# Chapter 10

# Sensing miRNA: Signal Amplification by Cognate RISC for Intracellular Detection of miRNA in Live Cells

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## Abstract

The ability to detect miRNA expression in live cells would leave these cells available for further manipulation or culture. Here, we describe the design of a miRNA sensor oligonucleotide whose sequence mimics the target mRNA. The sensor has a fluorescent label on one end of the oligo and a quencher on the other. When inside the cell, the sensor is recognized by its cognate miRNA-RISC and gets cleaved, setting the fluorophore free from its quencher. This results in fluorescence "turn on." Since cleavage by the RISC complex is an enzymatic process, the described approach has a very high level of sensitivity (nM). The rate of nonspecific cleavage of the sensor is very slow permitting the collection of meaningful signal over a long period of time.

**Key words** miRNA, Sensor, RISC, Intracellular detection, Fluorescence, Turn on, Live cells

## 1 Introduction

miRNAs are powerful regulators of gene expression [1]. They function by guiding a multienzyme complex, named the RNA-induced silencing complex (RISC), to their cognate mRNA transcripts. This primes the mRNA for cleavage and degradation or translational repression, depending on the level of complementarity between the miRNA and the mRNA [2, 3]. In this work, we focus on microRNA-10b (MIR10B) because of its proven role in metastasis [4]. Chemically, the sensor represents a single-stranded RNA with a Cy5 dye molecule covalently linked to its 5′ end and an Iowa Black RQ quencher to the 3′ end [5]. Iowa Black RQ is an efficient quencher of Cy5. Thus, the sensor molecule, by itself, has very low background fluorescence. The sequence of the sensor is completely complementary to the sequence of miRNA-10b. Once inside the cell, the sensor is recognized by the MIR-10b-RISC complex and is cleaved, releasing Cy5 from its quencher.

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This results in fluorescence "turn on" that can be detected by fluorescence readers, FACS machines and confocal microscopes. Potentially, the cells detected in this manner can be sorted out for further studies. Since the sensor is chemically similar to the native mRNA and uses the same pathways of degradation as mRNA, it is expected that the sensor will not affect cell phenotype.

## 2 Materials

All media and solutions were prepared in DNase- and RNase-free water, aliquoted, and stored frozen. Handling was minimized to reduce contamination with RNases. Thawing was done on ice to prevent degradation. Certified DNase- and RNase-free pipette tips, tissue culture flasks and multiwell plates were used throughout this study.

1. Sensor oligo: The oligo is a single-stranded RNA whose sequence is complementary to miR10b. On its 5′ end, it is conjugated to Cy5 (fluorophore). On its 3′ end, it is conjugated to Iowa black RQ (quencher). No other chemical modification is introduced, as it might affect the cleavability of sensor. The construct was custom synthesized by Integrated DNA Technologies (Coralville, IA). The sequence of the final construct is: 5′Cy5/rCrArCrArArArUrUrCrGrGrUrUrCrUrArCrArGrGrGrUrA/IAbRQSp-3′.
2. Predesigned, pre-validated miRCURY LNA™ microRNA inhibitors (antisense oligos, ASO) of MIR10B and scrambled controls were purchased from Exiqon (Woburn, MA). These oligos are made up of locked nucleic acids (LNA) that are chemically stable in a wide variety of biological environments. The antisense oligo designed to block MIR10B-RISC activity is labeled as MIR10B-ASO and its scrambled control is labeled as SCR-ASO.
3. Human breast cancer cell line, MDA-MB-231-luc-D3H2LN: The cell line is known for its metastatic potential in an orthotopic mouse model of breast cancer and was purchased from Caliper Life Sciences (Waltham, MA).
4. Dulbecco's modified Eagles medium: The cell line was maintained in Dulbecco's modified Eagles medium supplemented with heat-inactivated fetal bovine serum (10 % final). Antibiotics (penicillin and streptomycin; 1 % final concentration) were only present during regular culture.
5. Lipofectamine LTX-plus (Invitrogen) and Lipofectamine RNAiMAX (Invitrogen): The sensor incubated with these

reagents did not exhibit any appreciable increase in fluorescence after 24 h of incubation indicating that the reagents at hand were free of nonspecific RNase activity.

6. Prolong gold (Invitrogen) mounting medium containing DAPI and an anti-fade was used to mount cells for fluorescence and confocal microscopy.

## 3 Methods

### 3.1 Preparation of Cells

1. Exponentially growing cells were collected by trypsinization and counted using a cell counter.
2. Cell concentration was adjusted to 150,000 cells/ml in DMEM. 200 μl/well were plated in 96-well clear bottom black tissue culture plates. While performing these steps, care must be taken to avoid cell rupture during trypsinization and subsequent cell culture handling steps as this may result in release of RNase activity. Plates were incubated in a $CO_2$ incubator.
3. After 24 h, all wells were observed under the microscope for any contamination or cell clumps. Over- or under-seeded wells were marked and excluded from the study. Also all wells on the edge of the plate were excluded from the study because of the "edge effect" that leads to uneven evaporation of media from these wells.
4. The media in the 96-well plates was replaced with 100 μl of fresh DMEM without FBS or antibiotics (*see* **Notes 1** and **2**). Care was taken to not dry the cell layer during this step. Two different multichannel pipettes were used. One for removing and one for adding media.
5. Plates were returned to the incubator and allowed to equilibrate for a few hours.

### 3.2 Transfection of Cells

#### 3.2.1 Premise

Two rounds of transfections will be performed on the same cells. First, the cells will be transfected with a chemically stable (locked nucleic acid) MIR10B-ASO to block the endogenous MIR10B-RISC activity. For control, another set of cells will be transfected with SCR-ASO. After 2–4 h, a second round of transfection will be performed with the sensor oligos. Assuming 100 % efficiency, the MIR10B-ASO treated cells will not be able to bind and degrade the sensor and fluorescence activation will not occur. By contrast, in the control wells, in which the cells are treated with SCR-ASO, the MIR10B will bind and prime the sensor for degradation by the RISC, leading to fluorescence activation.

### 3.2.2 Transfection with Antisense Oligonucleotide

Transfection with an excess of antisense oligos (3.8 μM final concentration) was performed to ensure complete inhibition of MIR10B-RISC activity.

1. Dilutions of MIR10B-ASO and SCR-ASO were prepared by diluting 5 μl of stock (5 mM) solution into 125 μl of serum-free media.
2. Two sets of dilutions of lipofectamine were prepared by mixing 13 μl of lipofectamine in 120 μl of serum-free media (*see* **Notes 3** and **4**).
3. Next, the ASO and lipofectamine were mixed (final volume 130 + 133 = 263 μl) and allowed to incubate at room temperature for 30 min (*see* **Note 5**).
4. 10 μl of MIR10B-ASO + lipofectamine mix was added to 3 sets of 7 wells (21 wells total). Final concentration of ASO was 3.8 μM.
5. Similarly, SCR-ASO + lipofectamine mix was distributed in another set of 21 culture wells.
6. The culture plate was incubated for 2 h in a $CO_2$ incubator.

### 3.2.3 Transfection with Sensor Oligo

1. First, 140 μl of 12 μM sensor solution was prepared by mixing 11.2 μl of sensor stock solution with 110.8 μl of serum-free media and 18 μl of lipofectamine (*see* **Note 6**).
2. Then this solution was serially diluted to get 70 μl each of 12, 6, 3, 1.5, 0.75, and 0.375 μM sensor dilutions.
3. Then, 10 μl of each dilution was added in triplicate to cells pretransfected with either MIR10B-ASO or SCR-ASO. Final concentration of sensor in culture wells was 1000, 500, 250, 125, 62.5, and 31.25 nM.
4. Plates were incubated for 3 h.
5. After incubation, transfection media was replaced with 200 μl of DMEM supplemented with 10 % heat inactivated FBS. No antibiotics were added to this media.
6. After replacing media, fluorescence was immediately recorded on an IVIS-spectrum imaging station with filter sets for Cy5 dye. Wells for 125 nM sensor concentration were shown in Fig. 1a. Note that there is minimal background fluorescence at this time (Fig. 1a).
7. Fluorescence "turn on" was measured by reading the plate at specified time points (Fig. 1a). For this cell line, fluorescence peaked at 16 h after which no appreciable increase in MIR-10B-RISC-mediated fluorescence was observed (Fig. 1b).

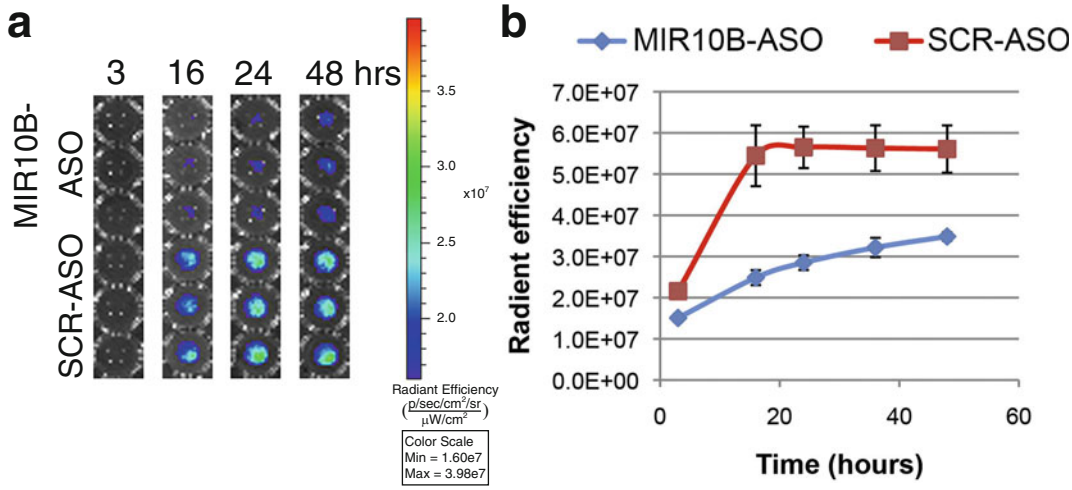


**Fig. 1** Sensor activation. (**a**) Sensor activation at a fixed concentration of 150 nM was followed for 48 h on IVIS-spectrum imaging station. Data were recorded using filter sets for Cy5 dye. (**b**) Quantitative analysis of signal observed in Fig. 1a. (This figure is reproduced with permission from Elsevier. Original article appeared in Chemistry & Biology 21, 1–6, February 20, 2014)

8. Nonspecific cleavage of sensor in MIR-10B-ASO-treated cells was slow and even after 48 h of incubation, fluorescence did not reach maximum (Fig. 1b).

### *3.3 Fluorescence and Confocal Microscopy*

1. For fluorescence microscopy, cells were grown in 8-chambered slides.
2. After 24 h of growth, they were transfected with either MIR10B-ASO or SCR-ASO as described above and incubated for 2 h.
3. After 2 h, cells were transfected with a single sensor concentration of 125 nM.
4. After 24 h, cell layers were washed with PBS, fixed in 4 % formaldehyde for 10 min, mounted in mounting medium, and observed under a fluorescence microscope with filter sets for Cy5 (Fig. 2a).

### *3.4 Flow Cytometry*

1. For flow cytometry and sorting, cells were grown in 6-well plates. The concentrations of ASO and sensor were kept constant as described above. Volumes were increased to match the increased area of 6-well plates.
2. After 16–24 h of incubation, cell were collected by trypsinization and kept on ice for sorting or fixed with 4 % formaldehyde for FACS analysis. No further washing steps were performed (Fig. 2b).

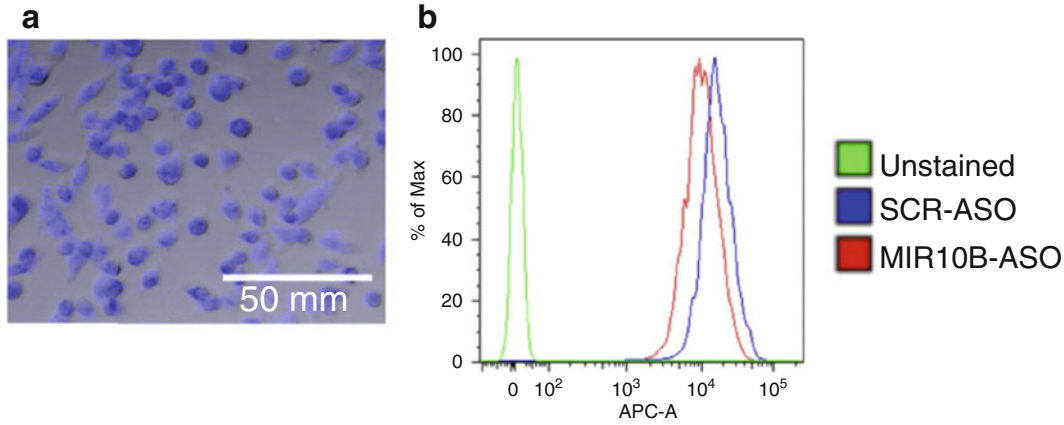


**Fig. 2** Sensor activation in live cells. (**a**) MIR10B-RISC activity was first blocked by treatment with MIR10B-ASO and then cells were transfected with the sensor. Fluorescence accumulation due to sensor activation was observed after ~16 h under a confocal microscope. (**b**) Flow cytometry of intact cells. (This figure is reproduced with permission from Elsevier. Original article appeared in Chemistry & Biology 21, 1–6, February 20, 2014)

## 4 Notes

1. Presence of antibiotics can be detrimental to cell survival during transfection.
2. Removal of FBS during transfection ensures efficient transfection and reduces RNase activity that might be present in FBS. Keep in mind that the sensor being used in subsequent steps of this experiment is an unmodified RNA oligonucleotide. The dye and quencher present on either end do not protect the sensor oligos from nonspecific RNase activity.
3. Lipofectamine is detrimental to cell survival. Amounts tolerated vary from cell line to cell line. Hence, the researcher must have a good idea of the amounts of lipofectamine that will be tolerated by their cell line. We recommend titration of lipofectamine at hand with the cell line to be tested.
4. Ratio of oligos to lipofectamine is important for efficient uptake. Adhere to manufacturer's recommendation.
5. Since in this protocol, two rounds of transfection will be performed on the same cells, the total amount of lipofectamine that is used should be less than the dose that can be easily tolerated by the cells.
6. Once the sensor is removed from the −80 freezer, exposure to bright light must be minimized.

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