

Application Forum

A Novel cellular microRNA target selection system

Nandita Sarkar, Fangting Wu, and Travis J. Antes, System Biosciences (SBI)
1616 N. Shoreline Blvd., Mountain View, CA 94043 USA

Introduction

microRNAs are a class of small, noncoding RNAs that control gene expression by translational repression or messenger RNA (mRNA) degradation through binding to target sites within 3' UTRs. More than 900 microRNAs are expressed in humans, and up to 30% of human genes may be regulated by microRNAs. Although a number of tools have been developed for identifying and profiling microRNAs, cataloging microRNAs and their expression patterns does not provide a complete picture of their cellular roles. In order to gain such an understanding, it is important to identify which mRNAs are regulated by specific microRNAs. Imperfect base pairing of microRNAs to target RNAs complicates the prediction of microRNA targets based on sequence alignment alone (1). Current experimental approaches to verifying predicted interactions are laborious and time-consuming. We have developed a unique technology for identifying the microRNAs that bind to 3' UTRs using a cellular selection system.

Our miR-Selection lentivector features a dual reporter system with firefly luciferase (Fire) and a cytotoxic sensor (Ctx). The miR-Selection platform captures the 3' UTR to microRNA binding event using a survival screen by modulating the reduction of the cytotoxic sensor. Quantitative validation is made simple using the built-in luciferase reporter. This powerful and elegant technology enables the accurate identification of microRNA targets (Figure 1).

How to use the miR-Selection system

First, clone the 3' UTR of interest into the multiple cloning site (MCS); this will place the expression levels of the dual reporter Fire-Ctx sensors under the control of microRNA binding sites present in this 3' UTR. Next, create a stable screening cell line using the constitutive EF1-Puro selection cassette built in to the system. Once the cell line is established, the selection can begin. Add the Ctx drug to the cell culture medium. The cells will die rapidly and be completely wiped out after 4 days unless a valid microRNA to 3' UTR binding event occurs. Cells surviving the Ctx drug treatment is due by detecting direct microRNA–3' UTR interactions when overexpressing a microRNA or pools of microRNAs during the selective screen.

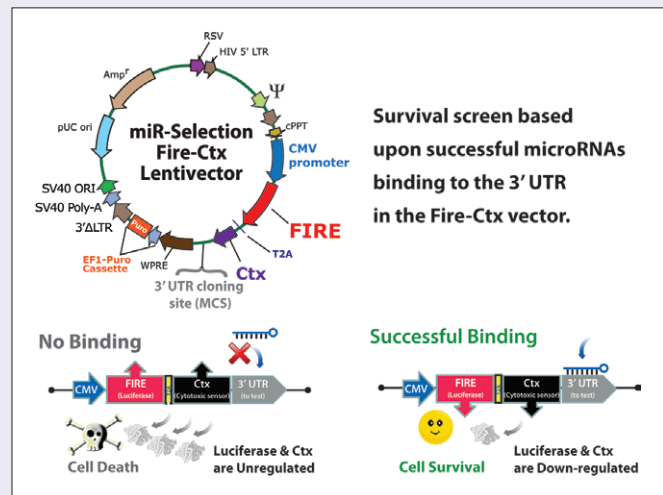


Figure 1. How the miR-Selection system works. The miR-Selection lentivector features an EF1-Puro cassette to create stable cell lines for screening (orange in vector map). The 3' UTR under study is cloned into the multiple cloning site (MCS) where it influences the expression of the upstream Firefly Luciferase (*Fire*) and the Cytotoxic sensor (*Ctx*) genes. If there is no binding site in the 3' UTR clone for the microRNA being tested, the cells do not survive the selection (skull and bones) but will thrive if microRNAs bind to the 3' UTR in the construct (happy face).

Materials and methods

Establish the miR-Selection screening cell line

HEK 293 cells were transduced with either the empty Fire-Ctx control (no UTR) or with the c-*Myc* 3' UTR lentivirus at an MOI of 5. The cells were allowed to recover for 72 hours and puromycin was added to the media at 1 mg/mL to create the stable cell lines. Medium and growth conditions were standard for HEK 293 cells (DMEM + 10% FBS, 37°C at 5% CO₂).

Performing the miR-Selection screen with Ctx and Luciferase assays

Stable HEK 293 cell lines harboring the control or c-*Myc* 3' UTR constructs were seeded in 6-well plates at a density of 80,000 cells per well. The next day, the cells were transduced with Lenti-miR-145 lentivirus at an MOI of 5 and allowed to recover for 72 hours. The cells appeared green since the Lenti-miR-145 virus coexpresses GFP as a marker as well as the miR-145 precursor. The cells were split 1:2

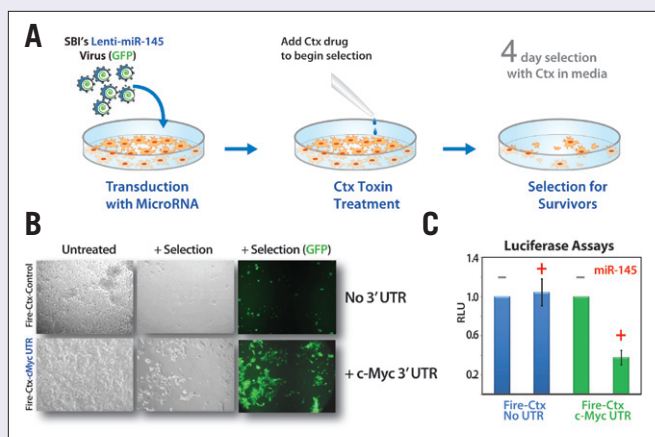


Figure 2. Validation of the miR-Selection system using miR-145 and c-Myc 3' UTR sensor. The overall schematic of the 4 day selection screen is shown in **A**. The stable cell lines expressing the Fire-Ctx control (No UTR) or c-Myc 3' UTR were transduced with Lenti-miR-145. After 4 days of selection in Ctx, the cells were imaged and counted for survivors **B**. The cell lysates from the cell lines with Fire-Ctx control (No UTR) or c-Myc 3' UTR were tested for luciferase activities without Ctx selection and the quantitative results are graphed and shown in **C**.

and transferred to a 6-well dish at a density of 80,000 cells per well. The 40 \times Ctx solution was added to a final 1 \times in the appropriate wells the next day. After 3 days, the media was changed with fresh Ctx where appropriate. The cells were visualized and surviving cells were counted at day 4. For the luciferase assays, cells were lysed and luminescence levels were measured in triplicate according to manufacturer's instructions (Promega, Inc.).

Results and discussion

We have built a dual reporter system that can detect microRNA binding activities in a cellular screen. The system features an innovative selection marker for cell survival screens as well as the gold standard luciferase reporter to quantitate microRNA binding. To test and validate this technology, we used the known microRNA binding activity of miR-145 to the 3' UTR of *c-Myc* in our tests (2). We made a stable cell line of the no-UTR control miR-Select construct and a stable cell line for the miR-Select construct that has the *c-Myc* 3' UTR cloned downstream of the dual reporters. Both cell lines were transduced with equal amounts of Lenti-miR-145 virus. The Ctx selection was initiated by adding the drug directly to the medium and allowing the cells to undergo selective pressure for 4 days. Stable cell lines expressing the Fire-Ctx control transduced with miR-145 before and after selection demonstrate the powerful selection technology with <3% of cells surviving the Ctx selection whereas overexpressing miR-145 enables over 65% survival (Figure 2). If microRNAs bind to the 3' UTR being tested, then the expression levels of both luciferase and the Ctx sensors will be greatly reduced. Lowering the amount of the Ctx sensor is what will enable the cells to survive in the presence of the Ctx drug. This interaction between microRNAs and the 3' UTR is key to the

selection system and is what is being measured during the screen. We also measured the luciferase (Fire) activities of the +/- *c-Myc* 3' UTR in the miR-Selection vector infected with or without Lenti-miR-145 virus without Ctx selection. Our results show that luciferase levels were unchanged in the no-UTR controls as expected; we also observed a 63% reduction in luciferase activity in the *c-Myc* 3' UTR plus Lenti-miR-145 cells. We validated the miR-Selection system with known binding partners of miR-145 and the 3' UTR from *c-Myc* using both a cellular selection and luciferase reporter assays. The power of the technology will be as a discovery tool to identify and validate, without prejudice, microRNAs that can bind and regulate a 3' UTR of interest.

Conclusion

To address this critical and growing need of a reliable high-throughput method to identify microRNA regulatory networks, we have developed a miR target selection platform that can accurately capture the microRNA–3' UTR target interaction in a cellular system. The miR-Selection system consists of a dual reporter system which constitutes a luciferase reporter (Fire) coupled with a cytotoxic sensor (Ctx). The 3' UTR sequence under study is fused downstream of the dual reporters, thus conveying direct control of the reporters via 3' UTR regulation. When microRNAs bind the 3' UTR, it suppresses expression of the reporters. This suppression can be monitored by luciferase measurements and, more importantly, captured through the cytotoxic sensor that will lead to cell survival in the presence of Ctx selection. The major advantage of using the cytotoxic sensor is that this screen can be performed in a high-throughput manner using cell survival as the outcome. This simple and clean approach will enable screens to simultaneously identify numerous targets of a single microRNA. Once the 3' UTR targets are identified, they can be validated using the same miR-Selection vector's luciferase reporter feature to quantitatively measure the strength of the repressive interaction. Thus, our technology will provide a rapid, efficient and quantifiable high-throughput screening tool to accurately identify microRNA targets. More information on the miR-Selection system can be found online at www.systembio.com/microrna-research or by phone at 650-968-2200.

References

1. Wang, W.X., B.R. Wilfred, K. Xie, M.H. Jennings, Y. Hu, A.J. Stromberg, and P.T. Nelson. 2010. Individual microRNAs (miRNAs) display distinct mRNA targeting "rules". *RNA Biol.* 7(3).
2. Sachdeva, M., S. Zhu, F. Wu, H. Wu, V. Walia, S. Kumar, R. Elble, K. Watabe, and Y.Y. Mo. 2009. p53 represses *c-Myc* through induction of the tumor suppressor miR-145. *Proc. Natl. Acad. Sci. USA.* 106:3207-3212.

Sponsored Paper. *BioTechniques* 48:XXX-XXX (June 2010)
doi 10.2144/000113449