Video Article

RNA Catalyst as a Reporter for Screening Drugs against RNA Editing in Trypanosomes

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Abstract

Substantial progress has been made in determining the mechanism of mitochondrial RNA editing in trypanosomes. Similarly, considerable progress has been made in identifying the components of the editosome complex that catalyze RNA editing. However, it is still not clear how those proteins work together. Chemical compounds obtained from a high-throughput screen against the editosome may block or affect one or more steps in the editing cycle. Therefore, the identification of new chemical compounds will generate valuable molecular probes for dissecting the editosome function and assembly. In previous studies, *in vitro* editing assays were carried out using radio-labeled RNA. These assays are time consuming, inefficient and unsuitable for high-throughput purposes. Here, a homogenous fluorescence-based “mix and measure” hammerhead ribozyme *in vitro* reporter assay to monitor RNA editing, is presented. Only as a consequence of RNA editing of the hammerhead ribozyme a fluorescence resonance energy transfer (FRET) oligoribonucleotide substrate undergoes cleavage. This in turn results in separation of the fluorophore from the quencher thereby producing a signal. In contrast, when the editosome function is inhibited, the fluorescence signal will be quenched. This is a highly sensitive and simple assay that should be generally applicable to monitor *in vitro* RNA editing or high throughput screening of chemicals that can inhibit the editosome function.

Video Link

The video component of this article can be found at https://www.jove.com/video/51712/

Introduction

The process of RNA editing, a post-transcriptional mRNA modification, was first discovered in trypanosomatids[1]. Since then, substantial work has been conducted in studying the mechanism behind RNA editing in *Trypanosoma brucei*[2,3]. In a series of enzymatic reactions, the editosome, a core complex of about 20 proteins, creates mature mitochondrial mRNAs for multiple components of the energy generating oxidative phosphorylation system. The order of catalytic events is endonucleolytic cleavage, uridylate (U) addition or deletion, and ligation, as dictated by guide RNAs (gRNAs)[4].

In addition to the core editosome complex proteins, a number of accessory factors have also been identified[5-7]. These proteins are mostly seen grouped in independent complexes. However, the order of protein assembly in the core editosome complex and the interaction patterns of the core complex with the accessory complexes are yet to be determined. Targeting the RNA editing process in trypanosomatids may provide chemical dissectors that aid in studying the assembly and function of the editosome complex. Furthermore, functional studies on several editosome proteins have shown essentiality across different life stages, indicating their potential as drug targets[8-12]. Therefore, the found inhibitors of the editosome may also act as lead compounds against trypanosomatids. This is timely, as drugs currently available against diseases caused by trypanosomatid are toxic, inefficient and expensive[13,14].

An efficient and convenient *in vitro* assay is necessary to explore the chemical universe for specific inhibitors that block RNA editing. Three assays have been developed and used to monitor editosome activities: (a) full round *in vitro* RNA editing assay[15], (b) pre-cleaved *in vitro* RNA editing assay[16,17], and (c) hammerhead ribozyme (HHR)-based assay[18]. The first two assays rely on direct visualization of the edited product (ATPase 6 mRNA) with the help of radioactivity. The HHR-based assay uses a modified version of the ATPase 6 mRNA that is modeled to behave as a ribozyme upon editing. The functional ribozyme then specifically cleaves a radiolabeled RNA substrate, serving as a reporter. Recently, Moshiri *et al.* developed a ‘mix and measure’ HHR-based *in vitro* reporter assay to monitor RNA editing where the radiolabeled RNA substrate is replaced with a fluorescence resonance energy transfer (FRET) substrate[19]. The principle advantages of this assay are: (a) it is a rapid and convenient mix and measure type of assay, as the production of active ribozyme and substrate cleavage occur simultaneously in the same tube in low volume (*i.e.* 20 µl), (b) it avoids the use of radioactively labeled materials, (c) sensitivity that is afforded by fluorescence

instrumentation in a micro-titer plate format, and (d) a high signal to noise ratio. Using this assay, the effect of known RNA editing ligase inhibitors against purified editosome was confirmed[19]. This experiment validated the assay for rapid identification of RNA editing inhibitors, primarily against whole editosomes from *T. brucei*.

Figure 1 is a detailed step-by-step schematic of the fluorescence-based *in vitro* RNA editing assay. This protocol can either be used for monitoring RNA editing *in vitro* or easily be adapted for screening compound libraries of various scales.

Protocol

The protocol below describes the procedure for performing the fluorescence-based RNA editing assay. The assay can be performed in a single PCR tube, 96-well, or 384-well plates depending on the scope of the experiment. Subsequently the fluorescence signal can be read on a suitable real time PCR detection system. The assay here is described in the context of 384-well plates.

1. Culturing *T. brucei* Cells

1. Prepare a growth medium for *T. brucei* procyclic form cells. For 1 L of medium:
    1. Dissolve 25.4 g SDM-79 powder in 800 ml miliQ water.
    2. Add 2 g of $NaHCO_3$ and pH to 7.3 with 10 M NaOH.
    3. Add nanopure water to a final volume of 900 ml, filter sterilize.
    4. Add Fetal Bovine Serum (FBS), penicillin-streptomycin solution and hemin (2.5 mg/ml) to final concentrations of 10% (v/v), 100 U/ml and 7.5 mg/L respectively.
2. Grow 300 ml of *T. brucei* 1.7A wild type (procyclic form) cells at 28 °C, shaking at 70 rpm to a density of 1.5 x 10^7 cells/ml. NOTE: This should produce 3 ml of active editosome with ~0.5 mg of total protein, sufficient for 600 editing reactions.
3. Harvest the cells by centrifugation at 6,000 x g for 10 min at 4 °C.
4. Wash the pellet with 50 ml of chilled PBSG buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 145 mM NaCl, and 6 mM glucose), and spin down the cells again by centrifugation at 10,000 x g for 10 min at 4 °C.

2. Isolation of Crude Mitochondria

NOTE: All the steps should be performed on ice or at 4 °C to preserve editosome activity.

1. Resuspend the harvested cells in 30 ml of DTE buffer (1 mM Tris-HCl pH 8.0 and 1 mM EDTA). Use a 40 ml sterile Dounce homogenizer (pre-chilled) to disrupt the cell membrane by stroking up and down at least 10 times on ice.
2. Immediately add 4.3 ml of 60% sucrose (w/v; *i.e.* 1.75 M) to the homogenate to a final concentration of 0.25 M. Centrifuge at 15,800 x g for 10 min at 4 °C, to preferentially bring down mitochondria.
3. Resuspend the mitochondrial pellet in 4.6 ml of STM buffer (20 mM Tris-HCl pH 8.0, 250 mM sucrose and 2 mM $MgCl_2$). Add 13.8 µl of 0.1 M $CaCl_2$ and 4 µl of RNase-free DNase I to final concentrations of 0.3 mM and 9 U/ml, respectively. Incubate the mixture for 1 hr on ice.
4. Add 4.6 ml of STE buffer (20 mM Tris-HCl pH 8.0, 250 mM sucrose and 2 mM EDTA) to inactivate the DNase I. Centrifuge at 15,800 x g for 10 min at 4 °C.
5. Resuspend the pellet in 400 µl of lysis buffer (10 mM Tris-HCl pH 7.2, 10 mM $MgCl_2$, 100 mM KCl, 1 µg/ml pepstatin, 1 mM DTT, and 1x complete EDTA-free protease inhibitor) and transfer to a microfuge tube.
6. Add 10% Triton X-100 to a final concentration of 1% and incubate the lysate for 15 min at 4 °C on a tube rotator.
7. Clear the mitochondrial lysate by centrifuging twice at 17,000 x g for 15 min at 4 °C; retaining the cleared supernatant each time.

3. Editosome Purification

1. Pour a 10 ml 10%-30% (v/v) linear glycerol gradient (**Table 1**) in an ultracentrifuge tube using 2x HHE gradient buffer (40 mM HEPES pH 7.9, 20 mM $Mg(OAc)_2$, 100 mM KCl, and 2 mM EDTA) and a gradient maker by following the instruction manual.
2. Carefully remove 500 µl of solution from the top of the glycerol gradient and gently load 500 µl of the cleared mitochondrial lysate. Spin at 178,000 x g for 6 hr at 4 °C using an ultracentrifuge.
3. Collect 500 µl fractions sequentially from the top to the bottom of the gradient at 4 °C. Then snap freeze the fractions using liquid nitrogen and store at -80 °C until usage.

4. RNA Preparation

1. Anneal the respective DNA template containing sequence complementary to T7 promoter sequence (**Table 2**) with a T7 promoter oligonucleotide (5'-TAATACGACTCACTATAGGG-3') in a 1:1 molar ratio by heating at 90 °C for 3 min and cooling at RT for at least 10 min.
2. Transcribe RNA using an *in vitro* transcription kit by following the instruction manual.
3. Stop the transcription reaction by adding equal volume of 7 M urea dye (7 M urea, 0.05% Xylene Cynol, and 0.05% Bromophenol blue). Run on a filter sterilized 9% denaturing polyacrylamide gel (9% acrylamide, 7 M urea, 1x TBE) .
4. Use the ultraviolet (UV) shadowing with a shortwave UV lamp to locate and excise respective RNA. Place the excised gel piece in a microfuge tube and add 400 µl of gel elution buffer (20 mM Tris-HCl pH 7.5, 250 mM NaOAc, 1 mM EDTA and 0.25% SDS). Elute overnight at RT on a tube rotator.
5. Precipitate the eluted RNA by adding 1 ml of cold 100% ethanol and incubating either at -80 °C for 30 min or -20 °C overnight.
6. Centrifuge at 16,000 x g for 30 min at 4 °C, to pellet down the RNA.
7. Wash the pellet with 1 ml of 75% ethanol. Centrifuge at 16,000 x g for 20 min at 4 °C.

8. Resuspend the RNA pellet appropriately in RNase free water to achieve the desired concentrations, as shown in **Table 2**.

5. Fluorescence-based RNA Editing Assay

1. For a single reaction, combine 1 pmol (1 µl) of preA6Rbz and 2.5 pmol (1 µl) of gA6Rbz (1:2.5 molar ratio) in a microfuge tube, incubate at 70 ºC for 3 min and let it sit at RT for at least 10 min.
2. Meanwhile prepare a master mix using **Table 3**, without the preA6Rbz and gA6Rbz, for the editing reaction containing 1x HHE buffer (25 mM HEPES pH 7.9, 10 mM $Mg(OAc)_2$, 50 mM KCl and 10 mM EDTA), 1 mM ATP, 5 mM $CaCl_2$, 16 ng/µl of Torula yeast RNA, 0.1% Triton X-100, and the purified editosome.
3. Add annealed preA6Rbz and gA6Rbz to complete the master mix.
4. Dispense 18 µl of the master mix (**Table 3**) into wells containing either 2 µl of RNase-free water (wells with no compounds) or 2 µl of 200 µM chemical compounds and include control samples in the plate according to **Figure 5**.
5. Seal the plate with a plate sealer and spin the plate down, to remove any air bubbles. Incubate at 28 °C for 4 hr.
6. Add 25 pmol (2 µl) of gA6Rbz competitor to each well. Place a fresh sealer, spin the plate down and place it on a real-time PCR machine. Program the following experiment:
   Step 1: 85 °C for 5 min; Step 2: 24 °C for 10 min; Step 3: Stop.
7. Add 15 pmol (1 µl) of FRET substrate to each well to a final volume of 23 µl. Seal the plate with a fresh sealer. Quickly spin the plate and place it back on the real-time PCR machine.
8. Program a new experiment with the following steps:
   Step 1: 37 °C for 1 min; Step 2: Read; Step 3: Go to Step 1, 40 times; Step 4: Stop.
9. Setup the plate by selecting all the wells that require reading and choose the FAM filter. Input volume as 23 µl and start the run.
10. Calculate the slope of the values obtained from each well/sample to obtain a kinetic measurement by plotting the slopes on a bar graph for analysis. NOTE: A kinetic read improves the signal-to-noise ratio between the sample and the background; as the background sample would have a slope close to zero. An end point reading has higher background noise.

Representative Results

To demonstrate the necessary steps required for setting up a large-scale screen, **Figures 2-5** are representative control experiments related to the quality of the assay. These are essential control experiments for a consistent assay over several days of screening or for comparison of different screens.

Assessing the Fluorescence Signal-to-noise Ratio

To ensure the stability and quality of the fluorescein-labeled oligoribonucleotide substrate in a large-scale setup, Z'-factor, defined as the difference between the assay background and the maximum signal was calculated using the active ribozyme molecule (A6Rbz). Assays with Z'-factor >0.5 are considered acceptable for high-throughput screen. **Figure 2** shows representative data using the FRET substrate labeled with 5' fluorescein (FAM; emitter) and 3' *N,N'*-tetramethylrhodamine (TAMRA; quencher) (A6Rbz_F/T). This substrate produced a Z'-factor of 0.64 when calculated using 72 replicates in the presence and absence of A6Rbz.

In this assay an alternative substrate using Iowa Black dark quencher (A6Rbz_F/Ib) can yield a better signal-to-noise ratio compared to TAMRA. This is because Iowa Black, unlike TAMRA, emits absorbed energy as heat and not light. The Z'-factor obtained using the FAM/Iowa Black (F/Ib)-labeled substrate was 0.68. The improvement in the Z'-factor for the F/Ib-labeled substrate over TAMRA-labeled substrate is because of its relatively lower background. These representative results show that both substrates are viable options for use in a high-throughput screen.

Determining the Editing Activities of Glycerol Gradient Fractions

To determine and select the most active editosome fractions for experiments, the glycerol gradient fractions were tested for *in vitro* editing using the fluorescence-based assay (Step V). These data show (**Figure 3**) fractions 7-12 as the most active fractions (≥50% editing activity; with the most active fraction as 100%). These fractions can be combined when more editosome is required.

Calculating the Z'-factor when the Editosome Fractions were Combined

To determine the effect of combinig the editosome fraction, the Z'-factor value of 0.6 was calculated when the most active fractions (F8+F9+F10) were used as the source of editosome. To calculate the Z'-factor 72 replicates were tested in the presence and absence of the editosome (**Figure 4**). The F/Ib substrate was used in this experiment. These data show that based on the Z'-factor, combining the glycerol gradient fractions have minimal effect on the quality of the assay.

Representative Control Experiment for the Assay

Representative data comparing different control samples were used to analyze the variables in the assay. As shown in **Figure 5**, reactions missing any RNA editing components (reactions 1-4) do not cleave the substrate. Cleavage of the substrate as measured by the fluorescence and relative editing activity is only observed in the presence of all components of the editing reactions in the absence of an inhibitor (reaction 5) or in the presence of all editing components and an inactive compound (reaction 6). In contrast, an inhibitory compound can block RNA editing and is used as a positive control (reaction 7). Here, Mordant black (MrB) and C53 compounds were used as the positive and negative controls, respectively.

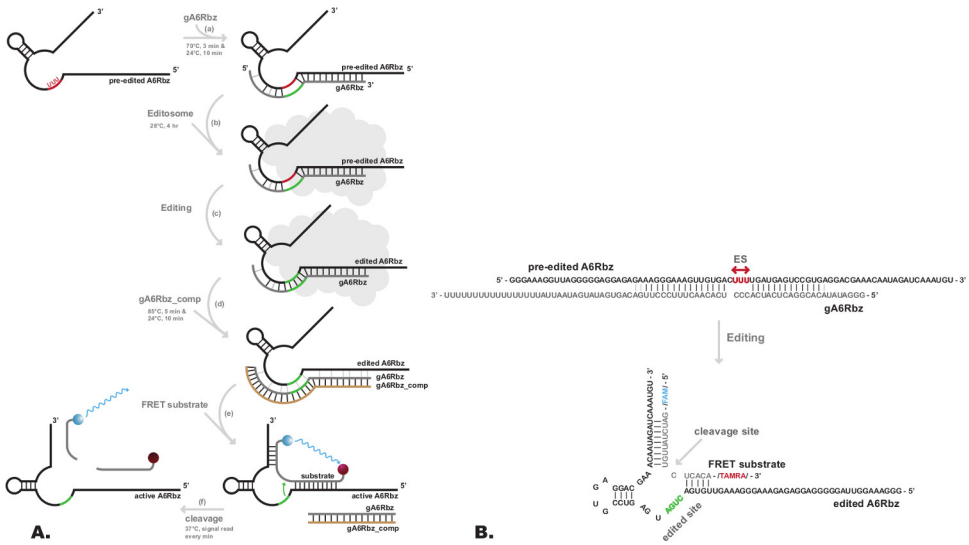


Figure 1. Hammerhead ribozyme-based *in vitro* RNA editing assay. A) Step-by-step schematic representation of the fluorescence-based *in vitro* editing assay: (a) Hybridization of the pre-edited hammerhead ribozyme (pre-edited A6Rbz) with its guide RNA (gA6Rbz). (b) Recognition and interaction of the RNA duplex by the purified editosome complex. (c) Deletion RNA editing catalyzed by the editosome. (d) Dissociation of the edited A6Rbz from the editosome and gA6Rbz by heating at 85 °C and addition of guide RNA competitor (gA6Rbz_comp). (e) Hybridization of the FRET substrate with the active A6 hammerhead ribozyme (active A6Rbz). (f) Detection of FAM signals following cleavage of the FRET substrate by the active ribozyme. **B)** The pre-edited hammerhead ribozyme (pre-edited A6Rbz) is shown in association with gA6Rbz that specifies the deletion of three Us (double-headed arrow) from the editing site (ES). As a result of RNA editing in the presence of functional editosome the inactive ribozyme is edited into its active form (edited A6Rbz) that can now cleave the FRET substrate (cleavage site indicated by an arrow). The conserved 5′-CUGA-3′ of the edited A6Rbz in the catalytic core essential for ribozyme activity (edited site) is highlighted (This figure has been modified from Moshiri[19]). Please click here to view a larger version of this figure.

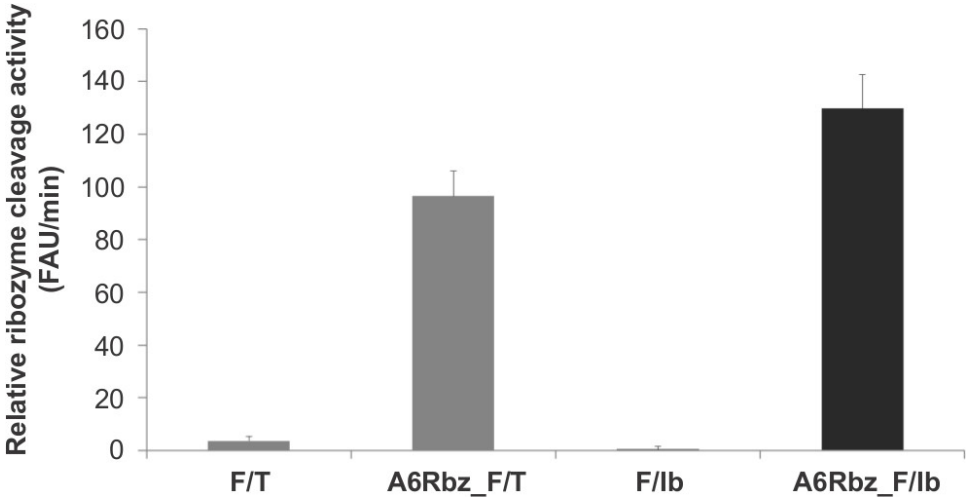


Figure 2. Signal-to-noise ratio comparison between FRET substrates harboring different quenchers. Ribozyme (A6Rbz) activity using FAM/TAMRA (F/T) and FAM/Iowa Black FQ (F/Ib) substrates. The y-axis represents fluorescence arbitrary unit (FAU) per minute.

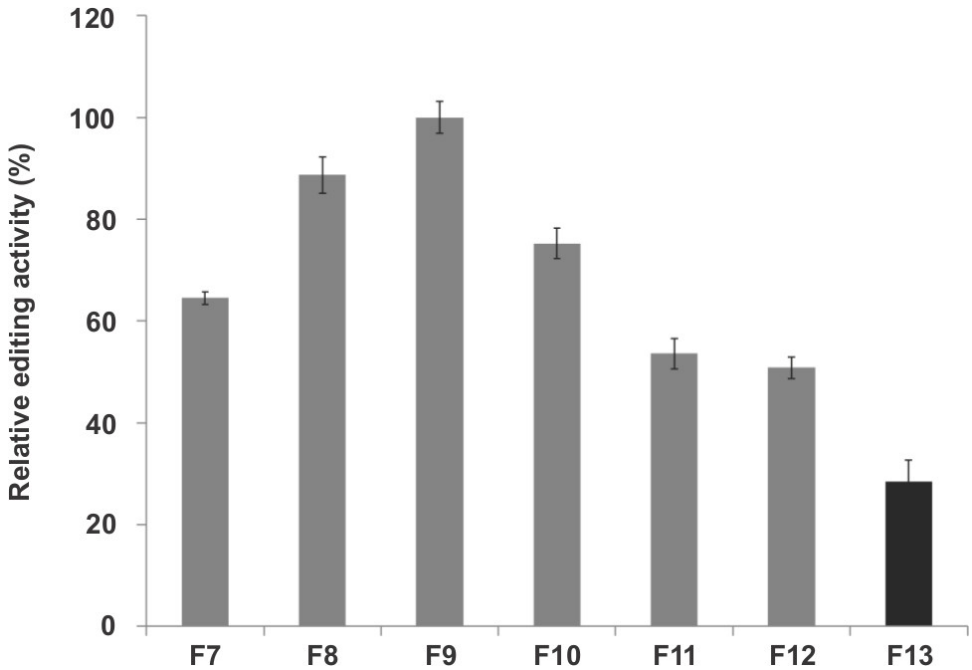


Figure 3. RNA editing activity of select fractions obtained from glycerol gradient centrifugation of the mitochondrial lysate. Fraction #9 (F9) has the highest activity. The y-axis represents relative editing activity in percentage, considering F9 as 100%.

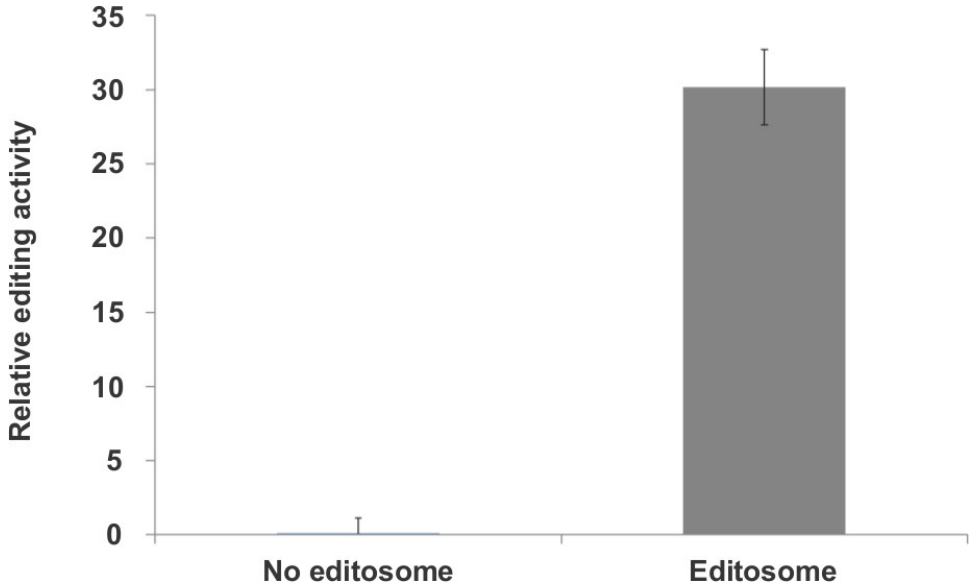
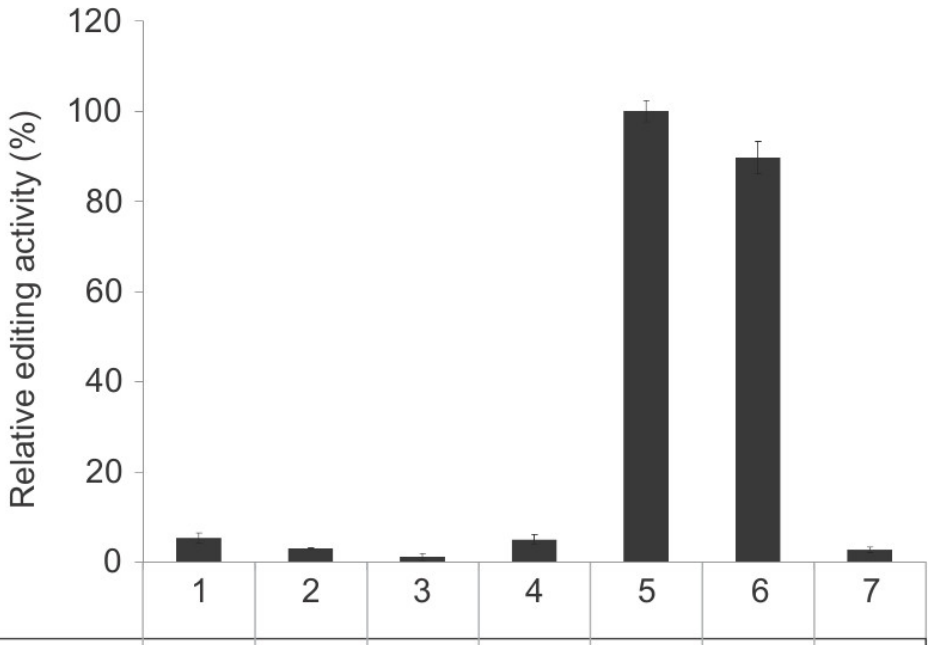


Figure 4. Z'-factor calculation using the most active fractions (F8+F9+F10) as the editosome source. Experimental variation was obtained from 72 replicates of each type of reaction. Z'-factor was calculated as 0.6. The y-axis represents relative editing activity.



	1	2	3	4	5	6	7
FRET substrate	+	+	+	+	+	+	+
preA6Rbz	-	+	-	+	+	+	+
gA6Rbz	-	+	-	-	+	+	+
gA6Rbz_comp	-	+	-	-	+	+	+
Editosome	-	-	+	+	+	+	+
Drug	-	-	-	-	-	C53	MrB

Figure 5. Representative experiment for the fluorescence-based RNA editing assay. Reactions were performed in a final volume of 20 µl per well and CFX384 TouchTM real-time PCR detection system was used for measuring the fluorescence signal. The graph presents various controls in addition to a complete editing reaction that contains all components for the assay (#5). The fluorescence was measured in the presence of FRET substrate alone (#1) to assess the integrity of the substrate. Sample #2 was performed in the absence of the editosome to monitor any ribozyme activity prior to editing. To assess the effect of denatured editosome on the substrate, the fluorescence was measured in the presence of the editosome and FRET substrate only (#3). To test the guide-directed editing specificity, a sample in the absence of gA6Rbz was used (#4). A non-inhibitory (C53, #6) and an inhibitory compound (MrB, #7) were used to test the effect on the editing assay. While RNA editing is inhibited significantly by MrB, C53 has negligible effect. The error bars correspond to an experimental variation (standard deviation) between 10 replicates. The y-axis represents relative editing activity in percentage, considering the complete reaction (#5) as 100%.

	10%	**30%**
2x HHE gradient buffer	5 ml	5 ml
Glycerol	1 ml	3 ml
DEPC H_2O	4 ml	2 ml
1 M DTT	10 µl	10 µl

Table 1. 10% & 30% Glycerol Solution (10ml each).

Pre-edited A6 Ribozyme (preA6Rbz)	
preA6Rbz DNA template	5'-ACATTTGATCTATTGTTTCGTCCTCACGGACTCA TCAAAAGTCACAACTTTCCCTTTCTCTCCTCCCCCTAACCTTT CCCCCTATAGTGAGTCGTATTA-3'
preA6Rbz RNA	5'-GGGAAAGGUUAGGGGGAGGAGAGAAAGGGAAA GUUGUGACUUUUGAUGAGUCCGUGAGGACGAAACAAUAGA UCAAAUGU-3'
RNA stock conc.	1 µM
Guide A6 Ribozyme (gA6Rbz)	

gA6RBz DNA template	5′-AAAAAAAAAAAAAAAAATAATTATCATATCACTGT CAAGGGAAAGTTGTGAGGGTGATGAGTCCGTGTATATCCCC CTATAGTGAGTCGTATTA-3′
gA6Rbz RNA	5′-GGGAUAUACACGGACUCAUCACCCUCACAACUU UCCCUUGACAGUGAUAUGAUAAUUAUUUUUUUUUUUUUUUUU-3’
RNA stock conc.	2.5 µM
Guide A6 Ribozyme Competitor (gA6RBz_comp)	
gA6Rbz_comp DNA template	5′-GGATATACACGGACTCATCACCCTCACAACTTTC CCTTGACAGTGATATGATAATTATTTTTTTTTTTTTTTTCCCTAT AGTGAGTCGTATTA-3′
gA6Rbz_comp RNA	5’-AAAAAAAAAAAAAAAAAAUAAUUAUCAUAUCACUG UCAAGGGAAGUUGUGAGGGUGAUGAGUCCGUGUAUAUCC-3’
RNA stock conc.	12.5 µM
Active A6 Ribozyme (A6RBz)	
A6Rbz DNA template	5′-ACATTTGATCTATTGTTTCGTCCTCACGGACTCAT CAGTCACAACTTTCCCTTTCTCTCCTCCCCCTAACCTTTCCCC CTATAGTGAGTCGTATTA-3′
A6Rbz RNA	5’-GGGAAAGGUUAGGGGGAGGAGAGAAAGGGAAA GUUGUGACUGAUGAGUCCGUGAGGACGAAACAAUAGAUCA AAUGU-3’
RNA stock conc.	1 µM
FRET substrate	
TAMRA quencher	5’-FAM-GAUCUAUUGUCUCACA-TAMRA-3' (Eurogentec)
Iowa Black quencher	5’-FAM- GAUCUAUUGUCUCACA-Iowa Black-3' (IDT)
RNA stock conc.	15 µM (for both)

Table 2. DNA Templates and RNA Substrates.

Composition	**Editing reaction (µl)**
10x HHE	1.5
0.1 M $CaCl_2$	1
100 mM ATP	0.2
10% Tritonx-100	0.2
500 ng/µl Torula Yeast RNA	1
Editosome	5
RNase free H_2O	7.1
1 µM preA6Rbz	1
2.5 µM gA6Rbz	1
Total	**18**

Table 3. Reaction Composition and Master Mix.

Discussion

A novel high-throughput screening method to identify inhibitors against the RNA editing complex of Trypanosomes was presented, providing a new tool for drug discovery to counter diseases caused by trypanosomatids. FRET-based ribozyme assay has been extensively used for different purposes[20-22]; however, we have utilized the capacity of FRET-based ribozyme assay for *in vitro* monitoring of RNA editing activity[19]. This assay could potentially be adapted to other types of RNA editing in eukaryotes, such as nucleotide substitution editing of nucleus-encoded RNAs of mammals[23].

The novelty of this assay is not in establishing FRET-based ribozyme assays per se, but in developing a method that incorporates this technique into an RNA editing assay that is amenable to high-throughput screening of chemicals against editosome. The ribozyme assay-based method has several advantages over other screens and assays currently utilized for this purpose. Specifically, the technique offers a sensitive and reproducible “mix-and-measure” assay relying on a fluorescent substrate as opposed to a radiolabeled one, thereby making it fit for high-throughput screening[19]. Real-time monitoring of a fluorescence signal after addition of the substrate instead of a simple end-point signal makes it possible to more accurately determine IC_{50} values of assayed compounds[19]. Furthermore, it permits testing of the inhibitory effects of compounds on the whole-editosome complex as opposed to individual recombinant proteins. Given the dynamic nature of interactions within

protein complexes, it can be suggested that this method allows identification of inhibitors against editosome proteins in a more biologically representative setting[24]. In addition, targeting the whole-editosome complex provides an opportunity to potentially arrest the progression of RNA editing at various transient steps that have not been previously identified. Thus, the technique will allow gaining a better perspective as to interactions and activities that are crucial to the process of RNA editing. This approach has been found to be successful in the past as exemplified by the elucidation of prokaryotic ribosome complex assembly and function utilizing antibiotics, such as viomycin and erythromycin, acting as inhibitors of the complex[24,25].

To ensure the successful completion of the method, certain adjustments in protocols may be necessary. First, selection of the proper glycerol gradient mitochondrial extract fraction is crucial. Here, fractions 7 through 11, corresponding to the ~20S region of the gradient, showed the highest editing activity, with fraction 9 exhibiting maximum activity. Although this fraction was selected for all of the tests presented, it is essential to test all fractions beforehand in order to assess in which fraction the editing activity peaks. Second, to validate fraction selection, it is critical to perform preliminary tests to assess the signal-to-noise ratio by calculating the Z'-factor value. If proper mitochondrial extract glycerol gradient fractionation has been completed, a Z'-value of 0.6 can be achieved. Next, it is recommended to re-evaluate the reaction volume of mitochondrial extract necessary to achieve maximal editing activity via titration[19].

An important limitation of the technique presented in this paper concerns the laborious and time-consuming process of mitochondrial extract glycerol gradient fractionation by which the editosome complex is purified[19]. Despite these disadvantages, this technique is preferentially suggested to obtain crude editosome fractions given its low cost versus yield potential, thereby qualifying it for application to high-throughput screening. In contrast, other methods such as TAP-tag purification, which are able to give more purified editosome fractions, are advisable for low to medium throughput assays such as in the case of secondary assays. Moreover, in order to ensure specific inhibitory effect of compounds, it is necessary to include controls to test them against the activity of ribozyme (A6Rbz) in the absence of the editosome. It should be noted that previous studies have highlighted that this method may be ineffective at calculating IC50 values for dye-like compounds due to the possible interference at high compound concentrations[19].

To further increase the sensitivity of the HTS technique presented, developing a similar fluorescence-based assay involving pre-cleaved pre-edited ribozyme is beneficial. This would allow bypassing the rate-limiting endonucleolytic cleavage step catalyzed by endonucleases in the editosome complex. Another advantage of this modified assay would be the application as a secondary screening tool to monitor the effect of the inhibitors identified through the primary screen on ExoUase, TUTase and ligation catalytic activities. The currently proposed assay is limited to the detection of inhibitors against the deletion type of RNA editing. Therefore, another avenue to explore would be the modification of the presented assay to enable monitoring of compound effects on the insertion type of RNA editing.

Disclosures

The authors have nothing to disclose.

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