

TECHNICAL NOTE:

High-throughput microRNA Target Screening: miR-122 Case Study

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Introduction

MicroRNAs (miRNAs) are important regulators of gene expression and have been shown to play a role in numerous biological processes such as cellular signaling (1), cell differentiation, growth, development, and apoptosis (2). Mutations and improper regulation of miRNAs have been linked to a variety of physiological disorders such as cancer and heart disease (3,4). In animals, miRNAs are usually complementary to one or more sites in the 3'UTRs of specific genes. Although current computational predictions of miRNA-UTR interactions provide important guidance for experimental analysis of miRNAs, little functional data exists on which to train prediction algorithms. Genome-wide transcript analysis can identify candidate target transcripts but cannot measure both the changes in a transcript's stability or translational efficiency attributable to miRNAs. We have created a genome-wide library of human 3'UTR-luciferase reporter constructs to enable researchers to screen thousands of potential miRNA targets in a high throughput fashion. Using this strategy, we sought to identify new targets of miR-122, an important regulator of cholesterol and fatty-acid metabolism in liver that has been suggested as a therapeutic target for metabolic disease (5).

Materials and Methods

SwitchGear Genomics has systematically identified human 3' untranslated regions (3'UTRs) and created a genome-wide collection of 3'UTR regions cloned into our optimized luciferase reporter vector system containing SwitchGear's RenSP reporter cassette (GoClone) as a component of the LightSwitch Luciferase Assay System. The RenSP reporter contains a PEST protein degradation sequence so that the destabilized luciferase protein has a half-life of approximately 1 hour compared to the ~3 hour half-life of the native luciferase protein, the CAT reporter protein half-life of ~50 hours, and GFP half-life of 25 hours. This modified form of luciferase greatly facilitates detailed kinetic studies, especially those focusing on repression, which might otherwise be obscured by reporter protein accumulation. Co-transfection controls were originally designed to remove variability due to transfection efficiency alone (1). Such controls were not designed nor are they adequate for comparing between different conditions or cell lines.

Cell culture

HT-1080 cells were cultivated in accordance with ATCC recommendations. The day before transfection, cells were seeded in 96-well plates so that they would be 80-100% confluent at the time of transfection.

Preparation of 3'UTR Reporter GoClone Constructs and transfection

Using the SwitchGear Genomics recommended protocol (see Figure 1), we co-transfected HT-1080 cells with 100ng of individual 3'UTR-luciferase GoClone reporter plasmids and either 20nM miR-122 mimic (Thermo Scientific, miRIDIAN Mimic C-300591-05) or a non-targeting mimic control (miRIDIAN microRNA mimic negative control CN-001000-01). All transfections were performed using the DharmaFECT Duo Transfection Reagent (Thermo Scientific, T-2010).

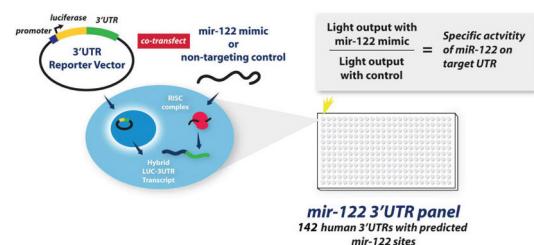


Figure 1: Experimental design for miR-122 functional screen

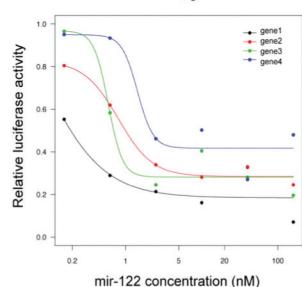
Measurement of luciferase activity and control measures

100 μ L of Luciferase reagent was added 24 hours after transfection to each well and the plate was incubated for 30 minutes in the dark before being read on a LmaxII-384 plate luminometer (Molecular Devices). Knockdown of activity (interaction of the miRNA with the 3'UTR) for each construct was quantified as the ratio of the luciferase signal observed with the miR122 mimic over the non-targeting control (luminescence = specific miRNA/non-targeting control). SwitchGear's housekeeping, random, and vector-only GoClone constructs were used in this assay to control for non-UTR specific treatment effects.

Results

After conducting a co-transfection experiment in HT1080 cells, we calculated the log₂ ratio of luminescence observed for each tested UTR reporter in the presence of the miR-122 mimic over the luminescence when transfected with the non-targeting control (Figure 2). Luminescence for 58/142 (40.8%) of the predicted targets was significantly different in the mimic co-transfection compared to the non-targeting control ($P<0.05$, t-test). Furthermore, of those 3'UTRs with significantly altered luminescence, 25/58 (43.1%) were repressed 2-fold or more by the miR-122 mimic. Thus, not only did this screen identify numerous novel targets of miR-122, but we also found a number of targets with more potent interactions with miR122 than were previously observed.

miR-122 dose response curve



EC50 (nM)	
Gene1	0.08
Gene2	0.80
Gene3	0.59
Gene4	1.44

Figure 3: Knock-down of 3'UTR luciferase changes in a dose-dependent manner

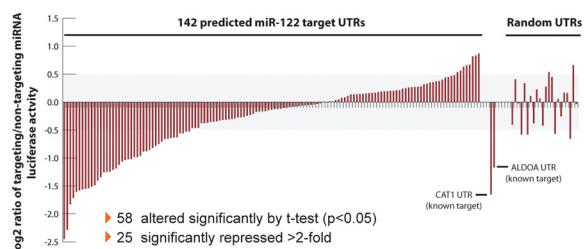


Figure 2: Summary of results from functional screen of predicted miR-122 targets in HT-1080 cells

In addition to performing a screen of putative targets using the 3'UTR GoClone reporter collection, we also performed a number follow-up studies to provide context for the initial results. We tested a number of the highly-repressed targets from our screen at miRNA concentrations ranging from 0.0625 to 100nM. The tested 3'UTR reporters responded to miR-122 mimic in a dose-dependent fashion, with EC50 at or below 1.5 nM (Figure 3). We also tested for the specificity of the miR-122 knockdown by selectively mutating 2-3 bases in the seed recognition sequence of the 3'UTR reporter. In 5/6 cases, mutating the miR-122 seed recognition sequence resulted in significantly decreased knockdown of luminescence in the presence of the miR-122 mimic (Figure 4). The remaining 3'UTR mutant reporter exhibited decreased knockdown, but the change was not statistically significant. Finally, we performed quantitative real-time PCR against endogenous 3'UTRs both in the presence of the miR-122 mimic and the non-targeting control to test for knockdown of the endogenous message. Upon plotting the log₂ ratio of mimic/non-targeting of both endogenous message and 3'UTR reporter luminescence we observed an overall correlation of R=0.78 (N=14) (Figure 5).

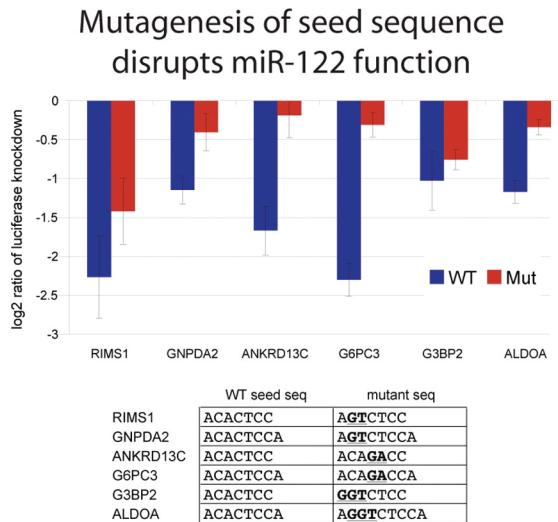


Figure 4: Mutagenesis of miRNA seed recognition sequence disrupts miRNA repression

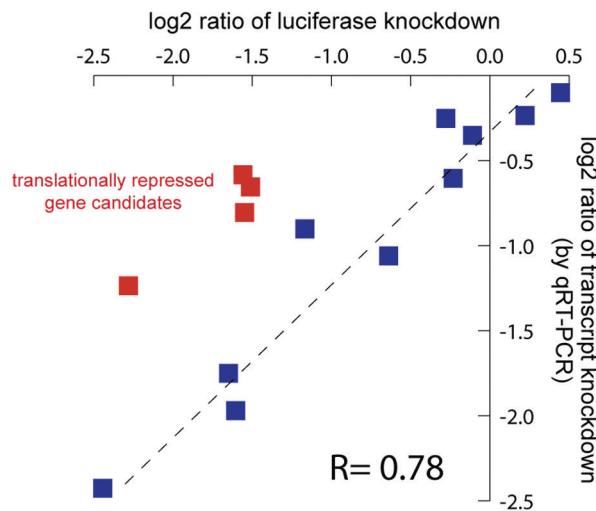


Figure 5: 3'UTR luciferase activity is highly correlated with endogenous transcript levels

Summary

Using a number of complementary approaches, we have demonstrated that the results from the 3'UTR high-throughput reporter screen were dose-dependent, specific, and reproducible. Computational predictions and transcript-based expression analysis alone cannot measure the functional roles of miRNAs, and our 3'UTR reporter screen clearly demonstrates the ability to measure actual miRNA function. Luciferase assays provide another advantage by measuring translational efficiency in addition to changes in message stability. In our screen, four of the 3'UTRs exhibited more repression by luminescence than by RT-PCR, highlighting transcripts that may be subject to translational repression. Our genome-wide library of human 3'UTR-luciferase reporter constructs enables researchers to screen thousands of potential miRNA targets and understand the roles of miRNAs in a single experiment.

References

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4. Chen JF, Murchison EP, Tang R, et al. (February 2008). Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. *Proc. Natl. Acad. Sci. U.S.A.* 105 (6): 2111-2116.