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IN SITU ANALYSIS OF RNAi USING AFFYMETRIX' VIEWRNA SYSTEM

A Major Qualifying Project Report

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ABSTRACT

RNA interference (RNAi) is a powerful mechanism for gene silencing that holds great promise for therapeutic applications. To design a drug, scientists must find siRNA sequences capable of strong mRNA silencing and effective methods to deliver the siRNA to target tissues. *In situ* hybridization techniques enable the visualization of the effectiveness of siRNAs. This report demonstrates the visualization of RNAi mediated gene silencing by using a new, highly sensitive bDNA probe-based *in situ* hybridization assay known as ViewRNA. The ViewRNA System showed in both a qualitative and semi-quantitative manner the reduction of SOD1 mRNA after cells were transfected with siRNA targeting the gene.

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BACKGROUND

Superoxide Dismutase-1, General Information

Superoxide Dismutase 1 (SOD1) is a cytosolic enzyme that is omnipresent throughout virtually all eukaryotic organisms, as well as some prokaryotic organisms. Human SOD1 (hSOD1) is a gene that is about 1kb long that translates into a protein that has a molecular weight of ~16 kDa. In its active form, the SOD1 enzyme exists as a homodimer. (**Figure-1**) (Valentine et al. 2005).

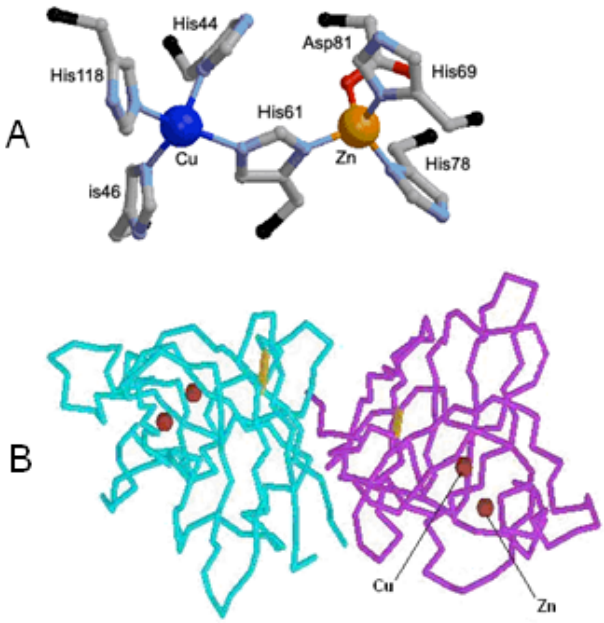

Figure-1: Structure of SOD1. (a) Each SOD1 subunit binds one Cu++ ion (denoted in blue) and one Zn++ ion (denoted in orange) to catalyze the dismutation of superoxide radicals. (b) The homodimeric structure of SOD1. (Valentine et al. 2005).

The normal function of the enzyme is to convert harmful superoxide radicals ($O_2^{-\cdot}$) to less toxic hydrogen peroxide (H_2O_2), using the reactions shown in **Figure-2**. When the enzyme is in its active homodimeric form, each subunit binds a Cu^{++} ion and a Zn^{++} ion.

This form of SOD1 is sometimes known as Cu/Zn-SOD1, to distinguish it from the manganese-binding mitochondrial form (Mn-SOD1). The copper ion drives the dismutation of one superoxide free radical (**Figure-2a**). The enzyme then takes a second superoxide free radical along with two hydrogen ions to form one oxygen molecule and one hydrogen peroxide molecule (**Figure-2b**) (Valentine et al. 2005). Hydrogen peroxide, which is still harmful to the cell, is readily converted to water and oxygen by catalases.

(a) $Cu^{++} + O_2^{-\cdot} \rightarrow Cu^{+} + O_2$
(b) $Cu^{+} + O_2^{-\cdot} + 2H^{+} \rightarrow Cu^{++} + H_2O_2$

Figure-2: The Reactions by Which SOD1 Converts Superoxide Radicals to Hydrogen Peroxide.

SOD1 has one of the highest turnover numbers of any known enzyme, which keeps oxidative damage from superoxide radicals to a minimum (Valentine et al. 2005).

SOD1 and Amyotrophic Lateral Sclerosis

SOD1 is a key anti-oxidative enzyme of interest in a variety of diseases induced by oxidative stress, but has recently become of particular interest due to its role in amyotrophic lateral sclerosis (ALS), sometimes referred to as Lou Gehrig's Disease or Charcot-Marie-Tooth disease (Valentine et al. 2005). ALS is a most often fatal neurodegenerative disease characterized by the atrophy and death of upper motor neurons in the brain, and lower motor neurons in the spinal cord. ALS affects roughly 1-2 people in every 100,000, with a female to male incidence ratio of roughly 2:3. The median age

of onset is 55, and, after diagnosis, patients typically survive only another three to five years (Boillee et al. 2006).

Two types of ALS exist: the familial form (fALS) and the sporadic form (sALS). SALS accounts for roughly 90% of all ALS cases, while fALS accounts for the remaining 10% (Boillee et al. 2006). Early symptoms of the disease typically include frequent cramping, twitching, stiffness, and muscle weakness. As the disease progresses, patients may lose weight and experience chronic fatigue, as well as difficulty controlling reflexes and maintaining coordination. Eventually, simple and vital tasks such as eating, speaking, moving, or even breathing become impossible under the patient's own power. Most often, respiratory failure or pneumonia (the occurrence of which is drastically augmented by the disease) is the final cause of death (Valentine et al. 2005). Diagnosing the disease in patients is often difficult, since there is no known biological marker unique to the disease. Doctors rely on other means to diagnose and track disease progression. Tests such as MRIs, myelograms, neurological exams, biopsies, and electromyography are commonly used for neurodegenerative diseases such as ALS (Valentine et al. 2005). While the disease is relatively rare, there is a great deal of research being conducted to bring it to an end (Valentine et al. 2005; Boillee et al. 2006).

The SOD1 enzyme was found to be responsible for about 2% of fALS cases through studies conducted with patients afflicted by the disease (Boillee et al. 2006). To date, more than 125 mutations have been documented on the SOD1 gene, though only 114 are related to ALS (Valentine et al. 2005). Of these 114 ALS-related mutations, all but 12 are missense mutations, with the remainder consisting of nonsense and deletion mutations. Many of these mutations reduce or eliminate enzymatic activity, but some

have no effect on catalytic activity at all. This suggests that there may be no relation between SOD1 enzymatic activity and the disease. Those mutations that do affect enzymatic activity could lead to ALS via a toxic gain of function. Studies have been conducted in animals that support this hypothesis. For example, mice lacking SOD1 activity entirely do not suffer from nervous atrophy or death (Valentine et al. 2005), but mice over expressing SOD1 go on to suffer from ALS-like symptoms (Bowling et al. 1993). For all that is known about fALS, the underlying molecular cause of the more common sporadic form of the disease (sALS) has yet to be confirmed. However, there is a strong focus on fALS in the hope that learning more about it could lead to potential treatments for sALS.

In the cases of the loss of physiological function, the explanation is that mutated SOD1 cannot compete effectively with the damaging effects of superoxide radicals, leading to oxidative damage from these harmful molecules (Valentine et al. 2005; Boillee et al. 2006). The gain of toxic function, however, consists of many more complex hypotheses that all provide models for how a mutant SOD1 can lead to ALS. The first hypothesis (**Figure-3,** diagram left side) points to a mutation affecting the active site of the enzyme. In these mutations, the channel leading to the active site is substantially larger than the wild-type enzyme's channel, allowing larger molecules to access the Cu^{++} and amino acids that catalyze the usual reaction (Valentine et al. 2005). By allowing larger molecules to access the active site, the enzyme can catalyze reactions that yield harmful molecules. Reactions such as peroxidation, which creates the dangerous OH^- ion responsible for unbalancing the delicate pH of the cell; tyrosine nitrosylation, which destroys the vital amino acid tyrosine; and reverse catalysis, in which SOD1 actually

creates more superoxide radicals rather than destroying them, are among the potential reactions that can occur from this variety of mutation. Only a select few of the documented SOD1 mutations, however, demonstrate these characteristics, making this hypothesis somewhat controversial.

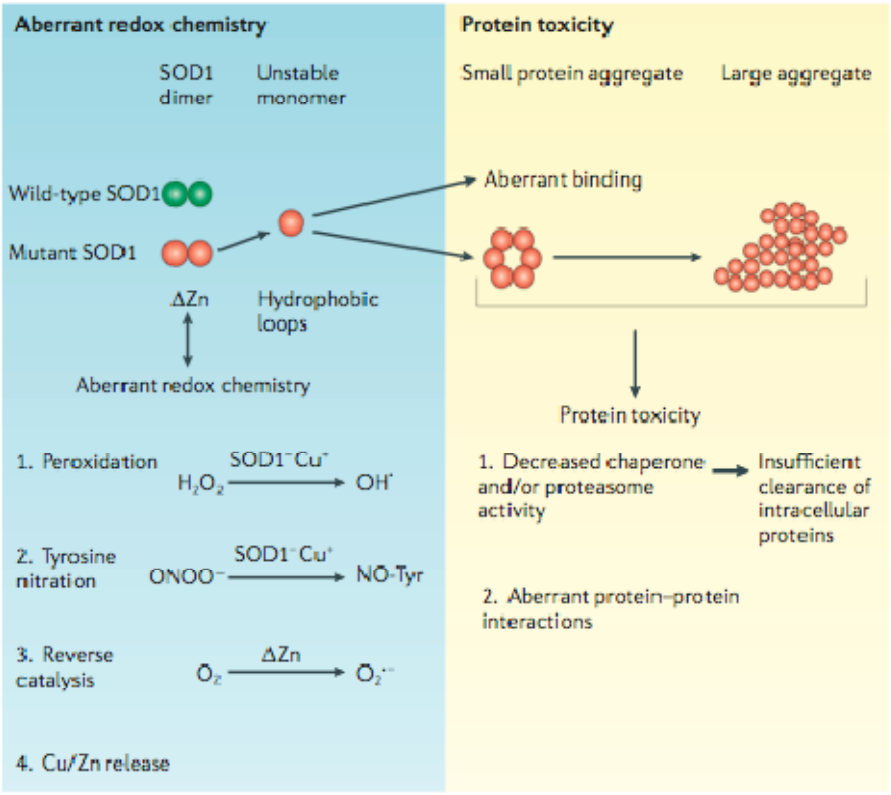

Figure 3: Diagram of the Two Main Hypotheses on How Mutant SOD1 Impairs Cellular Functions. Mutant SOD1 may cause ALS through either aberrant redox chemistry (left panel) or protein toxicity (right panel). In the case of aberrant redox chemistry, the mutant enzyme may generate such harmful products as hydroxide ions and nitrosylated tyrosine, or reverse its own reaction and generate superoxide radicals. In protein toxicity, mutant SOD1 molecules themselves may aggregate, overloading chaperone and proteasome function or creating aberrant interactions between proteins (Pasinelli and Brown, 2006).

The more widely accepted model (**Figure-3,** diagram right side) is that mutations lead to unstable versions of the SOD1 protein that can aggregate, resulting in a buildup of improperly-folded proteins that can interfere with cellular functions and have other adverse effects in unrelated pathways (Boillee et al. 2006). More support for the protein

aggregation model is shown in the link between aggregation and disease progression. Mice genetically engineered to express human SOD1 with mutations known to increase protein aggregation have been shown to come down with more severe cases of ALS. In addition to the interference with proteasome and chaperone activity mentioned earlier, aggregates can encapsulate necessary proteins that typically float freely in the cytoplasm, such as heat-shock protein-70 (hsp-70) (a protein that protects the cell from stress) or Bcl2 (a protein that prevents a cell from undergoing apoptosis), diminishing their activities (Boillee et al. 2006). Additionally, SOD1 aggregates can be very harmful to mitochondria, usually by disrupting cristae structure and the translocator outer membrane complex (Higgins et al. 2003). It is unclear whether these toxic function losses or gains are taking place within or outside of the mitochondrion.

RNAi and its Application in Treating ALS

Extensive research on SOD1 and its role in the onset of ALS has led to potential treatments for the disease. As outlined above, mutant SOD1 may be responsible for ALS; however, mice genetically engineered to not express SOD1 altogether show no developmental abnormalities (though their ability to recover from axonal stress is compromised) (Reaume et al. 1996). To try to treat ALS, many researchers have turned to a new technology in an attempt to treat this debilitating disease. In 2005, Dr. Craig Mello and Dr. Andrew Fire were awarded the Nobel Prize for their discovery of "small interfering RNAs" (siRNAs) (Fire et al. 1998). siRNAs are usually 18-25 nucleotides long RNA duplexes. They are designed to contain a sequence complementary to that of a target mRNA product of a target gene of interest. The siRNA is incorporated into a

protein complex called the RNA induced silencing complex (RISC). Once inside, the antisense strand of the duplex remains in RISC and directs the protein to the target mRNA The antisense strand can then hybridize with the mRNA and transcript and the protein can cleave it (Chu and Rana 2007). A protein called Argonaute is responsible for the catalytic activity of the RISC (Liu et al. 2004). This makes the mRNA susceptible to degradation by exonucleases, thus preventing the mRNA from being translated into protein and silencing the gene.

It is important to note that most companies researching RNAi use small, synthetic RNAs. These siRNAs are often chemically modified to prevent degradation by exonucleases (Chu and Rana 2007). The mechanism for natural RNAi in the cell begins with long, double-stranded RNAs (dsRNAs) expressed from endogenous genes (**Figure-4**). A molecule called Dicer cleaves dsRNA into smaller, double-stranded RNA fragments, usually with a 2nt overhang at the 3' ends (Zamore et al. 2000; Zhang et al. 2004; Du et al. 2008). The process then continues as above. Since RNAi is a potent, specific method of gene silencing, it is being developed by several companies for the treatment of a variety of diseases.

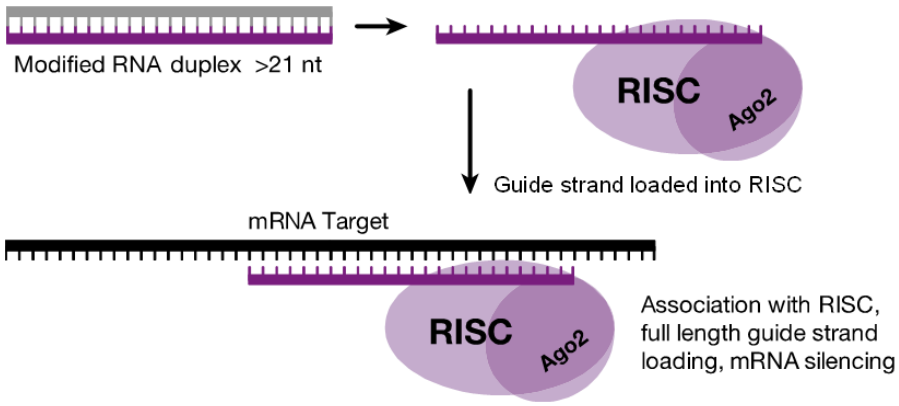

Figure 4: RNAi Silences Genes by Cleaving their mRNA. RNAi begins with a double-stranded RNA duplex (diagram upper left). Typically, companies studying RNAi simply inject modified duplexes that resemble Dicer products that have been modified to protect against exonuclease activity. The strands are unwound and loaded into the RISC, and the passenger (sense) strand is degraded (diagram upper right). The RISC can then bind to mRNAs with complementary sequences to its siRNA and Ago2 can cleave the mRNA (diagram lower), thus silencing a gene.

By delivering siRNA to the motor neurons of patients suffering from the disease, mutant SOD1 expression could be silenced. The hypothesis is that silencing will occur at such a level that no adverse side effects will come from the treatment, since as mentioned previously mice and flies survive when the SOD1 gene is not expressed. Another advantage of this new technology is that an siRNA can be designed to be specific, so off-target effects can be limited by design of the molecules.

ViewRNA: A Powerful New Tool in RNAi Study

As RNAi research advances, so must techniques that can be used in that research. Commonly, RNAi treatments focus on assays that allow the quantification of RNA in a cell, whether it is the amount of target mRNA or the levels of delivered siRNA. However, there are also have many questions regarding RNAi mechanisms, so there is interest in methods that allow for the visualization of different components for co-localization

experiments, and experiments to determine exactly where a therapy is being taken up (such as tissue type). Although many systems exist that allow RNA quantification within a cell, relatively few techniques allow scientists to visualize siRNA effectiveness.

In 2008, Affymetrix, Inc. released a system in which RNA could be visualized in the cell *in situ* through fluorescence microscopy. The system, known as ViewRNA, was an extension to the same company's QuantiGene system. QuantiGene is a branched DNA (bDNA) based assay that amplifies a signal by the hybridization *branching* of the probes, allowing several label enzymes to be attached to a single probe (thus is called bDNA) (Player et al. 2001). A luminometer is then employed to measure the amount of light generated by the reaction of enzymes covalently bound to the oligonucleotide probe. ViewRNA built upon this concept by using the same bDNA technology to allow for the hybridization of a fluorophore instead of an enzyme to the mRNA of interest *in situ*. Although more complicated, the ViewRNA technique had potential to enable a better understanding of other mechanisms of RNA silencing.

Both the ViewRNA and QuantiGene systems employ branched DNA (bDNA) technology to amplify the signal from one mRNA molecule. Unlike in QuantiGene, however, which requires cells be lysed beforehand to release all of the mRNA for sequence-based sorting, ViewRNA allows mRNA to be analyzed *in situ*. The procedure (diagramed in **Figure-5**) begins by culturing adherent cells in a 96-well plate or on a glass slide. The cells are transfected with an siRNA oligonucleotide, which will hopefully silence the mRNA of interest. The next day, the cells are fixed and the probe, which is a small single-stranded oligonucleotide, hybridizes with a complementary sequence on the target mRNA.

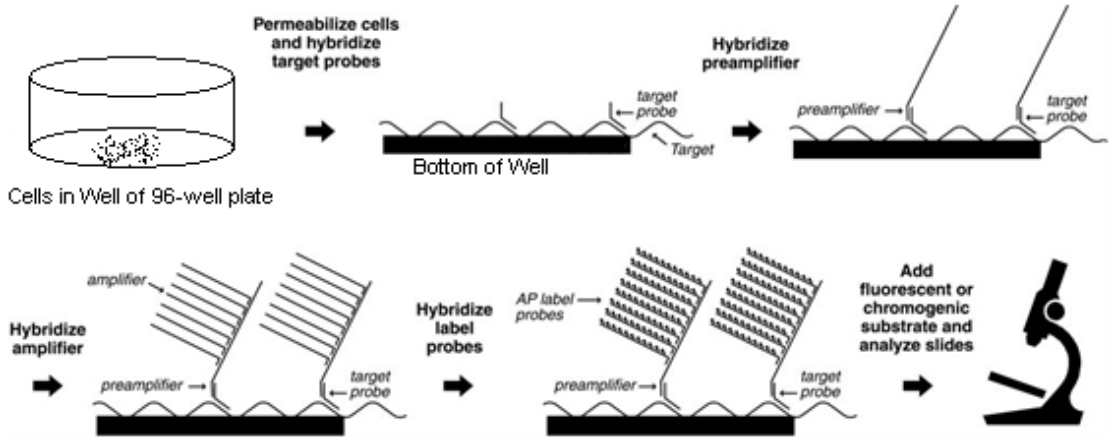

Figure 5: *In situ* bDNA Assays Can Show Individual Copies of RNA. *In situ* bDNA assays begin by fixing cells to a slide (upper left). The cells are then permeabilized and the probe is hybridized to the mRNA target (upper center). Several pre-amplifiers are then hybridized to each probe (upper right), and several amplifiers are hybridized to each pre-amplifiers (lower left). Finally, several fluorophores or label probes are hybridized to each amplifier (lower center). These will emit a fluorescent signal that can be seen under a microscope (lower right), showing individual copies of mRNA (Player et al. 2001).

The pre-amplifier, amplifier, and fluorophore, known as the Label Probe, are then added, amplifying the signal and emitting sufficient fluorescence to be seen under a microscope (Player et al. 2001). Finally, the cells are counterstained with DAPI and examined.

Under designated wavelengths of light, the fluorophores are excited, and emit a different wavelength of light that is visible under a microscope. The fluorophores used emit light in the green (FITC) and red (Texas Red) wavelengths. This is useful because it minimizes crosstalk between fluorophores hybridized to the target mRNA (SOD1, green) and the positive control mRNA (PPIB, red). In this way, each copy of the mRNAs of interest appears as a speck of light under a microscope. Multiple aspects of the RNAi mechanism can be studied in this way, including the effectiveness of the siRNA (through the diminishment in fluorescence) and localization (where the siRNAs and mRNA exist relative to one another in the cell) (Player et al. 2001).

ViewRNA has great potential for helping to learn more about RNAi. The assay has the potential to help scientists learn where siRNAs are being taken up in the body. In addition, it can show qualitatively how effective the siRNA is in a different region of a single organ; for example, how effective it is right next to a blood vessel versus farther away. In addition, it can be used in co-localization experiments with labeled siRNAs to show RNAi in real time providing crucial evidence for the technique. Reasons like this give ViewRNA great promise in the research of RNAi.

PROJECT PURPOSE

The goals of this project were to evaluate Affymetrix's ViewRNA Assay to examine its sensitivity and robustness. This, in turn, will be used to determine whether it can become an effective tool in visualizing siRNA silencing through the observation and localization of mRNA knockdown. Data gathered from this experiment will be used to optimize a protocol for use by RXi Pharmaceuticals in their research of RNAi, as well as play a role in deciding whether the company will invest in the protocol and utilize it in their research. The company hopes to use the assay in the analysis of tissue samples to pinpoint where siRNAs are being taken up *in vivo*. Currently, it is difficult to know where siRNAs are being taken up, and the hope is that ViewRNA will be able to solve this by showing where mRNA levels have been reduced. This could, in turn, lead to the development of an RNAi-based drug capable of treating myriad debilitating human diseases.

METHODS

Cell Culture and Transfection

HeLa cells obtained from the American Type Culture Collection (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were incubated at 37^{o}C with 10% CO_2 as recommended by the ATCC. Two separate 96-well plates were used, one for each assay. For the ViewRNA assay, cells were plated in a black plastic Nunc 96-well plate with transparent glass bottom coated with 0.01% poly-d-lysine at 20,000 cells per well. The plate was set up to perform each experimental condition in triplicate. For the QuantiGene 2.0 assay, cells were plated in a clear plastic Falcon 96-well plate at 10,000 cells per well. All siRNAs were synthesized by Thermo Scientific Dharmacon RNAi Technologies or TriLink Biotechnologies. RNA duplexes were re-constituted in 60 mM KCl, 6 mM HEPES (pH 7.5), and 0.2 mM $MgCl_2$, then stored at -20^{o}C until needed.

Cultured cells were reverse-transfected with 25 nM total RNA duplex containing 5 nM, 0.5 nM, or 0.05 nM siRNA 10015 (Dharmacon) or 0.5 nM siRNA 10132 (Dharmacon), the latter miRNA has a Cy3 fluorophore hybridized to the 5'end of the sense strand. 10015 consisted of a different sequence than 10132, but both bind SOD1 mRNA. The transfection was performed using Lipofectamine (Invitrogen) transfection reagent according to the manufacturer's instructions. Transfection complexes were prepared by adjusting the final concentration of total duplex to 25 nM with a non-targeting control duplex (luciferase). The transfection was performed with media

containing no antibiotics. Cells were then incubated under normal growth conditions for 24 hours.

ViewRNA Assay

Following transfection, cells were rinsed three times with 1X PBS (Gibco) and then fixed in 4% formaldehyde for thirty minutes. Following fixation, the cells were rinsed three more times with PBS, then permeabilized with a 1X detergent solution provided as part of the ViewRNA kit. Another three-rinse cycle with PBS was conducted, and the cells were then treated with a 1:16,000 dilution of Proteinase 24, also provided with the ViewRNA kit. The probe, which was made by Affymetrix, was then hybridized. The provided probe set was diluted 1:50 in Hybridization Buffer A (ViewRNA) and, following another rinse cycle with PBS, added to the plate, which was then incubated for three hours. One set of wells was only treated with the probe set for SOD1, one was only be treated with the probe set for PPIB, and the last was treated with both. Following probe hybridization the plate was rinsed three more times with Wash Buffer (ViewRNA), then stored overnight in Storage Buffer (ViewRNA). The next day, the pre-amplifier, amplifier, and label probes were hybridized. The plate was rinsed three times in Wash Buffer, and the pre-amplifier solution (ViewRNA) was diluted 1:250 in Hybridization Buffer B (ViewRNA), added to the plate, and incubated at 40^{o}C for one hour. Next, the amplifier (ViewRNA) was diluted 1:250 in Hybridization Buffer B, added to the plate following three rinses with Wash Buffer, and incubated at 40^{o}C for one hour. Finally, the Label Probe (ViewRNA) was diluted 1:250 in Hybridization Buffer C (ViewRNA), added to the plate after three rinses with Wash Buffer, and incubated at

40°C for one hour. The nuclei of cells were then counterstained with 4', 6-diamidino-2-phenylindole (DAPI). A 10 mg/ml solution of DAPI (Invitrogen) was diluted 1:5,000 in PBS, and then rinsed once with PBS before the wells were filled with PBS. At this point the plate was viewed under a Leica inverted microscope. Each photo was taken with the same parameters (including exposure time) within each channel (2 msec for phase contrast, 10 msec for DAPI, 600 msec for FITC, and 9 seconds for Texas Red). Photos were taken with a Leica cooled CCD camera.

Once taken and qualitatively analyzed, a quantitative method was attempted with an Area Density tool in LabWorks 4.6. Each photo was converted to an 8-bit image and the area density was calculated. Following this, the area density of each photo was normalized to the number of cells present in the in the DAPI image. The percent expression was determined by dividing the average normalized area density of photos in the same category (for example, all photos of cells transfected with 5 nM siRNA 10015) by the average normalized area density of the untransfected cells.

QuantiGene 2.0 Assay

Following transfection, the cells were lysed with a 1X lysis mixture, which was part of the QuantiGene 2.0 kit, and Protease K (Invitrogen). Lysis was performed for thirty minutes, and the lysate was stored at -80°C until the assay could be run. The probe and blocking reagent were diluted in 1X lysis mixture, and then combined with cell lysate in the capture plate and incubated at 55°C for eighteen hours. Following incubation, the plate was rinsed three times with Wash Buffer (QuantiGene). After the wash, the pre-amplifier was diluted in Amp/Label Probe Diluent (QuantiGene) and added to the plate,

then incubated at 55°C for one hour. Next, excess pre-amplifier was decanted, the plate was rinsed three times in Wash Buffer (QuantiGene), and amplifier, also diluted in Amp/Label Probe Diluent (QuantiGene), was added. The plate was then incubated for another hour at 55°C. Finally, excess amplifier was decanted, the plate was rinsed three more times with Wash Buffer (QuantiGene), and label probe, also diluted in Amp/Label Probe Diluent (QuantiGene), was added. The plate was then incubated for a final hour at 55°C. Once the label probe had been hybridized, excess label probe was decanted, the plate was washed three final times, and the substrate was added. The substrate was allowed to act for five minutes, and then the luminescence was measured on a Wallac Envision luminometer. The results were normalized to cyclophilin B (PPIB) expression, and then the percent expression relative to the untransfected cells was determined.

RESULTS

The SOD1 probe was designed to fluoresce green, and PPIB was designed to fluoresce red. The DAPI stain was included to show the location of nuclei for cell counting and identification, and also to localize mRNA relative to the nucleus. A phase contrast photo of the cell was also taken to ensure that the cells did not display any unusual morphology in response to any treatments. The photos showed a strong signal for SOD1 mRNA in negative control cells, and a diminished SOD1 signal in cells transfected with either targeting siRNA (10015 or 10132) **(Figure-6)**. The SOD1 knock down appeared to be dose-dependent for siRNA 10015; its efficacy was best at higher concentrations of siRNA. siRNA 10132 was as effective as 10015 at the 0.5 nM dose tested, indicating that the attachment of the Cy3 fluorophore did not hinder its binding to the SOD1 target mRNA. SOD1 mRNA exists in roughly the same abundance in the cell as PPIB mRNA, however the signal for PPIB was not as strong (not shown in Figure-6), and although it was stronger than the background, it was not strong enough to be able to display the ideal punctate pattern shown with SOD1. Thus individual mRNA copies were difficult to distinguish for PPIB (Appendix I).

With respect to Cy3 fluorescence, siRNA 10132 had a Cy3 fluorophore covalently attached to the 5' end of the sense strand. The hope was that this fluorophore would allow for co-localization experiments to be conducted between the SOD1 mRNA (green) and the transfected siRNA (red). Unfortunately, no fluorescence was observed from this fluorophore (Appendix II).

Figure-6: *In Situ* bDNA Assays Show siRNA Efficacy. This figure shows that the ViewRNA assay worked successfully and also shows that both targeting siRNAs (10015 and 10132) are effective for knocking down SOD1 mRNA. Efficacy of 10015 is shown to be better when higher concentrations of siRNA are transfected. 5 nM 10015 (top row) shows complete knockdown in all transfected cells. Only one cell in the field of view contains SOD1siRNA, and based on the amount it is unlikely that this cell was transfected. 0.5 nM siRNA (second row) shows that knockdown within the cells is no longer complete, but still very significant. Cells transfected with 0.05 nM 10015 (third row) show still less knockdown but SOD1 mRNA levels are still lower than in untransfected cells (bottom row) or cells transfected with a non-targeting control (fifth row). 0.5 nM 10132 (fourth row) showed comparable knockdown to 0.5 nM 10015. These images were taken at 20X magnification.

ViewRNA showed a semi-quantitative capability **(Figure-7)** when compared to QuantiGene **(Figure-8)**. The amount of SOD1 mRNA in cells transfected with siRNA designed to target SOD1 mRNA was lower than the amount of SOD1 mRNA in cells transfected with a non-targeting control or not transfected at all. In addition, as the concentration of active siRNA increased, SOD1 mRNA levels decreased. siRNA 10132 was approximately as effective as 10015 at the single 0.5 nM dose tested. These data show that the ViewRNA system provides semi-quantitative data whose trends closely match that of the well characterized QuantiGene system.

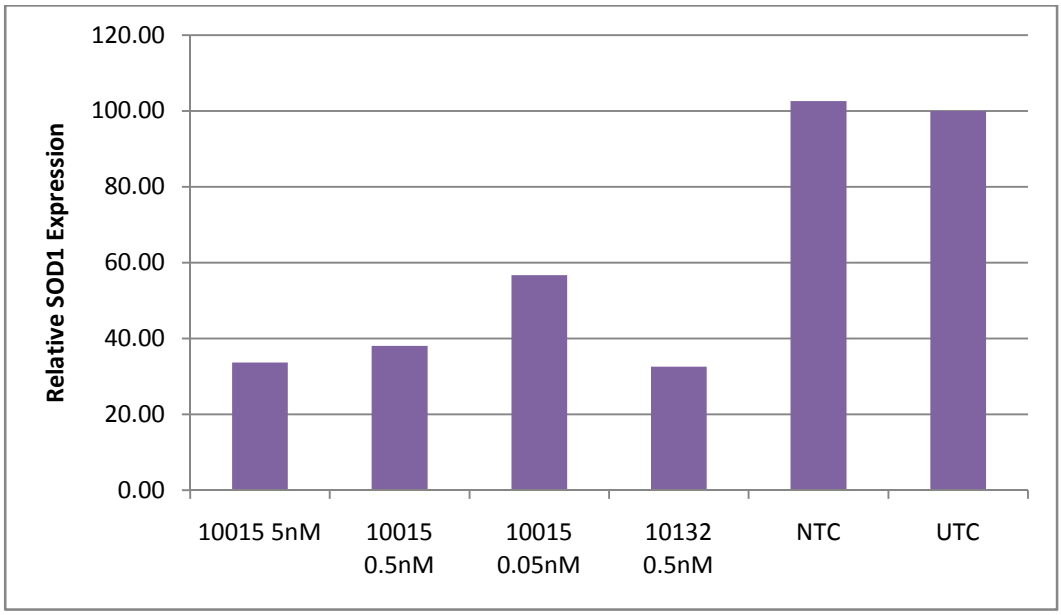

Figure 7: Quantitative Calculation of mRNA Using ViewRNA. This figure shows SOD1 mRNA levels in the cells from Figure-6 using the ViewRNA assay. SOD1 mRNA levels are lowest when the siRNA dose is highest (5 nM) and increase as the dose becomes smaller. The NTC and UTC showed expression levels of 100%. ViewRNA is a more qualitative method, but with special software the amount of fluorescence in a single image can be calculated to estimate the amount of mRNA present.

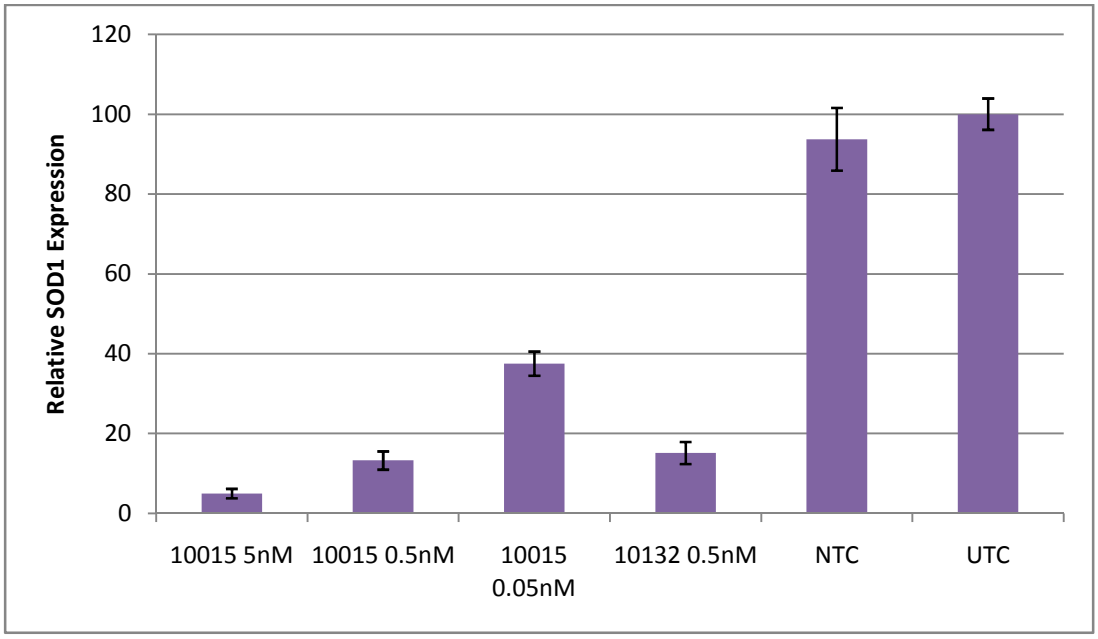

Figure 8: Quantitative Calculation of mRNA Silencing Using QuantiGene 2.0. This figure shows the results from the QuantiGene 2.0 assay. SOD1 mRNA levels are lowest at 5 nM 10015 (5% SOD1 expression) and highest when the cell was transfected with a NTC (94%) or untransfected (100%). As the dose of siRNA decreases, SOD1 mRNA levels rise. As a strictly quantitative assay, QuantiGene 2.0 serves as the benchmark for ViewRNA quantization.

DISCUSSION

The data from this project showed that the ViewRNA system produced results as expected, and could be used for detection of mRNA *in situ*. The system successfully showed a reduction in green fluorescence representing SOD1 mRNA expression from siRNA treatment. siRNA 10015 was shown to successfully reduce SOD1 mRNA levels in both the ViewRNA and QuantiGene assays, and the reduction was dose-dependent. siRNA 10132 showed approximately equal potency at the 0.5 nM dose tested, but the fluorophore to which it had been covalently attached did not show up under the microscope. Additionally, the fluorescence of the PPIB mRNA was very faint (Appendix II). Quantification experiments with the ViewRNA system conducted with the help of software capable of measuring area density showed this technique supports results obtained with the standard QuantiGene results, showing that ViewRNA is a powerful tool not only in the qualitative application but also in semi-quantitative manner. Overall, all project goals were met, the experiment was a success, and a protocol was developed for RXi's use of ViewRNA in their research of RNAi.

A big part of the assay optimization came in choosing the best type of cells to utilize. Initially, the choice of cell line was HEK293 cells, which are a very easy to transfect adherent cell line. However, the cells washed away during the fixation and protease treatment steps. Despite several attempts to optimize formaldehyde and protease concentrations, the HEK293 cells did not end up working. Instead, HeLa cells were used. Although a significant number of cells were still washed away in the aforementioned steps, HeLa cells showed much improvement in remaining adhered. Optimization tests

run at RXi, and recommendations from Affymetrix, allowed optimal conditions to be found at 4% formaldehyde and a 1:16,000 dilution of the Proteinase K solution that was included in the ViewRNA kit. For this reason, it is recommended that HeLa cells be used for studies that will include the ViewRNA assay as part of their research.

As part of the experiment, an siRNA had to be tested. The choice was siRNA 10015, which had been designed to hybridize with SOD1 mRNA. Studies conducted at RXi prior to this project (Salomon et al, unpublished data) showed that 10015 knocked down SOD1 mRNA very effectively, and thus it was chosen to demonstrate the sensitivity of the assay. Not surprisingly, 10015 knocked down SOD1 mRNA to nearly undetectable levels at a 5 nM dose. The dose-response demonstrated that, even at lower concentrations, 10015 was still very effective at knocking down SOD1 mRNA. Unlike in past studies, more about the mechanism behind 10015's effectiveness was learned by running ViewRNA in conjunction with QuantiGene 2.0. According to the data in **Figure-8**, the QuantiGene system shows that SOD1 expression was reduced to about 5% of its original expression level at the 5 nM siRNA dose. The QuantiGene 2.0 assay cannot determine the pattern of SOD1 expression, but by adding ViewRNA the pattern can be seen. The photo for 5 nM 10015 in **Figure-6** shows that only one cell in the image displays a detectable amount of SOD1 mRNA. All of the other cells in the image do not show any detectable fluorescence, showing that 5 nM is sufficient to knock down mRNA to almost undetectable levels in most cells. This data shows that most of the expression detected in QuantiGene 2.0 may actually be due to cells that, for whatever reason, may not be transfected with siRNA (for example, the cell in **Figure 6** appears to be dividing, and is showing full green fluorescence), and this combination of techniques can help

pinpoint ideal doses to find the best balance between effect and cost, as well as better understand the mechanism behind RNAi.

Another test that was run during this experiment was conducted with siRNA 10132. This siRNA also bound SOD1 mRNA, but contained a Cy3 fluorophore covalently attached to the 5' end of the sense strand. The hope was that this fluorophore would allow direct visualization of the siRNA *in situ*, and the localization of the siRNA in relation to the SOD1 mRNA transcripts. Although no fluorescence was observed, a comparable experiment could still be tried in the future. In this experiment, visualization was attempted immediately after the transfection incubation, and again after the ViewRNA protocol was completed. In future experiments this may be able to be performed by attempting visualization of the Cy3 fluorescence following the Day 1 procedure of the ViewRNA assay. At this point, the cells have been fixed and much of the background Cy3-hybridized RNAs removed. This time is also before the plate has been exposed to ambient light during the ViewRNA protocol, especially during Day 2. This may have resulted in bleaching of the Cy3 fluorophore, which is why it did not appear when the images were taken upon completion of the assay.

Another issue that was encountered while running the ViewRNA assay concerned the ability to analyze the positive control, PPIB. This gene, which was supposed to show up as red on the images, was very faint (only slightly higher than background) in all of the wells where it was tested (both wells where it was tested as the only probe set, and wells where it was tested alongside the SOD1 probe set) (Appendix II). The reasoning for this lack of signal for an mRNA whose abundance approximates that of SOD1 was not entirely clear. One possibility for the faint signal could be the filter set that was used

on the microscope. The Affymetrix protocol suggested using a Rhodamine filter set instead of a Texas Red filter set, which was used to obtain the images in Appendix I. Although it is somewhat unlikely since the two filter sets work in the same emission range, the slight difference between the two could account for the faint image, and simply changing the filter set could allow for PPIB to be a strong positive control. Another possible reason for the faint signal could be the probe set itself. PPIB mRNA exists in the cell in about the same copy numbers as SOD1 mRNA, so it should exhibit the same signal intensity. If the probe set is failing to hybridize, however, or is not hybridizing well with the mRNA, the signal could be less than optimal. The simplest correction would just be to run the experiment with a different positive control with a stronger signal, such as 18S rRNA. 18S exists in higher copy numbers than PPIB in the cell and is more commonly used as a positive control. For this reason, it is well-characterized and this allows companies to design better probe sets against them. Thus, the best way to improve the positive control may just be to choose a different gene.

With the initial challenge of getting the assay working complete, there are several future experiments that could be run. It is known that siRNA reduces mRNA levels, but mRNA levels are not always indicative of expression. The final protein product is what will determine whether an enzymatic reaction gets performed by the gene product, and as such, a future experiment could be to run an assay to determine whether SOD1 protein is still present in the cell. This could be done in several ways. The easiest ways would be a Western Blot or an ELISA. Both of these assays would detect SOD1 protein still present in the cells, and the ELISA has the added bonus of being able to quantitate the protein present. Additionally, the phenotype could be checked. Chemicals that generate

superoxide free radicals, like H_2O_2, could be added to a population of cells transfected with siRNA designed to knock down SOD1 mRNA, and the ability of the cell to confront oxidative stress could be observed. If the protein expression levels corroborate the mRNA levels, scientists could not only be more certain that RNAi has the ability to silence genes, but also that the bDNA assay used to analyze the mRNA levels in response to transfections with siRNA is yielding accurate results.

An addition to measuring protein levels, there are several other assays that could be run to further back up the ViewRNA assay results. While QuantiGene is a good measure of ViewRNA's capabilities, it may have many of the same issues as ViewRNA, since both assays employ the bDNA technology. Perhaps a better measure of ViewRNA (and even QuantiGene) results would be made with a comparison to a different assay, such as polymerase chain reaction (PCR). PCR is one of the most commonly used assays in the field. It is known to be a sensitive and accurate way to measure nucleic acid levels, including RNA (in which case RT-PCR or reverse-transcriptase PCR would be used). Another future experiment to back up the data obtained in this report might employ RT-PCR to show that ViewRNA and QuantiGene are both excellent assays for measuring mRNA levels.

In summation, the ViewRNA assay was optimized to be able to work at RXi. By the end of the project, it was working consistently and yielding quality results. The control, PPIB, only yielded faint fluorescence and needs more work before it can be used in the experiment. However, the *in* situ assay allows counting of the cells for normalization purposes, so this control is not necessary. siRNA 10015 was shown to be more potent as its concentration increased, and at a 5 nM dose reduced SOD1 mRNA

expression to undetectable levels in transfected cells. 10132 was also shown to be capable of reducing SOD1 mRNA levels; however, the fluorophore hybridized to its 5' end was not able to be visualized under the microscope. Despite the shortcomings, the primary goal of optimizing a protocol for the ViewRNA assay was met, making the project a success.

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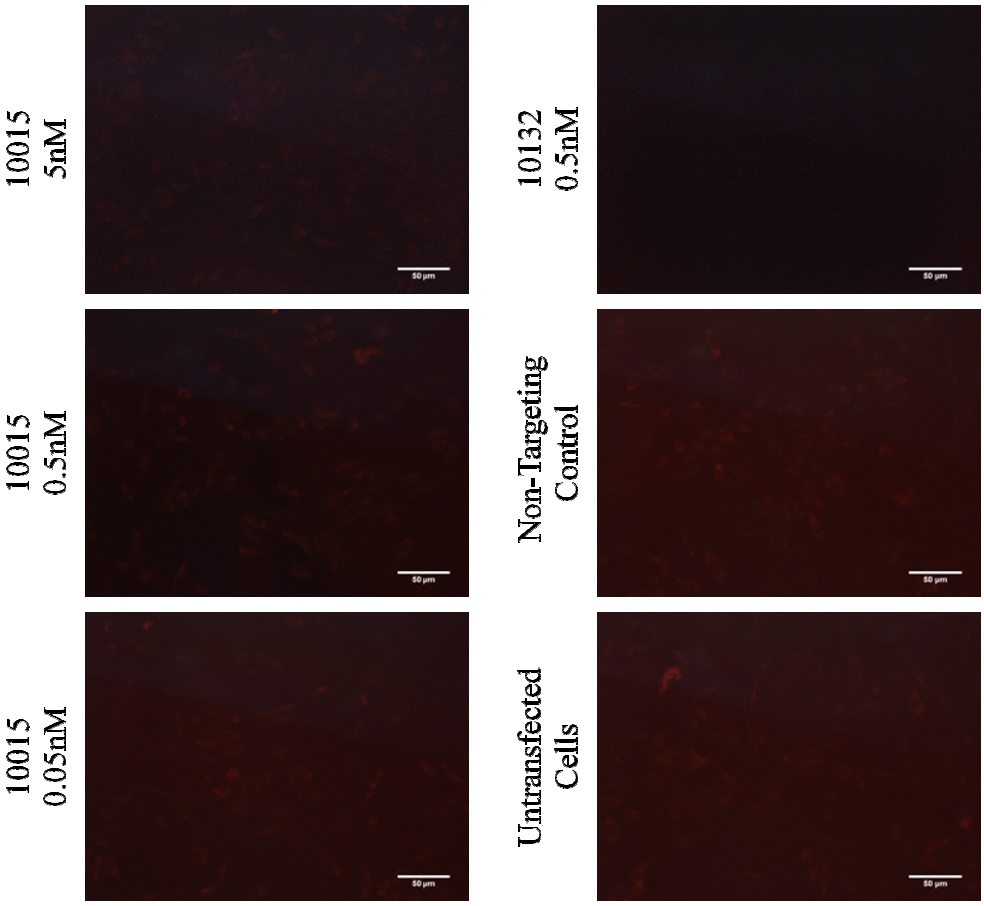
Appendix I: PPIB Fluorescence



This appendix shows the fluorescence noted through the Texas Red filter set. The fluorophore emitting at this wavelength was conjugated to PPIB. Although some fluorescence is noted that is higher than background, the signal is relatively faint compared to the green filter set. Additionally, the expected punctate pattern was not observed.

Appendix II: Cy3 Labeled siRNA Localization

This image shows the red channel of a well that only had the green (SOD1) fluorophore hybridized. This was to allow for the Cy3 label on siRNA 10132 to be seen without interference from the PPIB fluorophore. No fluorescence was noted in any of the wells for this channel, so the Cy3 labeled siRNA was not able to be visualized.

Appendix III: Optimized ViewRNA Protocol

I. Dilution using Filler non-targeting rxRNA

1. Prepare RNAi Compounds:
   a. Active siRNA stocks at 100 uM make the following:
      i. Make 0.3 uM working dilution in RNA Buffer (1:166.666 dilution)
         - Pre-dilution of 1:10 5 ul 100uM Stock + 45 ul RNA Buffer
         - For 100uM: 3ul 10uM Pre-dilition + 97 ul RNA Buffer
   b. RNA filler (11721) stock at 100 uM:
      i. Make 0.3 uM working stock in Opti-MEM (1:33.333 dilution)
         - 6 ul 100uM Stock + 1,994 ul OptiMEM

2. Preparation of Labeled diluted RNAi Compound: Prepare dilutions of RNAi compounds in 0.2ml PCR tubes:
   a. 5 nM Condition (dilute RNA to make 12X or 60nM active in filler RNA):
      i. Add 80 ul of diluted filler RNA to tube
      ii. Add 10 ul of Labeled working stock RNAi compound (0.3 uM) to tube with filler
      iii. Add 10 ul of unlabeled working stock RNAi compound (0.3 uM) to tube with filler
   b. 0.5 nM Condition (dilute RNA compound to 12X or to 6 nM active in filler RNA):
      i. Add 90 ul of diluted filler RNA to tube
      ii. Add 10 ul from 60nM RNAi active compound (5nM) to tube with filler
   c. 0.05 nM Condition (dilute RNA compound to 12X or to 0.6 nM active in filler RNA):
      i. Add 90 ul of diluted filler RNA to each tube
      ii. Add 10 ul from 6 nM RNAi active compound (0.5 nM) to each tube with filler

3. Preparation of unlabeled diluted RNAi Compound: Prepare dilutions of RNAi compounds in 0.2 ml PCR tubes:
   a. 5 nM Condition (dilute RNA to make 12X or 60nM active in filler RNA):
      i. Add 80 ul of diluted filler RNA to tube
      ii. Add 20 ul of unlabeled working stock RNAi compound (0.3uM) to tube with filler
   b. 0.5 nM Condition (dilute RNA compound to 12X or to 6 nM active in filler RNA):
      i. Add 90 ul of diluted filler RNA to tube
      ii. Add 10 ul from 60nM RNAi active compound (5nM) to tube with filler
   c. 0.05 nM Condition (dilute RNA compound to 12X or to 0.6 nM active in filler RNA):
      i. Add 90 ul of diluted filler RNA to each tube
      ii. Add 10 ul from 6 nM RNAi active compound (0.5nM) to each tube with filler

4. Make Bulk amount of diluted RNAiMAX.
   a. 2,205 ul Opti-MEM + 45 ul RNAiMAX
   b. Combine RNAiMAX and Opti-MEM, mix gently.
   c. Allow to sit at room temperature for about 5 minutes.

5. Complexing Plates (0.2ml PCR wells, each well contains enough for duplicates per dose):
   a. Add 65 ul of diluted RNAiMAX to each well.
   b. Add 65 ul of diluted RNAi compound from RNA plate to each well.

6. For Filler controls (25 nM concentration):
   a. Add 100 ul of diluted RNAiMAX to appropriate wells.
   b. Add 100 ul of diluted RNA filler to each well.

7. After adding RNA to each well mix by pipetting up and down 3 times, gently.

8. Allow complexing to take place for at least 15 minutes. Complexes are stable at room temperature.

9. While complexing occurs prepare cells to make a suspension at $1x10^5$ cells/ml. $1x10^4$ cells are needed for each well in 100ul.

10. After 15 minutes add 20 ul of complexed RNAi to each well using a multi-channel pipettor to each well of 96-well tissue culture treated.

11. Add 100 ul of cell suspension to make final concentration of cell per well at $1x10^4$ and final concentration of RNAi compound will be at appropriate dose.

12. Incubate for 24 hrs at 37° C, 10% CO_2.

II. ViewRNA Day 1

Before you begin:

- Prepare 9 mL of 4% formaldehyde by adding 1mL 37% formaldehyde to 8 mL 1x PBS. Vortex to mix.
- Warm Hybridization Buffer A by placing it in the 40°C incubator 30 minutes prior to use.

1. Cell Fixation
   a. Aspirate the cell culture media from the plate and wash the cells two times with 200µL of 1x PBS per well.
   b. Aspirate the final 1x PBS rinse and add 60µL of 4% formaldehyde solution. Incubate for 30 minutes at room temperature.
   c. Aspirate the 4% formaldehyde solution and rinse the plate three times with 200 µL of 1x PBS per well.

2. During the fixation incubation:
   a. Prepare 6 mL of 1x Detergent Solution by adding 4 mL of 1x PBS to 4 mL of 2x Detergent Solution. Invert gently to mix.
   b. Prepare Protease 24 Working Solution by adding 1µL of Protease 24 stock solution to 8 mL of 1x PBS. Vortex to mix.
   c. Prepare12 mL of 1x Protease 24 Stop Buffer by adding 9 mL of stock Protease 24 Stop Buffer to 3 mL of 1x PBS. Vortex to mix.

3. Permeabilization Treatment
   a. Add 60 µL of 1x Detergent Solution to each well and incubate for three minutes at room temperature.
   b. Aspirate the 1x Detergent Solution and rinse the plate two times with 200 µL of 1x PBS per well.

4. Protease Digestion
   a. Remove the 1x PBS from the plate and add 60 µl of Protease 24 Working Solution to each well. Incubate for ten minutes at room temperature.
   b. Aspirate the Protease 24 Working Solution and rinse the plate three times with 200µL of 1x PBS per well.
   c. Add 60µl of 1x Protease 24 Stop Buffer to each well. The plate is stable in Stop Buffer for several hours.

5. Hybridization with Target Probe Set
   a. Prepare 1x Working Hybridization Buffer A by adding 4mL of the 1x Stop Buffer prepared in Step 2 to 4mL warmed 2x Hybridization Buffer A.
   b. Prepare Target Probe Hybridization Solution by adding 80 µl of each target probe (1 and 2) to 8mL of 1x Working Hybridization Buffer A. Vortex to mix.
   c. Add 60 µL of Target Probe Hybridization Solution to each well. Pipette up and down twice to mix.
   d. Incubate plate at 40^{o}C for 3 hours.

6. During the Incubation:
   a. Prepare 200 mL Wash Buffer by adding 600 µL Wash Buffer Component 1 and 1mL Wash Buffer Component 2 to 198.4 mL RNAse-free water.
   b. Prepare 1x Storage Buffer by adding 4mL of stock Storage Buffer to 4 mL of RNase-free water. Vortex to mix.

7. Plate Wash
   a. Aspirate the Target Probe Hybridization Solution.
   b. Gently add 200 µL of Wash Buffer to each well.
   c. Aspirate the wash buffer and repeat two more times with fresh Wash Buffer.

8. Plate Storage
   a. Add 60 µl of 1x Storage Buffer to each well.
   b. Store the plate at 4^{o}C. The plate must be processed within 24 hours.

III. ViewRNA Day 2

Before you begin:

- Warm Pre-hyb Buffer, Hybridization Buffer B, and Hybridization Buffer C by placing each in the 40°C incubator for 30 minutes prior to use.
- Maintain the Pre-hyb Buffer at 40°C during the entire assay process or the precipitate will form.
- Prepare 1x Working Pre-hyb Buffer by adding 6.5 mL stock 2x Pre-hyb Buffer to 6.5 mL RNase-free water. Vortex to mix.
- Prepare 1x Hybridization Buffer B by adding 8 mL stock 2x Hybridization Buffer B to 8 mL 1x Working Pre-hyb Buffer. Vortex to mix.
- Prepare 1x Hybridization Buffer C by adding 4 mL stock 2x Hybridization Buffer C to 4 mL 1x Working Pre-hyb Buffer. Vortex to mix.

1. Hybridization of Pre-Amplifier
   a. Wash plate twice with 200 µl Wash Buffer per well.
   b. Prepare Pre-Amplifier Hybridization Solution by adding 80 µl of each Pre-Amplifier (1 (SOD1) and 2(PPIB)) to 8mL of 1x Hybridization Buffer B. Vortex to mix.
   c. Add 60 µl of Pre-Amplifier Hybridization Solution to each well.
   d. Incubate plate at 40°C for 60 minutes. The plate is stable at room temperature for 30 minutes before and 30 minutes after the incubation.

2. Plate Wash
   a. Aspirate the Pre-Amplifier Hybridization Solution.
   b. Gently add 200 µL of Wash Buffer to each well.
   c. Aspirate the wash buffer and repeat two more times with fresh Wash Buffer.

3. Hybridization of Amplifier
   a. Wash plate twice with 200 µl Wash Buffer per well.
   b. Prepare Amplifier Hybridization Solution by adding 80 µl of each Amplifier (1 and 2) to 8mL of 1x Hybridization Buffer B. Vortex to mix.
   c. Add 60 µl of Amplifier Hybridization Solution to each well.
   d. Incubate plate at 40°C for 60 minutes. The plate is stable at room temperature for 30 minutes before and 30 minutes after the incubation.

4. Plate Wash
   a. Aspirate the Amplifier Hybridization Solution.
   b. Gently add 200 µL of Wash Buffer to each well.
   c. Aspirate the wash buffer and repeat two more times with fresh Wash Buffer.

5. Hybridization of Label Probe
   a. Wash plate twice with 200 µl Wash Buffer per well.
   b. Prepare Label Probe Hybridization Solution by adding 80 µl of each Label Probe (1 and 2) to 8 mL of 1x Hybridization Buffer C. Vortex to mix.

c. Add 60 µl of Amplifier Hybridization Solution to each well.
d. Incubate plate at 40°C for 60 minutes. The plate is stable at room temperature for 30 minutes before and 30 minutes after the incubation.

6. Plate Wash
   a. Aspirate the Label Probe Hybridization Solution.
   b. Gently add 200 µL of Wash Buffer to each well.
   c. Aspirate the wash buffer and repeat two more times with fresh Wash Buffer.
   d. Replace Wash Buffer with 80 µl of PBS. The plate is stable for several hours at this stage.

7. Counterstaining of Nuclei with DAPI Solution
   a. Make DAPI Working Solution by adding 1 µL of 10 mg/mL DAPI stock solution to 10 mL of 1X PBS.
   b. Add 60 µl of DAPI Working Solution to each well and incubate for 1 minute.
   c. Aspirate the DAPI Working Solution and wash with 200 µl of 1x PBS.
   d. Remove 1x PBS and replace with 200 µl of fresh PBS.
   e. The plate is now ready for imaging. It may also be stored at 4°C for several days. The plate should be sealed using adhesive seal when stored at 4°C.

8. Scan Plate Using DAPI, Alexa 488, and Alexa 546 Filters
   a. Magnification: 400-fold image magnification is typically sufficient and can be achieved through the combined use of 10x eyepieces and a 40x oil immersion fluorescence objective with numeric aperture equal to or greater than 0.75.
   b. Filters: To visualize signals, the following multi-bandpass fluorescence microscope filter sets are required:

Nucleic Acid	Fluorophore	Excitation	Emission	Purpose
Target RNA 1	Alexa488	495	519	For detection of 1 copy/cell.
Target RNA 2	Alexa546	546	573	For detection of 1000 copies/cell. Use for mid-expression housekeeping gene such as B-actin.
Nuclear DNA	DAPI	358	461	For focusing and cell identification