Analytical Biochemistry 443 (2013) 1–12

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A quantitative method to identify microRNAs targeting a messenger RNA using a 3′UTR RNA affinity technique

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ARTICLE INFO

Article history:
Received 26 April 2013
Received in revised form 17 July 2013
Accepted 1 August 2013
Available online 9 August 2013

Keywords:
miRNA
mRNA
miRNA:mRNA binding
Gene targeting
Gene modulation

ABSTRACT

The identification of specific microRNAs (miRNAs) that target a given messenger RNA (mRNA) is essential for studies in gene regulation, but the available bioinformatic software programs are often unreliable. We have developed a unique experimental miRNA affinity assay whereby a 3′UTR RNA is end-labeled with biotin, immobilized, and then used as a bait sequence for affinity pull-down of miRNAs. After washes and release, cloning and sequencing identify the miRNAs. Binding affinity is quantitated by quantitative polymerase chain reaction (qPCR), comparing released and original input concentrations. As an initial demonstration, the *TCF8*/*ZEB1* mRNA affinity pull-down yielded miR-200 family member miRs in the majority of clones, and binding affinity was approximately 100%; virtually all copies of miR-200c bound the immobilized mRNA transcript. For validation in cells, miR-200c strongly inhibited expression of a *TCF8* luciferase reporter, native *TCF8* mRNA, and protein levels, which contrasted with other recovered miRNAs with lower binding affinities. For *Smad4* mRNA, miR-150 (and others) displayed a binding affinity of 39% (or less) yet did not inhibit a *Smad4* reporter, native *Smad4* mRNA, or protein levels. These results were not predicted by available software. This work demonstrates this miRNA binding affinity assay to be a novel yet facile experimental means of identification of miRNAs targeting a given mRNA.

MicroRNAs (miRNAs)[1] are small noncoding RNAs functioning in gene regulation [1]. In animal cells, miRNAs bind to target sites in the 3′UTR of messenger RNAs (mRNAs), causing posttranscriptional repression or degradation of mRNAs or blocking translation [2,3]. Therefore, the identification of specific miRNAs targeting a specific mRNA transcript is essential for studies in gene regulation and spin-off diagnostic and therapeutic approaches.

There are two general approaches to investigate miRNA:mRNA interaction. The first general approach uses bioinformatic target prediction tools, such as MicroCosm, PicTar, and TargetScan, to predict miRNA:mRNA interaction [4–6]. However, their predictions are notoriously inconsistent [7–9], overcalling some miRNA:mRNA interactions and undercalling others. The second general approach uses experimental methods. In general, there are several kinds of experimental methods to detect miRNA:mRNA interactions. One is to detect the interaction between a known candidate miRNA and a known candidate mRNA by miRNA overexpression or knockdown and correlating that with mRNA luciferase reporter assay, quantitative reverse transcription polymerase chain reaction (qRT–PCR), and Western immunoblot [10]. Another is to comprehensively detect the interactions of all retrievable mRNA-coupled miRNAs using RISC or Ago complex immunoprecipitation, such as HITS–CLIP [11] and PAP–CLIP [12], and then probe for those specific interactions of interest. A third approach is to comprehensively screen the mRNA targets of a known miRNA, such as TAP-tar [13], isolating mRNA targets using biotinylated synthetic miRNAs [14]. A final approach is to comprehensively screen for miRNAs that target a known mRNA.

Recently, two specific methods falling under the last category, using mRNA as bait, have been developed. One method uses MS2 binding protein to capture miRNA:mRNA complexes involving fusion of mRNA 3′UTR of interest with an MS2 tag and expression of MS2-tagged 3′UTR in cells [15]. Another method uses a biotinylated antisense oligonucleotide capture affinity technique to isolate and identify specific miRNAs targeting an mRNA [16]. Because both of the methods capture miRNA–RISC (RNA-induced silencing complex)–mRNA complexes from cell lysates, neither method can capture miRNAs unassembled into miRNA–RISC–mRNA complexes or, of course, miRNAs unexpressed in that cell. Furthermore,

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[1] *Abbreviations used*: miRNA, microRNA; mRNA, messenger RNA; qRT–PCR, quantitative reverse transcription polymerase chain reaction; RISC, RNA-induced silencing complex; NHBE, normal human bronchial epithelial; cDNA, complementary DNA; *Ct*, cycle threshold.

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.08.002

the method using MS2 protein [15] still needs viable cells and, therefore, is not amenable to human tissue analyses.

Prior to the publication of those latter two reports, we embarked on an effort to generate a simple mRNA-based screening approach to identify targeting miRNAs, outside of the cell-based transfection context, and to also make it applicable to miRNAs derived from all sources, including archived tissues and clinical specimens, resulting in the miRNA affinity assay reported here. This report is of an miRNA affinity assay for identification of miRNAs targeting a given mRNA.

The approach of this miRNA affinity assay is that the 3′UTR of an mRNA transcript is used as bait to fish out the specific miRNAs that target this mRNA transcript. Briefly, a 3′UTR RNA is coupled using a biotin end label, immobilized on a solid phase, and then used as a bait sequence for an unknown pool of miRNAs. After stringent washes and then thermal release, cloning and sequencing are used to identify released miRNAs. Those miRNAs with high cloning rates and binding rates are candidates for targeting the given messenger RNA in cellular systems. Luciferase reporter assay, qRT–PCR, and Western blotting are used to validate the interaction of the miRNA candidates and the given mRNA in cellular systems. The work described below has demonstrated this miRNA affinity assay to be a novel yet facile experimental means of identification of miRNAs targeting a given mRNA.

Materials and methods

miRNA affinity pull-down protocol

The principle of the miRNA affinity pull-down protocol is depicted in Fig. 1. First, the 3′UTR is produced by T7-tagged reverse transcription of mRNA and in vitro transcription and biotin labeling, best at the 3′ end. Then, biotin-labeled 3′UTR RNA is immobilized on the streptavidin beads and hybridized with the miRNA pool. Finally, the nonspecifically bound miRNAs are washed away from the beads and the specifically bound miRNAs are released and analyzed. The resulting released miRNAs are cloned and sequenced for identification, and then miRNA real-time PCR is used on input and released miRNA to ascertain quantitative miRNA binding rates.

Total RNA and small RNA isolation

Total RNA was extracted using Trizol (Life Technologies, cat. no. 15596-018) from NHBE (normal human bronchial epithelial) cells and mouse liver tissue according to the manufacturer's instructions. Small RNA fractions were isolated from total RNA using the miRVana miRNA Isolation Kit (Life Technologies, cat. no. AM1560) according to the manufacturer's instructions.

TCF8 and Smad4 3′UTR production

Complementary DNA (cDNA) was prepared by the method of Hurteau and Spivack [17]. Briefly, a universal reverse transcription primer was employed to generate a universal sequence-tagged cDNA. Then, PCR was employed. The forward primer contained T7 promoter sequence in order to later generate RNA by in vitro transcription. *TCF8* 3′UTR was amplified using AccuPrime Taq DNA Polymerase High Fidelity (Life Technologies, cat. no. 12346086) from the cDNA templates of NHBE cells. The forward primer of *TCF8* 3′UTR is 5′ TAATACGACTCACTATAGGGAGACAAATG AAGCCTAATCGT 3′ (where the boxed area represents T7 promoter), and the reverse primer of *TCF8* 3′UTR is 5′ CATTTTATTGTGAGATGGGAGTC 3′. *Smad4* 3′UTR

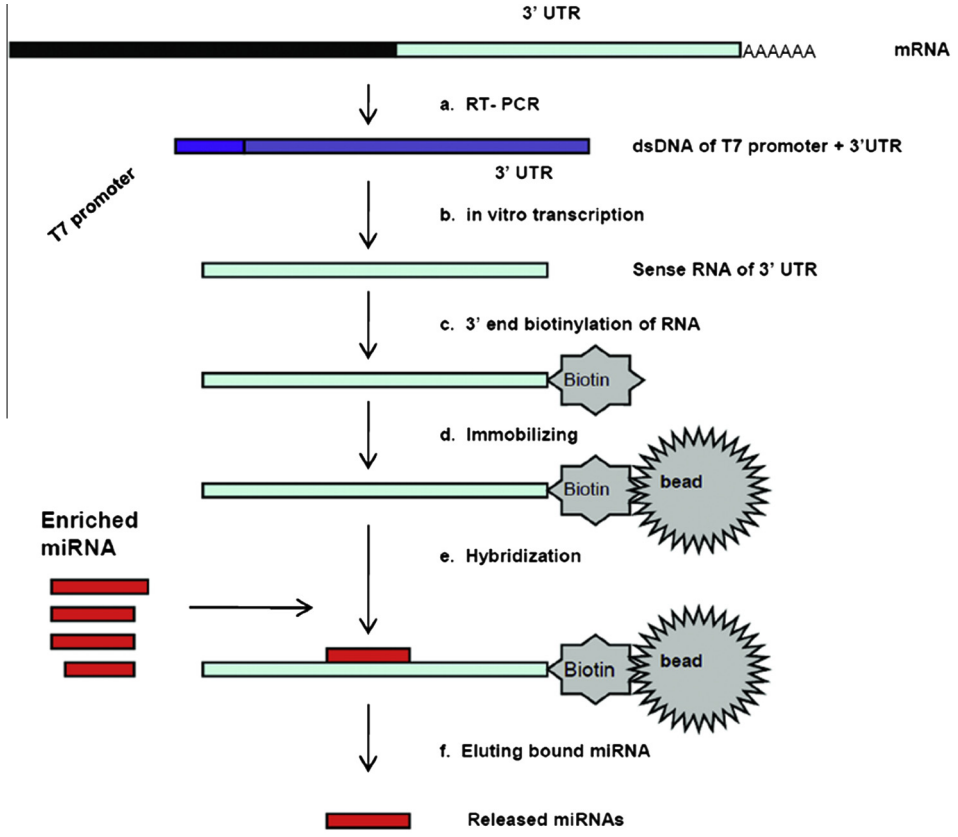

Fig.1. Overview of miRNA affinity pull-down assay. (a) produce T7-tagged ds 3'UTR by conventional mRNA reverse transcription; (b) synthesize sense RNA of 3'UTR by in vitro transcription; (c) label biotin at the 3'end of 3'UTR RNA; (d) immobilize the 3' end biotin 3'UTR RNA to streptavidin beads; (e) add the enriched miRNAs to the beads solution, and hybridize with the 3'UTR RNA on the beads; (f) remove the non-specifically bound miRNAs and elute the specifically bound miRNAs on beads.