0.15 mM LiCl.

2.2. Reagents

1. 0.5 mm RNase-free glass beads (SS Scientific, Eastbourne, UK)
2. Dynabeads MyOne Streptavidin C1 (Life Technologies, Grand Island, USA)
3. Paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA)

4. RiboSafe RNase Inhibitor (Promega, Madison, WI, USA)
5. Complete Mini Protease Inhibitor Cocktail Tablets (Roche Applied Science, Penzberg, Germany)
6. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA)
7. Deoxyribonuclease I (DNase I) (Sigma-Aldrich, St. Louis, MO, USA)
8. 100X Tris-EDTA buffer solution (Sigma-Aldrich, St. Louis, MO, USA)
9. Biotin-triethylene glycol (TEG) 5'-modified single-stranded DNA oligonucleotides (Eurofins MWG Operon, Ebersberg, Germany)
10. Dulbecco's Phosphate-buffered saline (DPBS) (Lonza Group Ltd, Basel, Switzerland)

2.3. Equipment

1. DynaMag-2 (Life Technologies)
2. FastPrep-24 (MP Biomedicals)
3. Shake 'n Stack hybridisation oven (Hybaid)

3. Method

Perform the method at room temperature unless otherwise stated.

3.1. Antisense Oligonucleotide design

DNA oligonucleotide design is based on similar principles used for FISH probes, PCR primers and oligonucleotide affinity purification protocols. Besides single-stranded DNA oligonucleotides, locked nucleic acid (LNA) or morpholino probes could be used for improved specificity and hybridisation.

Oligonucleotide hybridisation to any specific target mRNA is performed in high-salt conditions at 37°C. Under these conditions, the secondary structures of both the oligonucleotides and the target can significantly impede efficient hybridisation, as the self-hybridised double-stranded regions of the two sequences will inhibit oligonucleotide binding. Oligonucleotide capture efficiency can be tested prior to the miR-CATCH protocol (*see* Note 1).

An optimal capture oligonucleotide will have little ability to form harpins due to low self-complementarity , a high hybrid melting temperature and will hybridise with a single-stranded region of its target with high specificity (*see* Note 2 and 3). An oligonucleotide design strategy should use the following criteria:

- The oligonucleotide must be modified at the 5’ terminal residue with biotin connected to a TEG spacer.
- Oligonucleotides should be 18-25 residues in length.

- The oligonucleotide-target hybrid should have a melting temperature >50°C in 500 mM monovalent salt conditions.
- The target region shall be a single stranded at 37°C in 500 mM monovalent salt conditions as defined by RNA/DNA secondary structure prediction software. We used the mfold software **(7)**.
- The target region overlaps with no established or highly predicted binding regions for miRNAs or RBPs.
- The oligonucleotide should have a melting temperature <37°C in 500 mM monovalent salt conditions as defined by RNA/DNA secondary structure prediction software.
- The oligonucleotide should not contain stretches of more than three G or C residues.
- The hybridisation energy of oligonucleotide dimers should be <15% of that of the hybrid.
- Oligonucleotide complementarity with non-specific endogenous RNA sequences should be <15 bases.
- For long RNA targets, multiple oligonucleotides to distant regions can improve yield.

3.2. Sample preparation and cross-linking

This protocol is designed for a cell pellet of approximately 100 mg or about 10^7 cells. Adjustment of the volumes used is recommended for a different cell quantity. Cross-linking conditions can be optimised (*see* Note 4)

1. From paraformaldehyde, prepare a fresh 10 ml stock of methanol-free 2% paraformaldehyde in DPBS at room temperature.
2. Harvest and wash 10^7 cells in a 50 ml tube, resuspend in 10 ml normal growth medium at room temperature.
3. Add 10 ml 2% formaldehyde to the cells and mix on a plate rocker for 10 minutes at average speed.
4. Quench the formaldehyde cross-linking reaction by adding 1.33 ml 3 M glycine (~0.2 M final concentration) and mix on the plate rocker for 5 minutes.
5. Centrifuge at 2000 g for 5 minutes at 4°C.
6. Discard all supernatant and resuspend in 50 ml ice cold PBS and centrifuge at 2000 g for 5 minutes at 4°C.
7. Repeat step 6 once.
8. Resuspend the pellet in 0.6 ml ice cold PBS. Transfer the cells suspension to a 1.5 ml screw-cap microtube and transfer any remaining cells in the 50 ml tube with another 0.6 ml ice cold PBS.
9. Centrifuge the cells at 2000 g for 5 minutes at 4°C and discard the supernatant. With a 20 µl pipette, remove the last drop of supernatant.
10. Flash-freeze the cell pellet in liquid nitrogen.

    Stopping point: flash-frozen cell pellet can be stored at -80°C until use.

3.3. Cell Lysis

The lysing protocol has been designed for the FastPrep-24 cell homogeniser. Alternative lysing methods can be optimised for this protocol (*see* Note 5).

1. Prepare 1.4 ml FA lysis buffer with 1X protease inhibitor cocktail, 6 μl PMSF and 120U RNasin.
2. On ice, thaw the pellet in 0.4 ml prepared FA lysis buffer.
3. Add 0.4 ml glass beads and homogenise the cells on the FastPrep 24 homogeniser 4 times for 30 seconds at speed 5.5. Ensure the lysate remains on ice during the 5 minute cool down periods.
4. Separate the lysate from the glass beads by puncturing a hole in the bottom of the screw-cap microtube and centrifuging it into another microcentrifuge tube at 3500 g for 5 minutes at 4°C.
5. Wash the beads by adding 0.4 ml FA lysis buffer and repeat the centrifugation as per step 3.3.4.
6. Add 0.6 ml FA lysis buffer to the lysate, resuspend the pellet and add 120U RNasin.
7. **Optional:** Perform DNase treatment by adding to the lysate 25 mM $MgCl_2$, 5 mM $CaCl_2$ and 120 μg DNase I. Incubate the lysate at 37°C for 15 minutes.
8. Add 0.5 M EDTA to the lysate to a final concentration of 20 mM and briefly mix.
9. Centrifuge at 18,000 g for 5 minutes at 4°C, transfer the supernatant to a new microtube and keep on ice until use (*see* Note 6).

3.4. Magnetic bead preparation and oligonucleotide immobilisation

Washing of MyOne Streptavidin C1 magnetic beads and immobilisation of the biotin-TEG modified oligonucleotide proceeds as per the manufacturer's protocol with slight modifications.

1. Resuspend the magnetic beads in the vial by vortexing for >30 seconds.

2. For one capture, transfer 200 µl magnetic beads to a 1.5 ml microtube and add 1 ml 1X B&W Buffer at room temperature.

   For multiple captures, up to 400 µl magnetic beads can be added per 1.5 ml microtube.

3. Place the microtube on the magnetic stand for 2 minutes and aspirate off the supernatant.

4. Remove the microtube from the magnet and resuspend the beads in 1 ml 1X B&W Buffer.

5. Repeat step 3-4 twice, for a total of 3 washes.

6. Wash the beads twice in 1 ml Solution A for 2 minutes and capture with a DynaMag-2 (or similar apparatus) for 2 minutes after each wash.

7. Wash the beads once in Solution B and magnetically capture for 2 minutes before aspirating off the supernatant.

8. Resuspend the magnetic beads in 400 µl 2X B&W Buffer per 200 µl original volume of beads.

9. To 400 µl beads, add 400 µl 2 µM oligonucleotides in 1X TE buffer and tumble the microtubes for 15 minutes to facilitate oligonucleotide immobilisation to the beads.

10. Briefly wash the beads twice in 1 ml Hybridisation Buffer.

11. Per capture, in a microtube, resuspend the beads in 0.7 ml Hybridisation buffer and 0.5 ml 1X TE buffer, both prewarmed to 37°C.

12. To this add 0.2 ml previously prepared lysate and agitate for 90 minutes at 37°C (*see* Note 7)

13. While kept at 37°C, capture the beads with a magnet, discard the supernatant and resuspend in 1 ml Wash Buffer A prewarmed to 37°C.

14. Agitate the beads for 5 minutes at 37°C and capture the beads at 37°C for 2 minutes with a magnet. Discard the supernatant.

15. Repeat steps 13-14 twice times with Wash Buffer A for a total of three washes, and perform twice with prewarmed Wash Buffer B.

3.5. Elution and cross-link reversal

1. To elute the target mRNA, vigorously resuspend the bead pellet in 30 µl 0.1X TE buffer (pH ≥7.5) preheated to 60°C.

2. Incubate the bead mix at 60°C for 5 minutes and pellet the beads on the magnetic stand for 10-15 seconds. Transfer the supernatant to a new, labelled tube ensuring no beads are transferred. Also incubate a volume (>200 µl) of original lysate for use as a reference sample.

3. To reverse the formaldehyde cross-links, incubate the eluent at 70°C for 45 minutes. Also incubate the volume of lysate from step 2 (*see* Note 8).

4. Notes

1. To test the capture efficiency of the oligonucleotides, we strongly recommend performing a capture with samples which have not undergone formaldehyde cross-linking. This can be performed by excluding steps 3.2.1, 3.2.3-3.2.4, 3.2.6-3.2.7.
2. To establish the enrichment of the target gene of interest, qRT-PCR should be performed and should include at least one or multiple non-specific genes to measure the enrichment of the target gene over the background. The lysate can be used as a reference sample, which should undergo total RNA purification.
3. To assess specificity of the oligonucleotide, we recommend performing a capture with non-targeting oligonucleotides with mismatch or scrambled sequences. To test selectivity, we recommend analysing the expression of non-targeted genes with the highest degree of complementarity besides the intended target.
4. Careful preparation and timing of the cross-linking step is crucial as overtreatment will result in increased non-specific capture, while undertreatment will result in low miRNA co-purification. Depending on the sample, both formaldehyde concentration and incubation time can be optimised.
5. Formaldehyde cross-linking makes cells more resistant to lysis. Mechanical disruption with the aid of FA lysis buffer is advised, but not limited to the method used in this protocol. Care must be taken to preserve RNA integrity. Sonication is not recommended as this frequently results in RNA fragmentation.
6. Multiple simultaneous captures can be performed from the same lysate. Freezing and thawing lysate reduces capture efficiency.
7. For efficient capture with minimal RNA degradation, the hybridisation incubation time (step 3.4.12) can be optimised (30 minutes – 4 hours).

8. The eluent should be pure enough for further downstream analyses. Further RNA purification could result in the loss of low expressed miRNAs. However the lysate should always undergo RNA purification for further analysis.

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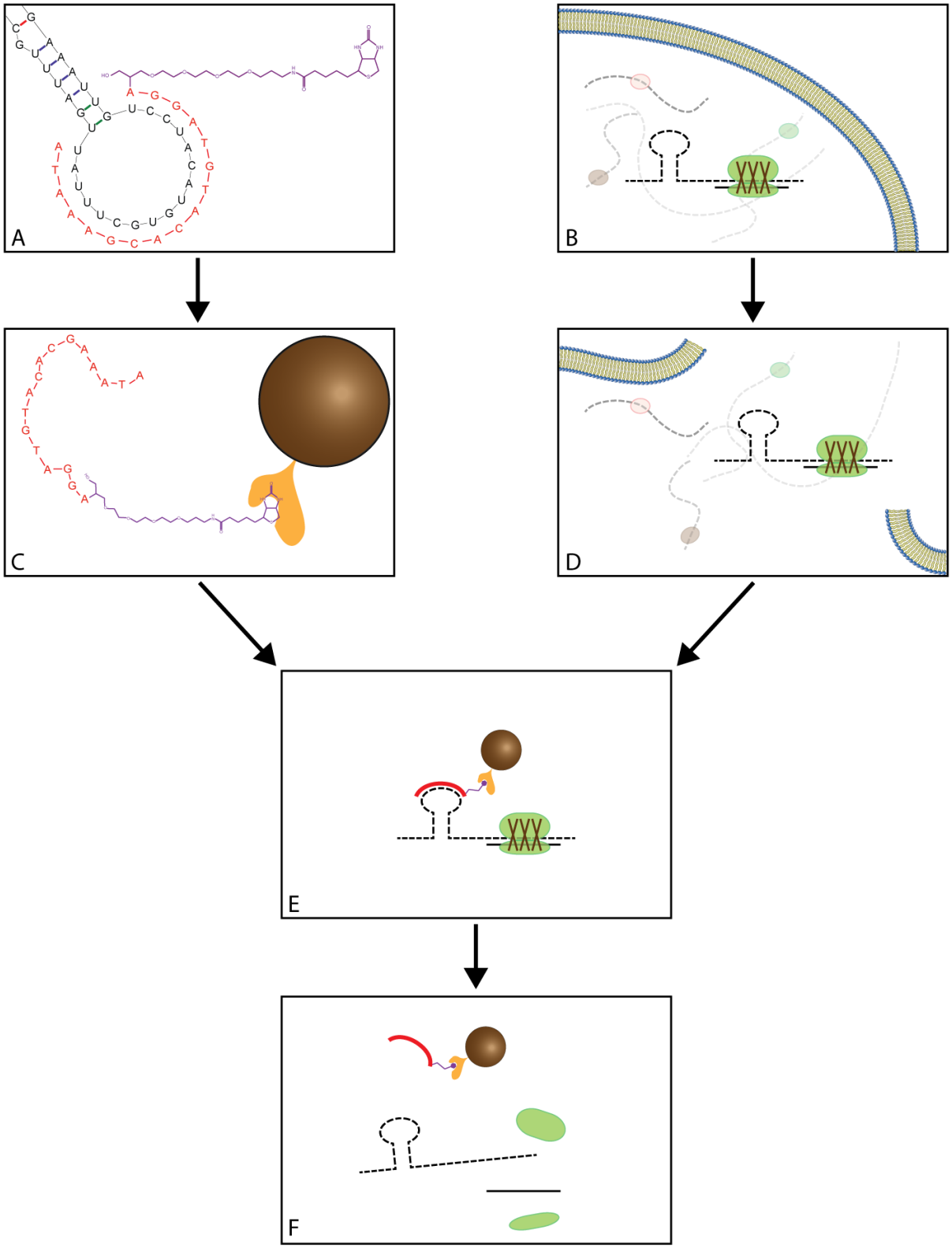
Figure Captions

Figure 1:

The miR-CATCH workflow. (A) Oligonucleotide synthesis: a biotin-TEG-modified oligonucleotide is designed and synthesised for a predicted single-stranded region of the target RNA. (B) Formaldehyde cross-linking: miRISCs are cross-linked to their targets. (C) Oligonucleotide immobilisation: capture oligonucleotides are immobilised to magnetic streptavidin-coated beads. (D) Cell lysis: cells are lysed and precipitates removed. (E) Target capture and purification: the oligonucleotide-bead complexes capture the target of interest, while background is washed away. (F) Cross-link reversal: the formaldehyde cross-links are reversed by heat, releasing the target of interest and its associated miRNAs.

Figure 2:

Expected target enrichment. This graph represents typical target enrichments achieved from cross-linked cells (+ cross-linking) and from untreated cells (- cross-linking). Total RNA was used as a reference sample and several non-targeted genes were used to assess background. Higher background is expected in cross-linked samples compared to untreated samples, resulting in a lower, but still significant enrichment.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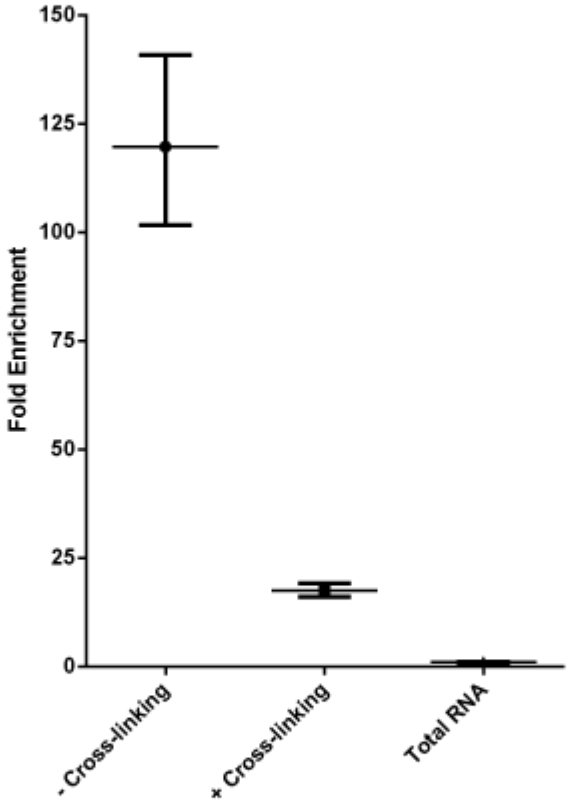
150
125
100
75
50
25
0
Fold Enrichment
- Cross-linking
+ Cross-linking
Total RNA