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Use of target protector morpholinos to analyze the physiological roles of specific miRNA-mRNA pairs in vivo

Alison A. Staton¹ and Antonio J. Giraldez^{1,2}

¹ Department of Genetics, Yale University School of Medicine, New Haven, CT 06510

² Yale Stem Cell Center, Yale University School of Medicine, New Haven, CT 06520

Abstract

MicroRNAs (miRNAs) regulate gene expression by pairing to complementary sequences in the 3'UTRs of transcripts. Though the molecular mechanism underlying miRNA biogenesis and activity is becoming better understood, determining the physiological role of the interaction of a miRNA with its target remains a challenge. A number of methods have been developed to inhibit individual miRNAs, but it can be difficult to determine which specific targets are responsible for any observed phenotypes. To address this problem, we use Target Protector morpholinos interfere with a single miRNA-mRNA pair by binding specifically to the miRNA target sequence in the 3'UTR. In this protocol, we detail the steps for identifying miRNA targets, validating their regulation, and using target protectors to interrogate their function in zebrafish. Depending on the biological context, this set of experiments has been completed in 6-8 weeks.

Introduction

miRNAs are short RNAs (~22 nucleotides) that specifically regulate gene expression at the mRNA level¹. Developmental, homeostatic, and disease processes are modulated by miRNA-mediated repression of transcripts^{2, 3}. This selective repression by miRNAs is generally conferred by bases 2-8 of the mature miRNA. This region, known as the “seed,” binds complementary sequences in the 3'UTRs of mRNA transcripts⁴. A single miRNA may regulate hundred of targets. Additionally, miRNA genes are abundant in the genomes of animals, from *C. elegans* to humans, with hundreds identified in the genomes of mice and humans⁵. Because of the vast number of miRNAs and the limited sequence required for targeting, miRNAs are estimated to regulate between 25 and 60 percent of human transcripts, based on the evolutionary pressure to maintain the target sequences in evolution^{6, 7, 8}. Thus to understand the role of each miRNA in an organism's development and physiology, it is fundamental to know which putative miRNA-target regulatory interactions are physiologically relevant in the biological context under study. This protocol describes the methods available to test whether a target is regulated by a miRNA and to interrogate the physiological role of this regulation in vivo using target protectors. This approach had provided insight into the role of miR-430 in targeting the morphogen *nodal*⁹ and the chemokine signal *sdf1a*^{9, 10}.

To whom correspondence should be addressed antonio.giraldez@yale.edu Tel: 203.785.5423 Fax: 203.785.4415.

Author Contributions

A.A.S. and A.J.G. designed experiments and interpreted results. A.A.S. performed all experiments and prepared the manuscript with input from A.J.G.

Competing Financial Interests

AJG is an author on a patent to use TP technology to block Regulation of target mRNAs

To further dissect the roles of individual miRNAs while avoiding non-specific effects, many studies since have removed individual miRNAs. The first of these mutants, *lin-4* and *let-7*, were identified in *C. elegans* genetic screens for defects in developmental timing^{11, 12}. In mice and *Drosophila*, mutants have been made through deletion or targeted mutation of the miRNA gene Bantam (for instance, miR-17¹³ and miR-451¹⁴ in mice, and miR-1¹⁵, miR-278¹⁶, miR-309¹⁷, and miR-8¹⁸ in *Drosophila*). In other systems where knockout technology is not as advanced, transient techniques are used (**Figure 1**). Morpholinos have been used extensively in zebrafish and *Xenopus* to inhibit translation of mRNAs^{19, 20}. By designing a morpholino to bind the mature miRNA, the hairpin structure of the pre-miRNA is disrupted, preventing processing to make a mature miRNA²¹. This is particularly useful in studying early embryonic phenotypes because the morpholino is injected at the one-cell stage and is effective for the first five days of development²²⁻²⁴. Other antisense oligonucleotides, such as antagomirs, have been optimized for use in cell lines^{25, 26}. An alternate approach, rather than impeding processing, aims to bind all mature miRNAs. By stably overexpressing an mRNA with multiple miRNA binding sites, the miRNA binds this ectopic transcript rather than its endogenous target. Because they are used to soak up the mature miRNA, these mRNAs are called miRNA sponges²⁷⁻²⁹.

While deleting or blocking individual miRNAs provides a genetic way to dissect the overall function of a given miRNA, these approaches also have a number of shortcomings. Technically, generating these knockouts can be difficult because many miRNAs are members of large miRNA families and may be present in multiple copies in the genome. Additionally, removing the miRNA causes upregulation of all the transcripts it regulates. Because a direct relationship cannot be drawn between a miRNA and a specific target, it can be difficult to gain insight into the molecular mechanism underlying a specific phenotype. Furthermore, the effects might be caused by secondary targets that are up- or downregulated. For instance, loss of regulation of a targeted transcription factor will also increase the expression of genes it controls, though these are not directly targeted by the miRNA.

In order to examine the importance of a miRNA repressing a particular target, we have developed target protectors to specifically interfere with this interaction. Target protectors are antisense oligonucleotides designed to bind perfectly to the region of the 3'UTR complementary to the miRNA⁹. We have successfully used this technology to examine the role of a particular miRNA, miR-430, in regulating the chemokine Sdf1 during primordial germ cell migration in zebrafish¹⁰. This approach has now been used in a number of other contexts including cell culture³⁰, zebrafish¹⁰, *Xenopus*^{31, 32} and *Drosophila* (through transgenic delivery)³³. In zebrafish and *Xenopus*, morpholinos have been used. The modified backbone of these oligos increases their stability³⁴ while preventing them from being loaded into the RNA-induced silencing complex (RISC) and triggering an RNAi response. Currently, the only other method for disrupting a specific miRNA-target pair is through creating a genetic mutation of the target site in the 3'UTR using current mutagenesis techniques^{35, 36}, which requires a great deal of time and resources.

The widespread regulatory role of miRNAs has become apparent in the last decade. Using Target Protectors allows examination of the effects miRNAs have on individual targets. This directed approach is broadly applicable. For instance, use of Target Protectors can aid those interested in examining the physiological role of individual miRNA targets. Additionally, researchers who are new to the miRNA field can study the regulation of their genes or pathways of interest and test whether the regulation by different miRNAs plays an important role in their biological system. From a therapeutic standpoint, this approach of interfering with the miRNA-mediated repression of a single target can have a number of applications. If a disease is caused by lowered expression of a gene, using a Target Protector to interfere with its regulation by miRNA could restore expression to the normal level. This strategy takes

advantage of ability of Target Protectors to increase expression by a modest amount and only in cells where the gene is already expressed. Similarly, Target Protectors could be used to increase the expression of a gene with a beneficial or disease prevention role. Finally, if a mutation creates a new miRNA binding site that results in lower expression of an important gene, treatment could occur by protecting this new target site from the mutation⁹.

Several potential limitations of Target Protectors should be considered. While morpholinos bind with a high level of specificity to their target sequence, if similar sequences are present in the genome, it is possible that off-target effects could be observed. This would be particularly problematic if the sequence surrounding the miRNA seed is similar to that of other targets of that miRNA. Additionally, if the Target Protector does not bind efficiently to the target sequence, interference with miRNA targeting may be incomplete, and masking the full effect of miRNA regulation. Finally, morpholinos have the limitations that they are generally only active for the first five days of development, and they can cause non-specific effects due to toxicity.

In this protocol, we describe the process for (i) determining if a 3'UTR confers miRNA-mediated repression, (ii) designing Target Protectors, and (iii) confirming the specificity of the TP-induced phenotype. While the protocol described here is focused on zebrafish, many of the principles can be easily adapted to other organisms or even cell culture.

Experimental Design

After identifying a putative target mRNA, the ability of its 3'UTR to confer regulation in vivo is determined using a reporter vector. This reporter is injected in zebrafish embryos in the presence or absence of the miRNA of interest. Additionally, a reporter is made in which the putative target site in the 3'UTR is mutated. Once regulation is confirmed, a TP and control-TP are designed and ordered. The TP is injected in embryos to determine an appropriate concentration and then co-injected with the reporter mRNA to confirm that it relieves miRNA-mediated repression. The TP is then injected in wild-type embryos and the resulting phenotype is characterized. The specificity of this phenotype is confirmed by comparison to the control-TP phenotype and by rescue with a translation-blocking morpholino. Below, we describe a few topics to consider when designing the experiments.

Prediction of target sites

Several approaches can be taken to identify the 3'UTR of a gene of interest. The publicly available UCSC or Ensembl genome browsers can be used to locate the coding sequence. In many cases, an annotated 3'UTR based on EST data is available. It is important that the entire UTR be identified to ensure that functioning regulatory sites are not missed. Three ways of identifying the UTR are looking at Refseq sequences, examining mRNA sequencing data, and using 3' Rapid Amplification of cDNA ends (RACE). In most cases, Refseq is sufficient, but it is important to note that a stretch of A's in the 3'UTR sequence may cause mispriming by oligo dT and can result in an underestimation of the 3'UTR length.

Predicting which miRNAs target a 3'UTR remains a challenge in the field. Many software programs are available to predict which miRNAs might target a given UTR (PicTar, TargetScan, etc.; reviewed in ^{37, 38}). These algorithms consider many factors including match to the seed sequence, 3' compensatory binding, and conservation among species. In deciding which miRNAs to study, it is also important to focus on those that are expressed in the tissue and developmental stage being studied. MiRNA expression data in zebrafish is available³⁹.

If a particular miRNA is being studied, the 3'UTRs of potential targets can first be searched for 7- or 8-nucleotide matches to the seed sequence. However, it is important to note that the

seed is not always sufficient for repression⁴⁰, and non-canonical miRNA seeds can also be targeted¹²⁴¹⁻⁴⁴. Because of these difficulties in predicting miRNA targets, a number of approaches have been used to identify targets experimentally. Several studies have used gene expression analysis to find mRNAs that are upregulated when miRNA function is blocked or downregulated upon miRNA overexpression^{6, 45-47}. These expression studies have been followed by experiments examining the changes in protein expression that occur in the presence and absence of miRNAs^{48, 49}. Additionally, knowledge of how target regulation occurs has allowed biochemical experiments to pull down RISC proteins and determine which miRNAs and mRNAs are associated⁵⁰⁻⁵⁷. Using a combination of these in silico predictions and available data sets, putative miRNA-target interactions can be identified. Initial experiments using TPs demonstrated that protection of a particular miRNA site in the 3'UTR does not affect regulation of a separate site in the same 3'UTR⁹. This is important to keep in mind, as 3'UTRs may be regulated by more than one miRNA, or the same miRNA may target a transcript at several sites. In either case, separate TPs, one for each target site, would be required to completely relieve this regulation.

Primer design

Once the 3'UTR is identified, design primers to amplify the entire sequence. Primer3 or other primer design software can be used to find a forward primer that begins immediately after the end of the coding sequence. Include an XhoI restriction site at the beginning of this primer to allow cloning into the expression vector (5'- caactcgag-template sequence -3'). The reverse primer should be as close to the end of the 3'UTR as possible and should include an XbaI restriction site (5'- CAATCTAGA-sequence complementary to end of UTR- 3'). It is important to check if XhoI or XbaI restriction sites are present in the 3'UTR sequence. If these sites are encoded, other restriction sites (Sall in the place of XhoI and NheI in the place of XbaI) can be used to clone into the expression vector.

To mutate the miRNA target site, design a set of primers to the region including the seed. One primer binds 3' to 5' and acts as a reverse primer, and the other binds 5' to 3' and will act as a forward primer. When aligned, at least ten nucleotides of these primers should overlap. In these primers, change three nucleotides in the middle of the seed (for instance, TGA to ACT). Amplify the 3'UTR using the original forward primer with the mutated reverse primer and the original reverse primer with the mutated forward primer. Then combine the PCR products of these reactions for a PCR with the original forward and reverse primers. This product can be cut and cloned as above. More details on this mutagenesis can be found in ⁵⁸.

Validation of miRNA-mediated regulation

To study regulation of a particular target, it is important to first establish that the 3'UTR confers regulation by a particular miRNA. miRNAs may speed degradation or slow translation of their targets, but repression caused by either mechanism can be assessed by measuring protein output of a reporter^{12, 45, 59, 60}. Reporter assays were initially designed with β -galactosidase but have more recently been adapted to use a fluorescent protein (GFP or luciferase). GFP allows direct visualization of down-regulation, which can be particularly useful in examining tissue-specific regulation. On the other hand, luciferase allows for more precise quantification of expression levels, making it more suitable for measurement of subtle differences and for high-throughput applications⁴⁶. Both types of reporters share the same basic design, using a vector encoding the reporter gene followed by the 3'UTR sequence (**Fig. 2a, b**). For comparison, a second reporter that lacks a regulated 3'UTR is expressed in the same cells. In zebrafish, these reporters are frequently in vitro transcribed and injected as mRNA. This allows for ubiquitous expression of the reporter.

DNA reporter constructs have also been used^{46, 61}. These constructs contain both the reporter and the control in the same plasmid, driven by separate promoters (Fig. 2c). Expression of the control but not the reporter indicates repression by the miRNA. Because inheritance of this construct is uneven, embryos express the DNA reporter in a mosaic pattern, and individual cells can be evaluated. This type of reporter can be particularly useful for addressing tissue-specific regulation of a target. For instance, in looking at the regulation provided by muscle-specific miR-1, muscle cells will only express the green control while nearby non-muscle cells will be both green and red.

In this protocol, we will describe use of a GFP reporter and dsRed control mRNA. The protocol can easily be adapted for luciferase mRNA, as shown in **Box 1**. For more details about the dual promoter DNA reporter, refer to ^{46, 61}. Once regulation of a reporter has been established, predicted target sites can easily be mutated to verify that the target sequence is required for repression. Two or three nucleotides in the sequence complementary to the miRNA seed are mutated using site-directed mutagenesis.

Design and Test Target Protectors

To block the regulation of an endogenous miRNA target, target protectors are designed to bind the target site in the 3'UTR. We design our target protectors to be 25 nt. long, the standard length for morpholinos. We follow several parameters to create a TP that binds well: includes the entire sequence (7-8 nt.) complementary to the miRNA seed, contains a G or C at the 3' end, and has an overall GC percentage of 40-60%. We consider recommendations for effective morpholinos, such as avoiding sequence self-complementarity and stretches of four or more contiguous G's. We do not typically focus on including sequences upstream of the seed, even though this may be an area of partial binding for the miRNA. We have successfully developed TPs that extend upstream or downstream from the seed. However, our experience includes a limited number of examples, and we have not carried out an exhaustive analysis to determine whether binding the sequence complementary to the 3' end of the miRNA affects the efficiency of the TP. Finally, once a suitable TP sequence is identified, we run a Blast search to look for other regions of the genome where binding could occur. Upon finding a unique sequence, the morpholino can be ordered from Gene Tools.

Some 3'UTRs may contain multiple miRNA target sites. This may include multiple sites targeted by one miRNA, or single target sequences for several miRNAs. In either case, reporter assays can be used to determine if all sites confer repression, as described above. This will narrow down the number of Target Protectors to be designed. Individual Target Protectors for each validated target site can then be co-injected to achieve complete protection of the target, as we did in protecting the two miR-430 target sites in the *cxcr7a* 3'UTR¹⁰. The limitation of this approach is that the concentration of the Target Protectors must be adjusted so that they do not have a toxic effect.

Controls

Before examining the phenotypic effect of using the TP, it is important to test whether the designed TP can relieve miRNA-mediated repression without causing off-target effects. To this end, we first inject the TP at a range of concentrations and determine the highest concentrations that do not cause non-specific defects such as general cell death or embryo lethality. This sets the concentration range for the following experiments. Second, we co-inject the TP with the GFP reporter mRNA and determine the lowest concentration of TP required to relieve GFP reporter repression to a level similar to that of the mutant reporter or the reporter in the absence of the miRNA (*MZdicer* mutants). Third, we test that the injection of the TP does not cause an increase in expression of the GFP reporter in the

absence of the miRNA. This indicates that a change in wild-type reporter expression is due to interference with miRNA targeting and not general stabilization of the 3'UTR. Fourth, to confirm that the TP was indeed acting in this manner, we design a control TP that binds part of the 3'UTR sequence that lacks any predicted miRNA target sites. If the TP is functioning simply by blocking miRNA-mediated repression of the target, the control-TP, which binds outside the target site, should not alter the expression of the wild-type reporter.

Phenotype Analysis

To assess the role of a particular miRNA-target interaction, we inject the TP in wild-type embryos and determine the resulting phenotype. This part of the protocol can be adapted for the process being examined. This may include *in situ* hybridization, antibody staining, or live imaging⁶²⁻⁶⁵. Previous studies examining the target of interest can provide insight into the potential phenotypic effect. For instance, in studying *squint* and *lefty*, expression of downstream signaling genes and changes in morphology known to accompany disturbance of the levels of these transcripts were assayed⁹. Similarly, overexpression and knockdown of *sdf1a* and *cxcr7b* had previously been shown to disrupt germ cell migration^{66, 67}. In examining regulation of *sdf1a* and *cxcr7b*, we used *in situ* hybridization for *nanos* to examine the location of germ cells¹⁰. Alternatively, if the physiological effects of altering gene expression levels are unknown, the endogenous expression can be used to identify cell types or tissues affected by inhibiting miRNA-mediated repression.

Once a phenotype is identified, it can be compared to the phenotype observed in the absence of the miRNA. Two additional experiments can test the specificity of the observed phenotype. First, injection of a control TP outside the miRNA complementary region should not cause a phenotype different from wild type. Second, we test whether the phenotype observed is a direct consequence of increasing the translation of the endogenous gene by asking whether reducing the translation of the target in the presence of the TP restores the wild-type phenotype. To this end, we inject low amounts of a morpholino that blocks the translation of the target mRNA (directed against the translation start codon, AUG MO). To design the AUG MO, the same criteria used in create the TP (described above) can be used. Alternatively, if ordering through Gene Tools, the sequence including the start site can be provided and a recommended MO sequence will be returned. Reducing the overall translation of the transcript can counteract the upregulation that was caused by removing the miRNA-mediated repression, rescuing the TP-induced phenotype. This rescue experiment requires careful titration of the AUG MO, as injecting too much will produce a knockdown phenotype. Additionally, this experiment is likely to be most effective when examining the effects of a broadly expressed miRNA. If the miRNA is only expressed in a subset of cells expressing the transcript, using of an AUG MO that will knockdown all transcripts may have additional phenotypes caused by the loss of function in domains where the target is required and the miRNA is not expressed.

Reagents

Zebrafish wild-type strains can be obtained from Zebrafish International Resource Center

- Caution: zebrafish lines should be maintained and bred in accordance with local and institutional guidelines.

Expression vector encoding GFP (pCS2+GFP)⁶⁸

Expression vector encoding dsRed (pCS2+dsRed)⁶⁸

Primers for amplifying 3'UTR (see Experimental design for further information on primer design)

Primers for mutating miRNA target site (see Experimental design for further information on primer design)

AmpliTaq (Applied Biosystems cat. no. N8080172)

10x Buffer II (provided with AmpliTaq)

25 mM MgCl₂ (provided with AmpliTaq)

10 mM dNTPs (Invitrogen, cat. no. 18427-013)

XhoI (NEB cat. no. R0146S)

XbaI (NEB cat. no. R0145S)

NotI Restriction Enzyme (NEB cat. no. R0189S)

Alkaline Phosphatase, Calf Intestinal (CIP) (NEB cat. no. M0290S)

10x NEBuffer 4 (provided with XhoI and XbaI enzymes)

10x NEBuffer 3 (provided with NotI enzyme)

100x BSA (NEB, provided with XhoI and XbaI enzymes)

Agarose (American Bioanalytical cat. no. AB00972-00500)

Ethidium bromide solution (Sigma-Aldrich cat. no. E1510)

- Caution: Ethidium bromide is a hazardous substance and appropriate safety precautions should be followed during handling and disposal.

1.5 mL centrifuge tube (USA Scientific cat. no. 720678)

Qiaquick gel extraction kit (Qiagen cat. no. 28704)

LB-Ampicillin plates (see Reagent Setup)

Luria Broth Base (Invitrogen cat. no. 12795-027)

Agar (Bacteriological) (American Bioanalytical cat. no. AB01185-00500)

Ampicillin (Sigma-Aldrich cat. no. A0166)

25 mm syringe filter, 0.22 µM, nylon (Fisher Scientific, cat. no. 09-719c)

One Shot® TOP10/P3 Chemically Competent *E. coli* (Invitrogen cat. no. C5050-03) or other chemically competent *E. coli* cells

S.O.C. medium (Invitrogen cat. no. 15544034)

T4 DNA ligase (NEB cat. no. M0202S)

T4 DNA ligase buffer (Supplied with ligase)

QIAprep Spin Miniprep Kit (Qiagen cat. no. 27104)

mMESSAGE mMACHINE® SP6 Kit (Ambion cat. no. AM1340)

RNeasy Mini Kit (Qiagen cat. no. 74104)

Nuclease-free water (Ambion cat. no. AM9937)

0.5% solution of Phenol Red (Gibco cat. no. 15100-043)

Glass capillaries with filament, 1mm x 4in (World Precision Instruments cat. no. TW100F-4)

Fish Water (water removed from the zebrafish facility system)

Methylene Blue (Sigma-Aldrich cat. no. M9140)

Blue Water (see Reagent Setup)

Protease (Roche cat. no. 165921)

6-well plates (VWR cat. no. 82050-842)

100 mm Petri dishes (BD Biosciences cat. no. F1029)

60 mm Petri dishes (BD Biosciences cat. no. F1007)

Agarose-coated plate (see Reagent setup)

Agarose injection plate (Rembold)

Fire-polished wide-bore glass pipette

Equipment

28° C and 37° C incubator

37° C shaking incubator

Microcentrifuge (Eppendorf 5424)

Electrophoresis apparatus for agarose gels

NanoDrop 2000 (Thermo Scientific) or other method of DNA quantification

Microinjection apparatus with stereomicroscope (as shown in⁶⁹)

Micrometer (1 mm divided into 0.01 mm units, Meiji Techno cat. No. MA285)

Stainless steel forceps, No. 5 (Fine Science Tools cat. no. 11252-20)

Thermocycler

Dissecting microscope (Zeiss Discovery V12) with filters for GFP and dsRed

Camera (Zeiss AxioCam MRc) connected to a computer

Adobe Photoshop or other imaging software

Reagent Setup

50x TAE—Add the following to 900ml distilled water: 242g Tris base, 57.1ml Glacial Acetic Acid, 18.6 g EDTA. Adjust volume to 1L with additional distilled water. This buffer can be made ahead of time and stored at room temperature (**approximately 25° C**).

1% agarose gel—Add 1 g of agarose to 100 mL of 1xTAE buffer. Microwave for approximately 1 minute until all agarose is in solution. Add Ethidium Bromide to the hot agarose to a final concentration of 0.5 µg/mL. Pour this mix in a gel apparatus, add a comb to create wells, and cool until solid. Remove the comb and add 1 x TAE buffer. Gels should be made fresh for each use.

Ampicillin Stock Solution—Dissolve 1 g of Ampicillin salt in 20 mL water filtered with 0.22 µm filter. Make 1 mL aliquots and store at −20° C for up to 1 year.

LB-Ampicillin Plates—To make LB plates, add 25 g Luria Broth base and 15 g bacteriological agar to 1 L of water. Autoclave for 30 minutes to sterilize. Cool to 55° C. Then add 1 mL of 50 mg/mL Ampicillin. Pour into 100 mm Petri dishes and cool until solid. Store upside down at 4° C for 1-2 months.

Injection Needles—Pull glass capillary tubes to create needles with tapered tips that are strong enough to poke into the cell. It may be necessary to optimize conditions to pull the best needles. Needles can be pulled ahead of time and held in a Petri dish in clay.

Blue water—Add 2 mL of 0.1% methylene blue to 1 L of fish water. Store at room temperature for 1 month.

Pronase—Prepare a stock solution by dissolving 0.1 g protease in 20 mL water. Prepare 2 mL aliquots and store at −20° C for several months. On the day of injection, add 2 mL of pronase stock to 8 mL of blue water, for a final concentration of 1 mg/mL.

Agarose-coated plates—Dissolve 1 g agarose in 100 mL Blue water. Microwave for approximately 1 min, until all agarose is in solution. Pour agarose in a Petri dish or in the wells of a 6-well plate to cover the bottom surface (~2-4 mm thick). Cool until agarose is solid, then fill the plate with blue water. Prepare new plates fresh for each use.

Procedure

Generating fluorescent reporters (1-2 weeks)

1. For each putative target being studied, set up a 50 µL PCR reaction as follows to amplify the 3'UTR from a cDNA library (see Experimental design for further information on identification of the 3'UTR and primer design). We use AmpliTaq (Applied Biosystems), but any Taq DNA polymerase can be used:

	Volume	Final
10× Buffer II	5 µL	1×
MgCl ₂ (25 mM)	5 µL	2.5 mM
dNTPs (10 mM)	2 µL	1 mM
Forward Primer (10 µM)	1 µL	0.5 µM
Reverse Primer (10 µM)	1 µL	0.5 µM

	Volume	Final
cDNA	2-3 μ L	
AmpliTaQ Polymerase (5 U/ μ L)	0.5 μ L	2.5 U
Sterile water	Up to 50 μ L	

- Run the PCR reaction using the following conditions:

Cycle Number	Temperature	Time
1	95° C	2 min
2-34	95° C	30 sec
	58° C	30 sec
	72° C	1 min/kb amplified
35	72° C	7 min

- Set up digestion of the PCR reaction with XhoI and XbaI. Mix the components and incubate at 37° C for 2 hours.

	Volume	Final
10× NEBuffer 2	5 μ L	1×
10× BSA	5 μ L	1×
PCR Reaction (from Step 2)	25 μ L	variable
XhoI (20 U/ μ L)	0.5 μ L	10 U
XbaI (20 U/ μ L)	0.5 μ L	10 U
Sterile water	14 μ L	

Critical Step: Check the sequence of the 3'UTR PCR product for XhoI and XbaI sites. If either is present, use other cloning sites, as described in the primer design.

- While cutting the PCR product above, digest the reporter vector with XhoI and XbaI. Setup the reaction shown below and incubate at 37° C for 2 hours.

	Volume	Final
10× NEBuffer 4	5 μ L	1×
10× BSA	5 μ L	1×
pCS2+GFP	5 μ L	variable
XhoI (20 U/ μ L)	0.5 μ L	10 U
XbaI (20 U/ μ L)	0.5 μ L	10 U
Alkaline Phosphatase (CIP)	0.5 μ L	5 U
Sterile water	33.5 μ L	

- Load 25 μ L of each digestion from Steps 3 and 4 on a 1% (wt/vol) agarose gel in 1 x TAE buffer. Discrete bands the size of the 3'UTR PCR product and digested vector should appear in the gel. Using a razor blade, cut these bands out of the gel and place each into a 1.5 mL centrifuge tube.

Caution: Wear gloves and goggles when cutting out gel.

Caution: Ethidium bromide is a mutagenic compound and appropriate precautions should be taken when handling the gel.

Troubleshooting

- 6 Use the Qiaquick Gel Extraction Kit to isolate DNA from gel slices, following the manufacturer's instructions. In the final step, elute the DNA in 30 μ L sterile water.
- 7 Combine the digested DNA in the ligation mix shown below. Incubate at RT for 15 minutes.

	Volume	Final
10 $\times$ T4 DNA ligase buffer	2 μ L	1 $\times$
pCS2+GFP (XhoI, XbaI digested from Step 6)	1 μ L	variable
3' UTR PCR (XhoI, XbaI digested from Step 6)	3-10 μ L	variable
T4 DNA ligase (400 U/ μ L)	1 μ L	400 U
Sterile water	Up to 20 μ L	

- 8 Transform 3 μ L of the ligation reaction in chemically competent DH5 α cells, as described previously in ⁷⁰. Plate 100 μ L of the transformation on an LB plate containing 50 μ g/mL ampicillin. Incubate at 37° C overnight.
- 9 Use a sterile pipette tip to pick 4-6 colonies and incubate in 2 mL LB media containing 50 μ g/mL ampicillin. Shake **at 250 revolutions per minute (rpm)** at 37° C overnight.

Troubleshooting

- 10 Follow the manufacturers instructions for the Qiagen Miniprep Kit to isolate the plasmid DNA. In the final step, elute the DNA in 30 μ L nuclease-free water.
- 11 Check for presence of correct vector by digesting 3 μ L of isolated plasmid DNA from Step 10 with XhoI and XbaI, as in Step 4. Run the reaction on an agarose gel and confirm that two bands are present: one that is the size of the 3'UTR and another the size of the vector containing GFP.

Pause point: The DNA plasmid can be stored at –20° C for months.

Troubleshooting

- 12 Once the reporter vector has been created, it can then be used to create a reporter with the miRNA target site mutated (mut-3'UTR). Any technique for site-directed mutagenesis may be used. We use the sequential PCR method, described in ⁵⁸, to mutate three nucleotides in the middle of the 3'UTR sequence complementary to the seed, as shown in ⁴⁵.

Pause point: The DNA plasmid can be stored at –20° C.

Prepare reporter mRNAs for injection (2-3 days)

- 13 Use a NanoDrop or other method of DNA quantification to determine the concentration of the plasmid DNA. Use the GFP reporter plasmids (wild-type and mutated) as well as the pCS2+dsRed control plasmid.

- 14** Mix the following components to linearize the reporter plasmid. Incubate at 37° C for 2 hours.

	Volume	Final Conc.
10× NEBuffer 3	5 µL	1×
10× BSA	5 µL	1×
Reporter plasmid	Variable	4 µg
NotI (10 U/µL)	1 µL	1 U
Sterile water	Up to 50 µL	

Critical Step: Check the sequence of the 3'UTR for NotI restriction sites. If this site is present, use another enzyme that has sites after the SV40 3'UTR (such as SacII, ApaI, or Asp718) and does not cut elsewhere in the vector. Change the buffer as necessary.

- 15** Load the entire reaction on a 1% agarose gel with 1x TAE buffer. Run the gel at 100 volts for approximately an hour. Use a razor blade to cut out the band of the linearized plasmid. Collect the gel slice in an RNase-free 1.5 mL microfuge tube.

Critical Step: From this point forward, all reagents should be kept free of RNase contamination.

- 16** Follow the manufacturer's instructions and use the Qiaquick Gel Extraction Kit to isolate DNA from gel slices. In the final step, elute the DNA in 30 µL RNase-free water.

Critical Step: This Gel Extraction kit should be kept separate from that used in cloning and reserved for use in preparing *in vitro* transcript templates in order to maintain it RNase free and prevent RNase contamination.

- 17** Use the mMESSAGE mMACHINE® SP6 Kit to *in vitro* transcribe capped RNA from the digested plasmid. Thaw the Reaction Buffer and NTP/CAP mix at room temperature. Add the following components to an RNase-free microfuge tube. Incubate at 37° C for 2 hours.

	Volume	Final
10× Reaction Buffer	2 µL	1×
2× NTP/CAP	10 µL	1×
Linearized DNA template (from Step 16)	8 µL	1 µg
Enzyme mix	2 µL	

- 18** Add 80 µL of nuclease-free water to the reaction and follow the manufacturer's instructions for RNA Clean Up using the RNeasy Mini Kit. In the final step, elute the RNA from the column with 30 µL nuclease-free water.
- 19** Determine the concentration of the RNA using a NanoDrop or other method of RNA quantification. We typically obtain 15-25 µg per reaction. Split RNA into 3 µL aliquots in nuclease-free tubes. Store at -80° C.

Troubleshooting

Critical Step: RNA aliquots should be made to avoid freeze-thaw cycles that will accelerate degradation of the RNA.

Pause Point: RNA can be stored at -80°C for months.

Injection and analysis of reporters (2-3 days)

- 20** On the evening before injections, set up mating pairs of wild-type zebrafish as described in⁷¹.
- 21** Obtain and dechorionate embryos as described in previous work^{69, 72}.
CRITICAL STEP For injection of zebrafish embryos, we dechorionate embryos and inject in the cell at the one-cell stage. In our experience this reduces injection variability.
- 22** On the morning of injection, prepare the following mix and keep on ice.

	Volume	Final
GFP reporter mRNA (from Step 19)	variable	100 ng/ μL
dsRed control mRNA (from Step 19)	variable	80 ng/ μL
1% Phenol Red	0.5 μL	0.1%
Nuclease-free water	Up to 5 μL	

- 23** Load the needle with 1-2 μL of the RNA injection mix. Place the needle in the microinjection apparatus and use forceps to break the tip of the needle.
- 24** Calibrate the injection volume by measuring the diameter of a drop expelled in mineral oil on a micrometer slide (**Fig. 3**) The micrometer divides 1 mm into 100 segments. Create a drop that has a diameter of 0.1 mm, producing a 500 pL drop. Adjust the injection volume by changing the injection time or breaking off more of the tip of the needle to make the opening slightly larger.

Critical step: For accurate measurement, ensure that the drop does not collapse on the slide.

- 25** Use a polished glass pipette to place dechorionated, wild-type embryos at one-cell stage in an injection plate. Insert the tip of the calibrated needle into the cell of a one-cell embryo. Inject 1 nL (two drops) in each embryo as described in previous work^{69, 72}. Inject groups of 15-20 embryos with either the wild-type or mutated reporter.

Critical Step: Dechorionated embryos must remain completely submerged in water. Because embryos without a chorion can adhere to plastic, all Petri dishes should be coated with agarose, as described in the Reagent Setup.

- 26** Transfer the injected embryos to the wells of a 6-well plate. Do not place more than 25-30 embryos per well. Place embryos in a 28°C incubator. After 6-8 hours, inspect embryos and remove all that are unfertilized or malformed.
- 27** Visualize reporter fluorescence in a dissecting microscope when embryos are either undergoing gastrulation (6-9 hours post fertilization, hpf) or at 24 hpf.

Troubleshooting

- 28** Select 3-4 embryos from each group of injections that show similar expression of the control (dsRed). Place these embryos in separated areas of an agarose-coated plate.

Troubleshooting

- 29 Use a 10 μ L plastic pipette tip to punch a straight line of holes in the agarose, creating a holding place for each embryo. Place the embryos in these holes, allowing the yolk to rest in the hole with the rest of the embryo flat on the plate. Be sure to keep all embryos from a group together so they can be correctly identified. Turn embryos so that they are in the same orientation.
- 30 Take pictures of the red and green fluorescence of all embryos.
- 31 Compare pictures of the reporters with a wild-type 3'UTR and a mutated 3'UTR. Quantification of repression is described in **Box 2**.

Designing and validating target protector (2-3 weeks)

- 32 Design and order TP and Control-TP (see Experimental Design)
- 33 Make a series of five dilutions of TP in nuclease-free water (0.1 mM to 2 mM) Inject 15-20 wild-type embryos **for each concentration (75-100 embryos total)** with increasing amount of target protector as described in Step 25. Leave a group of 15-20 embryos uninjected to act as a control.
- 34 Transfer embryos to the wells of a 6-well plate and store in a 28° C incubator. After 6-8 hours, remove all dead or unfertilized embryos. Note if high levels of lethality occur in any group. Examine embryos at 24 and 48 hpf for increases in embryo death or non-specific defects such as increased cell death. This will help determine which TP concentrations are toxic, and an amount just below this can be used for subsequent experiments.

Troubleshooting

- 35 Inject the fluorescent wt- and mut-3'UTR reporter (each with dsRed control) in wild-type embryos as described in Steps **22-25**. Inject 40-50 embryos per reporter.
- 36 Split the embryos injected with a given reporter into three groups, equal in number and inject one of these subgroups (as described in Step **25**) with the amount of TP determined above in Step 34, inject another with the same amount of the control-TP, and leave one subgroup that is not injected with any TPs.
- 37 Place these groups of embryos in separate wells of an agarose-coated 6-well plate. Store in a 28° C incubator, removing dead or unfertilized embryos after 6-8 hours. Compare the fluorescence of each subgroup as shown in Steps 24-28. Look for an amount of TP that relieves repression of the wt-3'UTR reporter so that its expression is comparable to that of the reporter with the mut-3'UTR (**Fig. 4**). **CRITICAL STEP** The wt-3'UTR reporter with TP should not show more fluorescence than the mut-3'UTR reporter. Injecting the TP with the mut-3'UTR reporter should not cause a change in expression. Additionally, injecting the control-TP with either reporter should not change the expression of GFP. Any of these three results indicate that the TP is enhancing protein expression in a miRNA-independent manner.

Troubleshooting

Identifying and quantifying target protector phenotype (1-4 weeks)

- 38 Inject the amount of TP or control TP determined above in Step 37 into 40-50 wild-type embryos at the one-cell stage as described in Step 25. Save 25-30 uninjected embryos for comparison.

- 39** Analyze the injected embryos for phenotypic effects. This may involve analysis of morphology or labeling specific cell types using in situ hybridization or antibody staining (**as described in Experimental Design**). As a starting point for analysis, the phenotype observed in the absence of the miRNA (miRNA knockdown or mutant) can be compared to the TP-injected embryos.

Troubleshooting

Rescue the TP phenotype to confirm specificity (2-3 weeks)

- 40** Order a morpholino that binds the start site of the target gene (AUG MO). Create a series of 5 dilutions from 0.5-2 mM in nuclease-free water and inject in the one-cell embryo as described in Step 25. Determine the concentration necessary to cause a complete knockdown. This can be accomplished by comparing the morpholino phenotype to published knockdown or mutant phenotypes. If non-specific defects such as widespread cell death are observed, use a lower concentration.
- 41** Inject the diluted AUG MO in wild-type embryo as described in **Step 25**. Inject at a concentration 10-20x lower than the amount required for a complete knockdown (as determined in Step 40). Identify the concentration range that produces a mild version of the phenotype observed in the complete knockdown (Step 40). For instance, if the complete knockdown causes all germ cells to mislocalize, a milder phenotype may be embryos with only 50% of cells in the wrong location.
- 42** Co-inject (as described in Step 25) the TP **at the concentration determined in Step 37** with a narrow range of low AUG MO concentrations **as determined in Step 41**. Collect embryos for analysis of the phenotype as in Step 36. Compare the phenotypes of Control-TP, TP, Control-TP + low AUG MO, and TP + low AUG MO. Because a low level of the AUG MO will reduce translation of the mRNA, co-injecting it with the TP should rescue the overexpression phenotype caused by protecting the mRNA from miRNA-mediated repression (**Fig. 5**).

Troubleshooting

Timing

Steps 1-12: Generating fluorescent reporters (1-2 weeks)

Steps 13-19: Prepare Reporter mRNAs for injection (2-3 days)

Steps 20-31: Injecting and analyzing reporters (2-3 days)

Steps 32-37: Designing and validating target protector (2-3 weeks)

Steps 38-39: Identifying and quantifying target protector phenotype (1-4 weeks)

Steps 40-42: Rescuing phenotype (2-3 weeks)

Note: For Steps 38-42, the timing can vary greatly depending on the nature of the phenotype. If the phenotype is subtle, it may take longer to identify. Additionally, the nature of the assay used to examine the phenotype will impact the timing of these steps.

Troubleshooting

Troubleshooting advice can be found in **Table 1**.

Anticipated Results

Using this protocol, we have confirmed targeting of a 3'UTR by a particular miRNA. **Fig. 4a** shows regulation conferred by the *sdf1a* 3'UTR in the presence of miRNAs. This repression was not seen when three nucleotides in the miR-430 seed sequence were mutated (**Fig. 4b**). Additionally, injecting a target protector designed to bind the miR-430 target site relieved repression of GFP (**Fig. 4b**). Finally, the physiological role of this interaction was determined by examining the location of germ cells (**Fig. 5**). Although each miRNA is predicted to target many mRNAs, only some are regulated *in vivo*. Using this procedure, it is possible to determine if a transcript is regulated by a particular miRNA and to dissect the effect this interaction has on an organism's biology.

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Box 1 Measuring Luciferase expression in zebrafish embryos. Timing 1 day

1. Inject Firefly Luciferase reporter and Renilla Luciferase control RNAs into the cell of a one-cell embryo. Inject 100 pL at a concentration of 10 ng/ μ L.
2. Split the injected embryos into two equal groups. In one group, inject the mature duplex of the miRNA of interest. Leave the other group without the miRNA.
3. Transfer the embryos to an agarose-coated plate with blue water and incubate at 28 for at least 8 hours. Measurement should be done after 8 hpf but before the endogenous miRNA is expressed.
4. Transfer 5-10 embryos into a transparent 1.5 mL tube. Make triplicates for each injection group. Remove as much water from the tube as possible.
5. Use the Dual-Glo Luciferase Assay System and prepare reagents according to the kit instructions. Perform the assay at room temperature.
6. Add 50 μ L of Dual-Glo Luciferase Reagent to each tube.
7. Homogenize embryos by vortexing for 5 seconds.
8. Wait 10 minutes.
9. Measure Firefly Luciferase luminescence.
10. Add 50 μ L of Dual-Glo Stop & Glo and mix briefly, and wait at least 10 minutes.
11. Measure Renilla luminescence.
12. Normalize the luminescence of the Firefly luciferase to the Renilla luciferase for each sample. Average the triplicates for each group and compare the expression in the presence and absence of the miRNA.

Box 2 Quantitation of GFP reporter expression

1. Inject embryos with GFP reporter and dsRed control, as described in Steps 21-30.
2. Take pictures of the embryos to be compared side-by-side.
3. Measure the average pixel intensity for GFP and dsRed using Adobe Photoshop or other imaging software.
4. For each embryo, the intensity is the pixel intensity of the background area next to the embryo subtracted from the pixel intensity of the embryo.
5. To compare the difference in expression, use the following calculation: Fold increase in intensity = $(\text{GFP Intensity}_{\text{No miRNA}} / \text{GFP Intensity}_{\text{miRNA}}) / (\text{dsRed Intensity}_{\text{No miRNA}} / \text{dsRed Intensity}_{\text{miRNA}})$

Figure 1. Interfering with miRNA processing can help reveal the functions of miRNAs
 miRNA genes are transcribed by RNA Polymerase II as long transcripts that can contain one or more miRNAs. These transcripts (pri-miRNAs) are cleaved first by Drosha to produce a hairpin structure (pre-miRNA) and then by Dicer, generating a double-stranded RNA molecule. One of these strands is loaded into the RNA-induced silencing complex (RISC) and guides the complex to target mRNAs¹. Protein output from these genes is reduced, either due to deadenylation and accelerated degradation or translational repression. Tools that inhibit these steps (shown in red) are useful in understanding the roles of miRNAs.

Figure 2. Several different reporters can be used for validating miRNA targets

a) Injection of GFP mRNA with the 3'UTR of interest. In the presence of the miRNA, GFP expression is lower than when the miRNA is absent. dsRed mRNA is injected as a control.

b) Firefly luciferase with the 3'UTR of the putative target is injected. Luminescence, a measure of luciferase expression, is higher in the absence of the miRNA. Renilla luciferase is used as a control.

c) A DNA construct containing both RFP and GFP is injected. The 3'UTR is after RFP. Integration of the plasmid is mosaic, so only some cells will be fluorescent. Lower expression of RFP than GFP indicates repression by the miRNA.

Figure 3. Calibration of the injection needle

a) The view through a microscope of a micrometer with injection bubbles of different volumes. The injection needle should be calibrated to allow accurate injection amounts. Use forceps to break the injection needle to the size required to create the desired volume. b) Inject the reporter into the cell, not the yolk, of a one-cell stage embryo. We find that this reduces variability.

Figure 4. Reporters demonstrating that the *sdf1a* 3'UTR confers repression by miR-430

a) Expression of GFP is reduced in the presence of miRNAs. GFP with part of the *sdf1a* 3'UTR was injected in wildtype (+ miRNA) and MZ*dicer*(- miRNA) embryos. dsRed was co-injected as a control. Note that there is no change in dsRed expression. b) GFP with the *sdf1a* 3'UTR (wild-type 3'UTR) or with the same 3'UTR with the miR-430 site mutated (mutated 3'UTR) were injected in wildtype embryos. There is a strong increase in GFP expression when the miR-430 target site is mutated. The wild-type reporter was then co-injected with a target protector designed to bind the miR-430 site (*sdf1a*-TP) or a control target protector (control-TP). Only *sdf1a*-TP relieves repression of GFP expression. Note that the expression of GFP with the mutated-3'UTR or in the presence of *sdf1a*-TP is similar. dsRed is used as an injection control. c) Sequence alignment of miR-430a and *sdf1a*-TP with the *sdf1a* 3'UTR. The seed region at the 5' end of the miRNA pairs perfectly

with the 3'UTR. Sdf1a-TP was designed to bind this sequence also, as illustrated in the schematic to the right. The control TP does not bind the sequence complementary to the miRNA. The mutations in the seed binding site made to generate the mutated reporter are shown in red. All zebrafish used for these experiments were cared for in accordance with university guidelines.

Figure 5. Using the *sdf1a*-TP reveals a role for miR-430 targeting in germ cell migration
 a) Scheme for injection of target protectors and morpholinos. Injection of the TP, but not the control-TP, should increase expression of the endogenous RNA. This increase is reduced by adding a low amount of the corresponding AUG morpholino (AUG MO). The endogenous transcript is shown in light green, with an increase in expression represented by a darker green. miRNA-mediated repression is shown by binding of RISC to the 3'UTR with the resulting deadenylation or translational repression (black lines). b) Illustration of phenotypic results. In looking at *sdf1a* regulation, we examined germ cell migration. The cartoon shows localization of the primordial germ cells (blue) at 24 hpf. Injection of *sdf1a*-TP increases the number of germ cells that are located outside of the region where the germ

cells should be. This mislocalization is rescued by injecting a low concentration of *sdf1a* AUG MO, indicating that the phenotype seen upon injection of the TP is specific to upregulation of *sdf1a*.

Table 1

STEP	PROBLEM	POSSIBLE CAUSE	SOLUTION
5	No band is visible	PCR reaction failed	Load undigested PCR on a gel. If a band is present, repeat digest. If no band is visible, check primer sequences and repeat the PCR using a gradient of annealing temperatures.
	Band is not the expected size	Restriction enzymes cut in the middle of the product	Check sequence for restriction enzyme sites. Redesign cloning to use enzymes that do not cut the product.
9	No colonies	Ligation failed	Check the concentration of vector and insert used. Check that the ligation buffer and enzyme are not expired and have not been thawed too many times.
		Wrong antibiotic selection	Be sure that transformation was plated on plates with Ampicillin selection.
	Too many colonies	No antibiotic selection	Make new LB plates with Ampicillin selection. Check that Ampicillin is not expired.
		Too many cells plated	Dilute the transformation and plate fewer cells.
11	No insert	Vector recircularized without insert	Prepare other colonies and check for insert
			Repeat digest of the vector, making sure to CIP treat, and repeat cloning using a higher ratio of insert to vector
19	No RNA or low RNA concentration	Wrong vector used	Sequence the vector to make sure that the SP6 promoter, coding sequence, and 3' UTR are present.
		RNA is degraded	Be sure that in vitro transcription kit is not contaminated and use all RNase-free products
27	No or little fluorescence is visible	RNA is degraded	Run the RNA on a gel. A smear or lack of a band indicates degradation. Repeat the in vitro transcription and be sure to aliquot the RNA to avoid freeze-thaw cycles, using RNase-free products.
		Fluorescent protein is absent or mutated	Sequence the vector to ensure that the GFP or dsRed is present and free of mutations.
28	Variability among embryos in an injected group	Variability in the amount of RNA injected	Calibrate the injection needle well. Be sure to inject the same volume in the center of the cell (not the yolk) of the embryo. Watch for any clogs in the needle.
		Embryos at different stages	Limit the number of embryos per well in 6-well plates to 20-25. Remove any dead embryos.
34	High level of lethality or generalized cell death	Concentration of injected TP is too high	Repeat injections using lower amounts of TP. It is best to inject an amount of TP that is just below the threshold for non-specific phenotypes. Necessary to relieve miRNA mediated repression of the GFP reporter.
		TP is toxic due to non-specific binding	Redesign TP, changing most of the sequence but maintaining binding in the seed region.
37	Variability among embryos in an injected group	Variability in the amount of RNA or TP injected	Calibrate the injection needle well. Be sure to inject the same volume in the center of the cell (not the yolk) of the embryo. Watch for any clogs in the needle.
	TP does not increase expression	TP concentration is too low	Increase the amount of TP injected, staying below the threshold for non-specific effects.
		TP is not effective at relieving miRNA targeting	Confirm the sequence of the TP. Redesign TP so that it binds to a different sequence that still includes the seed.
39	Control-TP shows phenotype	Phenotype is not specific to relief of miRNA binding	Lower the amount of TP and control-TP injected and test for the phenotype in both groups.
			Analyze injected embryos for other phenotypes that are specific to the TP-injected group.
		Control TP also stabilizes the transcript	Test whether the control-TP stabilizes the GFP reporter. Confirm that the control-TP does not bind to a potential miRNA binding site.

STEP	PROBLEM	POSSIBLE CAUSE	SOLUTION
42	TP phenotype is not rescued	AUG MO concentration is too high/too low	Test a range of AUG MO concentrations. Use ranges that are near the threshold of MO necessary to observe a phenotype.
		TP phenotype is not specific to misregulation of this gene	Test lower concentration of the TP to determine if they are sufficient to cause a phenotype that can be rescued with the AUG MO Analyze TP-injected embryos for other phenotypes that can be rescued by the AUG MO.