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TITLE:

High-throughput antiviral assays to screen for inhibitors of Zika virus replication

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SUMMARY:

In this work, we describe the protocols used in replicon-based and viral enzyme-based assays to screen for inhibitors of Zika virus replication in a high-throughput screening format.

ABSTRACT:

Antiviral drug discovery requires the development of reliable biochemical and cellular assays that can be performed in high-throughput screening (HTS) formats. The flavivirus non-structural (NS) proteins are thought to co-translationally assemble on the endoplasmic reticulum (ER) membranes, forming the replication complex (RC). The NS3 and NS5 are the most studied enzymes of the RC and constitute the main targets for drug development due to their crucial roles in viral genome replication. NS3 protease domain, which requires NS2B as its cofactor, is responsible for the cleavage of the immature viral polyprotein into the mature NS proteins, whereas NS5 RdRp domain is responsible for the RNA replication. Herein, we describe in detail the protocols used in replicon-based screenings and enzymatic assays to test large compound libraries for inhibitors of the Zika virus (ZIKV) replication. Replicons are self-replicating sub-genomic systems expressed in mammalian cells, in which the viral structural genes are replaced by a reporter gene. The inhibitory effects of compounds on viral RNA replication can be easily evaluated by measuring the reduction in the reporter protein activity. The replicon-based screenings were performed using a BHK-21 ZIKV replicon cell line expressing *Renilla* luciferase as a reporter gene. To characterize the specific targets of identified compounds, we established *in-vitro* fluorescence-based assays for recombinantly expressed NS3 protease and NS5 RdRp. The proteolytic activity of the viral protease was measured by using the fluorogenic peptide substrate Bz-nKRR-AMC, while the NS5 RdRp elongation activity was directly detected by the increase of the fluorescent signal of SYBR Green I during RNA elongation, using the synthetic biotinylated

self-priming template 3'UTR-U30 (5'-biotin-U30-ACUGGAGAUCGAUCUCCAGU-3').

INTRODUCTION:

The Zika virus (ZIKV) is an emerging arthropod-borne virus member of the genus *Flavivirus*, which includes the closely related Dengue virus (DENV), Japanese encephalitis virus (JEV) and Yellow Fever virus (YFV), that pose constant threats to public health [1]. The 2015-16 ZIKV outbreak in the Americas received global attention following its emergence in Brazil due to the association with severe neurological disorders, such as congenital ZIKV-associated microcephaly in newborns [2, 3] and Guillain-Barré syndrome in adults [4]. Although the number of infection cases declined throughout the next two years, autochthonous mosquito-borne transmissions of ZIKV were verified in 87 countries and territories in 2019, therefore, evidencing the potential of the virus to re-emerge as an epidemic [5]. To date, there are no approved vaccines or effective drugs against ZIKV infection.

Antiviral drug discovery requires the development of reliable cellular and biochemical assays that can be performed in high-throughput screening (HTS) formats. Replicon-based screenings and viral enzyme-based assays are two valuable strategies to test small-molecule compounds for inhibitors of ZIKV [1]. The flavivirus non-structural (NS) proteins are thought to co-translationally assemble on the endoplasmic reticulum (ER) membranes, forming the replication complex (RC) [6]. The NS3 and NS5 are the most studied enzymes of the RC and constitute the main targets for drug development due to their crucial roles in viral genome replication. NS3 protease domain, which requires NS2B as its cofactor, is responsible for the cleavage of the immature viral polyprotein into the mature NS proteins, whereas NS5 RdRp domain is responsible for the RNA replication [6].

Replicons are self-replicating subgenomic systems expressed in mammalian cells, in which the viral structural genes are replaced by a reporter gene. The inhibitory effects of compounds on viral RNA replication can be easily evaluated by measuring the reduction in the reporter protein activity [7]. Herein, we describe the protocols used for screening inhibitors of the ZIKV replication in a 96-well plate format. The replicon-based assays were performed using a BHK-21 ZIKV *Rluc* replicon cell line that we have recently developed [8]. To characterize the specific targets of identified compounds, we established *in vitro* fluorescence-based assays for recombinantly expressed NS3 protease using the fluorogenic peptide substrate, Bz-nKRR-AMC, whereas for NS5 RdRp we measured the elongation of the synthetic biotinylated self-priming template 3'UTR-U30 (5'-biotin-U30-ACUGGAGAUCGAUCUCCAGU-3'), using the intercalating dye SYBR Green I.

The ZIKV protease (45-96 residues of NS2B cofactor linked to residues 1-177 of NS3 protease domain by a glycine rich linker [G_4SG_4]) was obtained, as described for YFV [9], while the polymerase (276-898 residues of RdRp domain) was cloned and expressed, as detailed in [10]. Both enzyme sequences were derived from GenBank ALU33341.1. As primary antiviral screenings, compounds are tested at 10 µM and those showing activities ≥ 80% are then evaluated in a dose-dependent manner, resulting in the effective/inhibition (EC_{50} or IC_{50}) and the cytotoxic (CC_{50}) concentrations. In the context of representative results, the EC_{50} and CC_{50} values of NITD008, a known flaviviruses inhibitor [11], from replicon-based screenings are shown. For the enzymatic

assays, the IC_{50} values of two compounds from the MMV/DNDi Pandemic Response Box, a library composed of 400 molecules with antibacterial, antifungal and antiviral activities, are shown. The protocols described in this work could be modified to screen for inhibitors of other related flaviviruses.

PROTOCOL:

1. Luciferase activity assay

NOTE: Ensure that all procedures involving cell culture are conducted in certified biosafety hoods (see **Table of Materials**).

1.1. Prepare growth media consisting in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 500 µg/mL G418.

1.2. Prepare a 10 mM stock solution of tested compounds in 100% DMSO, and then dilute them to 1 mM in 100% DMSO.

1.3. Culture ZIKV *Rluc* replicon cells in growth media in a 75 cm^2 culture flask at 37 °C in a CO_2-humidified incubator (see **Table of Materials**) until they reach 70-90% confluency.

1.4. Discard the medium. Add 5 mL of trypsin-EDTA to the flask for 5 to 10 min and then centrifuge the cells at 125 x *g* for 5 min.

1.5. Discard the supernatant, resuspend the cells in 5 mL of DMEM 10% FBS and count 10 µL of resuspended cells at a hemocytometer.

1.6. Adjust the cells to 2 x 10^4 cells/well in DMEM 10% FBS and seed 100 µL of cells per well in a 96-well cell culture plate (see **Table of Materials**).

1.7. Incubate the plate for 16 h at 37 °C in a CO_2-humidified incubator (see **Table of Materials**).

1.8. Next, discard the medium with a multichannel micropipette and add 100 µL/well of DMEM 2% FBS to the plate.

1.9. Add 1 µL of the compounds per well to result in a final concentration of 10 µM 1% DMSO in assay medium. In the first column, add only 1% DMSO as a no inhibition control and NITD008 in the last column, as a positive control (100% inhibition).

1.10. Incubate the plate for 48 h at 37 ºC in a CO_2-humidified incubator (see **Table of Materials**).

1.11. Thaw the *Renilla* luciferase Assay System kit at room temperature, prepare a 1x *Renilla* luciferase Lysis Buffer working solution and an appropriate volume of *Renilla* Luciferase reagent (Assay buffer + substrate; 100 µL per well), according to the manufacturer's instructions.

1.12. Discard the supernatant from the cells with a multichannel micropipette and add 25 µL of 1x *Renilla* luciferase Lysis Buffer per well.

1.13. Incubate the plate at room temperature for 15 min and then transfer 20 µL of cell lysates with a multichannel micropipette to a white opaque 96-well plate (see **Table of Materials**) containing 100 µL/well of *Renilla* luciferase Assay Reagent.

1.14. Read the luminescent signals in a luminometer or in any equipment that has the option to read luminescence (see **Table of Materials**).

1.15. For each plate, calculate the Z-factor value [12], as follows: Z' = 1 – ((3SD of sample + 3SD of control)/|Mean of sample – Mean of control|); SD – standard deviation. A Z-factor between 0.5 and 1.0 means a good quality assay [12].

1.16. To determine the EC_{50} values of compounds, proceed as described in steps 1.3 to 1.8 and then add the compounds serially diluted to the cells , together with the negative (1% DMSO) and positive (NITD008 at 10 µM) controls. Perform the assay twice in duplicates.

1.17. Plot the average values of inhibition rates per compound concentration and use a graph analysis software to perform a sigmoidal fitting and obtain the EC_{50} values.

2. Cell proliferation-based MTT assay

2.1. Proceed as described in item 1 steps 1.1 to 1.8.

2.2. Add the compounds initially at 10 µM and the control 1% DMSO to the cells.

2.3. Prepare a 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution in phosphate buffered saline (PBS - 137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4, 1,8 mM KH_2PO_4; pH 7.4) and vortex until complete solubilization of MTT.

2.4. Add the MTT solution to the cells at one tenth of the well volume (10 µL/well).

2.5. Incubate the plate 37 °C in a CO_2-humidified incubator (see **Table of Materials**) for 3-4 h.

2.6. Discard the supernatant with a multichannel micropipette and add 100 µL of DMSO (100%) to each well.

2.7. Solubilize the formazan crystals by pipetting up and down and then read the absorbance at 570 nm in a spectrophotometer (see **Table of Materials**).

2.8. To determine the CC_{50} values of compounds, proceed as described in item 1 steps 1.1 to 1.8 and then add the compounds serially diluted to the cells, together the negative (1% DMSO)

control. Perform the assay twice in duplicates.

2.9. Plot the average values of inhibition rates per compound concentration and use a graph analysis software to perform a sigmoidal fitting and obtain the CC_{50} values.

3. NS2B-NS3 protease activity assay

3.1. Thaw a protein aliquot on ice.

3.2. Set the plate reader (see Table of Materials) temperature to 37°C.

3.3. Prepare the appropriated amount of protein diluted to 80 nM (5 µL/well). Final protein concentration is 4 nM.

3.4. Thaw the appropriate amount of Bz-nKRR-AMC substrate on ice (300 µM stock solution diluted in assay buffer, 10 µL/well).

3.5. In a 96-well white plate (see **Table of Materials**), dispense 84 µL of assay buffer (20 mM Tris pH 8.5, 5% glycerol and 0.05% Triton X-100) in each well.

3.6. To make the positive control reaction, to each well of the last column dispense 1 µL of Aprotinin to achieve final concentration of 1 µM (stock solution 100 µM diluted in water)

3.7. To make the negative control reaction, to the first column dispense 1 µL of DMSO (final concentration 1%).

3.8. To perform the compound screening, dispense 1 µL of each compound to achieve final concentration of 10 µM (1 mM stock concentration) excluding positive and negative control wells.

3.9. Dispense 5 µL of the protease solution.

3.10. Incubate the plate at 4 °C for 30 minutes.

3.11. To start the reaction, dispense 10 µL of Bz-nKRR-AMC stock solution (final concentration of 30 µM).

3.12. Set the excitation wavelength to 380 nm and emission to 460 nm and read the fluorescence for 30 min every 1 min in a microplate reader (see **Table of Materials**). Perform the entire experiment at 37 °C.

3.13. Calculate the mean values of the fluorescence for positive and negative control reactions. Set as 100% of protease activity the mean value of fluorescence for negative control reactions subtracted of the mean value of positive control and calculate the percentages of activity for

each compound.

3.14. For each plate, calculate the Z-factor value, as described in step 1.15.

3.15. Proceed with IC_{50} determination for compounds that exhibited an inhibition rate higher than 80%.

3.16. Perform the assay in triplicates as described in steps 3.1-3.13, using a serial dilution of the compound.

3.17. Plot the average values of inhibition rates per compound concentration and use a graph analysis software to perform a sigmoidal fitting and obtain the IC_{50} values.

4. NS5 RdRp elongation assay

NOTE: All materials used in this assay are RNase, DNase and pyrogenase free certified.

4.1. Prepare both the assay buffer (50 mM Tris pH 7.0, 2.5 mM $MnCl_2$, 0.01% Triton X-100) and the 200 mM ATP stock solution with 0.1% diethylpyrocarbonate (DEPC) treated water.

4.2. Anneal a 5 µL aliquot of 200 µM 3'UTR-U_{30} (5'-biotin-U_{30}-ACUGGAGAUCGAUCUCCAGU-3') in PCR treated water by incubating it for 5 minutes at 55 °C in a thermocycler.

4.3. Thaw the stock solution of NS5 RdRp, 200 mM ATP and x10.000 SYBR Green I on ice.

4.4. Dilute the protein to a final concentration of 250 nM in 3 mL of assay buffer.

4.5. Prepare substrate solution by diluting the stock solutions of the ATP, 3'UTR-U_{30} and SYBR Green I in 3 mL of assay buffer to a final concentration of 1 mM, 300 nM and 1X, respectively.

4.6. In a 96-well PCR plate (see **Table of Materials**), add 24.5 µL of diluted protein in columns 1 to 11 of each row. Add the same volume of assay buffer in the remaining wells.

4.7. For control and blank reaction, add 0.5 µL of DMSO in columns 1 and 12. Add 0.5 µL of compound diluted in DMSO to a final concentration of 10 µM 1mM stock solution).

4.8. Seal the plate with a sealing film and incubate at room temperature for 15 minutes.

4.9. Start the reaction by adding 25 µL of substrate solution and seal the plate again.

4.10. Incubate at 30 °C in a real-time PCR system (see **Table of Materials**) and monitor the fluorescence for 1 hour, measuring the fluorescence every 30 s with the FAM filter (Emission:494 nm/Excitation:521 nm).

4.11. For each plate, calculate the Z-factor value, as described in step 1.15.

4.12. Proceed with IC_{50} determination for compounds that exhibited an inhibition rate higher than 80%, as described in step 3.15.

4.13. Plot the average values of inhibition rates per compound concentration and use a graph analysis software to perform a sigmoidal fitting and obtain the IC_{50} values.

REPRESENTATIVE RESULTS:

All the protocols described herein were stablished in 96-well plates and allows the evaluation of 80 compounds per plate in a primary screening of a single concentration, including the negative and positive controls placed at the first and last column of the plates, respectively. The replicon-based screenings are represented in **Figure 1**, which includes the RNA construct developed to obtain the BHK-21-RepZIKV_IRES-Neo cell line (**Figure 1A**), the assays schematic representation (**Figure 1B**) and the dose-response curves of NITD008 (EC_{50} of 0.28 µM, CC_{50} > 10 µM) (**Figure 1C**). The EC_{50} and CC_{50} values of hit compounds are determined as the concentrations required to inhibit 50% of the *Rluc* activity and cause 50% cytotoxicity, respectively. With respect to the luciferase assay, DMSO 1% is used as a no inhibitor control (0% inhibition) and NITD008 is used as a positive control (100% inhibition), as previously described [8].

The NS2B-NS3 protease activity is measured by fluorescence monitoring of AMC released due to the proteolytic activity of the protease (**Figure 2A**). Aprotinin, a protein that acts as trypsin inhibitor and is already described as an inhibitor of flavivirus proteases [13–15], was used in this assay as an experimental positive control (IC_{50} of 0.13 ± 0.02 µM, data not shown). **Figure 2B** illustrates a dose-response inhibition curve of a molecule targeting the protease activity, the compound MMV1634402 (IC_{50} of 0.36 ± 0.08 µM). The elongation activity of NS5 RdRp is measured in real time by the increase in fluorescence intensity of SYBR Green I when intercalated with the synthesized dsRNA (**Figure 2C**). The dose-response inhibition curve of a hit molecule targeting ZIKV RdRp, the compound MMV1782220 (IC_{50} of 1.9 ± 0.8 µM), is showed in **Figure 2D**. Since nucleoside analog inhibitors, such as NITD008, are not suitable for enzymatic assays, as phosphates needs to be incorporated intracellularly to the molecule [16], we did not use any positive control for NS5 RdRp elongation assay. However, Clofazimine, a commercial antibiotic, which we recently identified as an inhibitor of viral polymerase [8], could be used as an experimental control in next assays.

FIGURE AND TABLE LEGENDS:

Figure 1: Replicon-based screenings. A) Schematic representation of the ZIKV replicon construct containing a *Rluc* sequence at the 5' UTR terminus and a Neo gene at the 3' UTR terminus, that we have developed to obtain the BHK-21-RepZIKV-IRES_Neo cell line [8]. **B**) Schematic representation of the luciferase activity assay and cell proliferation-based MTT assay performed to screen for inhibitors of ZIKV replication. **C**) The dose-response curves (EC_{50} and CC_{50}) of NITD008. The assay was performed in duplicates. Error bars represent standard deviations.

Figure 2: Viral enzyme-based assays. **A**) Schematic representation of NS2B-NS3 protease activity

assay. **B**) The dose-response inhibition curve (IC_{50}) of compound MMV1634402. **C**) Schematic representation of NS5 RdRp RNA polymerase activity assay. **D**) The dose-response inhibition curve (IC_{50}) of compound MMV1782220. The assays were performed in triplicates. Error bars represent standard deviations.

DISCUSSION:

The protocols described herein could be readily adapted for screenings in a 384 or 1536-well formats. For biochemical and/or cell-based screenings performed in HTS format, the Z' factor value, a statistical parameter, is calculate for each plate to ensure the sensitivity, reproducibility and accuracy of those assays [12]. A Z' factor value of 0.5 or above is expected for replicon-based screenings while a value of 0.7 or above is expected for the NS3 and NS5 activity assays. For the replicon-based HTS, we have developed the BHK-21-RepZIKV_IRES-Neo cells, a stable cell line harboring a replicative ZIKV replicon containing a *Renilla* luciferase (*Rluc*) sequence at the 5' UTR region and a neomycin phosphotransferase (Neo) gene driven by an internal ribosomal entry site (IRES) at the 3´UTR. We retained 38 residues of capsid and 30 residues of envelop genes that are required for the correct initiation of the RNA translation, to maintain comparable replication levels and drug sensitivity between cell passages [8]. Due to the lack of structural genes, replicons do not produce progeny virions, thus eliminating the risk of laboratory-acquired viral infection [17]. The antiviral assays using the ZIKV replicon cells consists in the luciferase activity and the cell proliferation-based MTT (cytotoxicity) assays performed in parallel. This is necessary to exclude false-positive hits, comprising molecules that interfere directly with the reporter protein expression and/or activity and those that adversely affect cell health [7]. Replicon systems allows the discovery of molecules that inhibit RNA replication but not those required for viral entry and virion assembly/release. Alternatively, replicons can be packaged to produce virus replicon particles (VRPs) by providing the structural proteins *in trans* [17]. The resulting single-round infectious particles (SRIPs) are infectious, but progeny virus cannot propagate as the package genome lacks structural genes. Therefore, VRPs could be used to test for inhibitors of viral entry/replication by measuring the levels of the reporter protein [7].

In addition to the replicon-based screenings, we also detailed herein the protocols used in viral enzyme-based assays for the recombinant NS3 protease and NS5 RdRp. The proteolytic activity of the viral protease was measured by using the fluorogenic peptide substrate Bz-nKRR-AMC, which contains the ZIKV protease recognition and cleavage sequence coupled with the fluorescent tag 7-amine-4-methylcoumarin (AMC). Due to the protease activity, the fluorescent tag is released and the reaction rate is directly measured by monitoring the fluorescence in a spectrophotometer [18, 19]. This assay is highly sensible, relatively cheap, quick and suitable for screening of large compound libraries [20, 21]. The major drawback is the possible quenching between tested compounds and the fluorophore that can lead to false-positive hits. However, this issue could be addressed by an additional fluorescence measurement in the presence of AMC. Also, compounds showing emission or absorption in the same wavelength of the fluorophore cannot be evaluated by this method [18, 20].

Regarding the NS5 RdRp, its elongation activity is directly detected by the increase of the fluorescent signal of SYBR Green I during the elongation of a self-priming 3'UTR-U_{30} template.

The protocol was adapted from assays with intercalating dyes such as Pico Green and SYTO 9 that have been widely used to evaluate compounds for different viral polymerases [22–26]. Even though we have used a self-priming biotinylated template[27] in the assay, other templates, such as poliU, can be used as well [25]. The main disadvantage of this method is the high number of false-positive hits that interact with the dye, either by interfering with the fluorescence or by decreasing the dsRNA intercalation [28]. Therefore, hit compounds need to be validated with counter-assays such as biophysics methods or by comparing the SYBR™ Green I fluorescence in dsRNA with and without the compound [29]. Nevertheless, the easy implementation, direct measurement and affordability are key points to the use of fluorescence-based methods as HTS platforms, in comparison to radio-labeled or coupled assays that are difficult to implement in medium/large scale campaigns [27, 30, 31].

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DISCLOSURES:

Authors declare no conflict of interest.

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Figure1

Click here to access/download;Figure;J1.tif

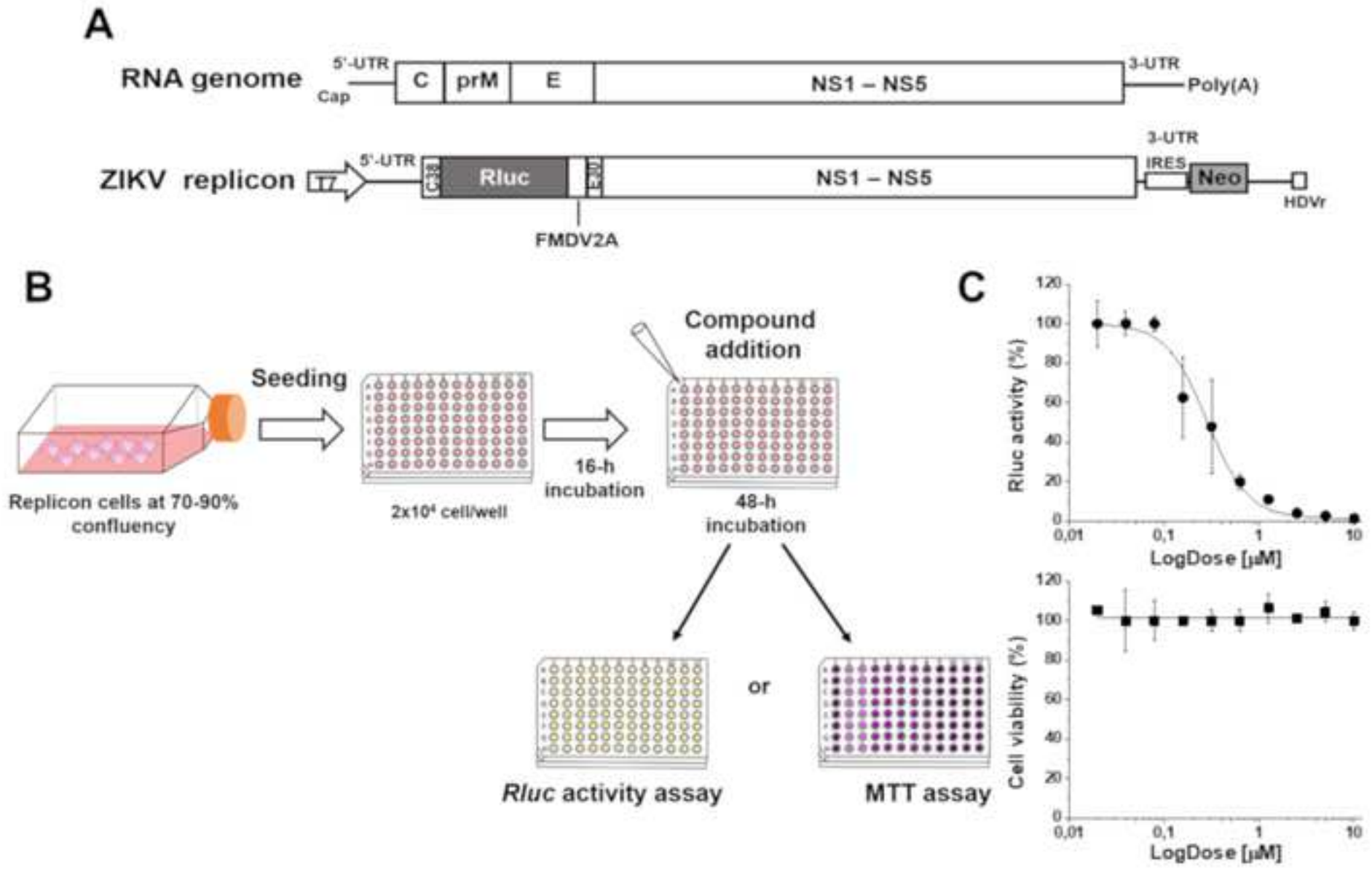
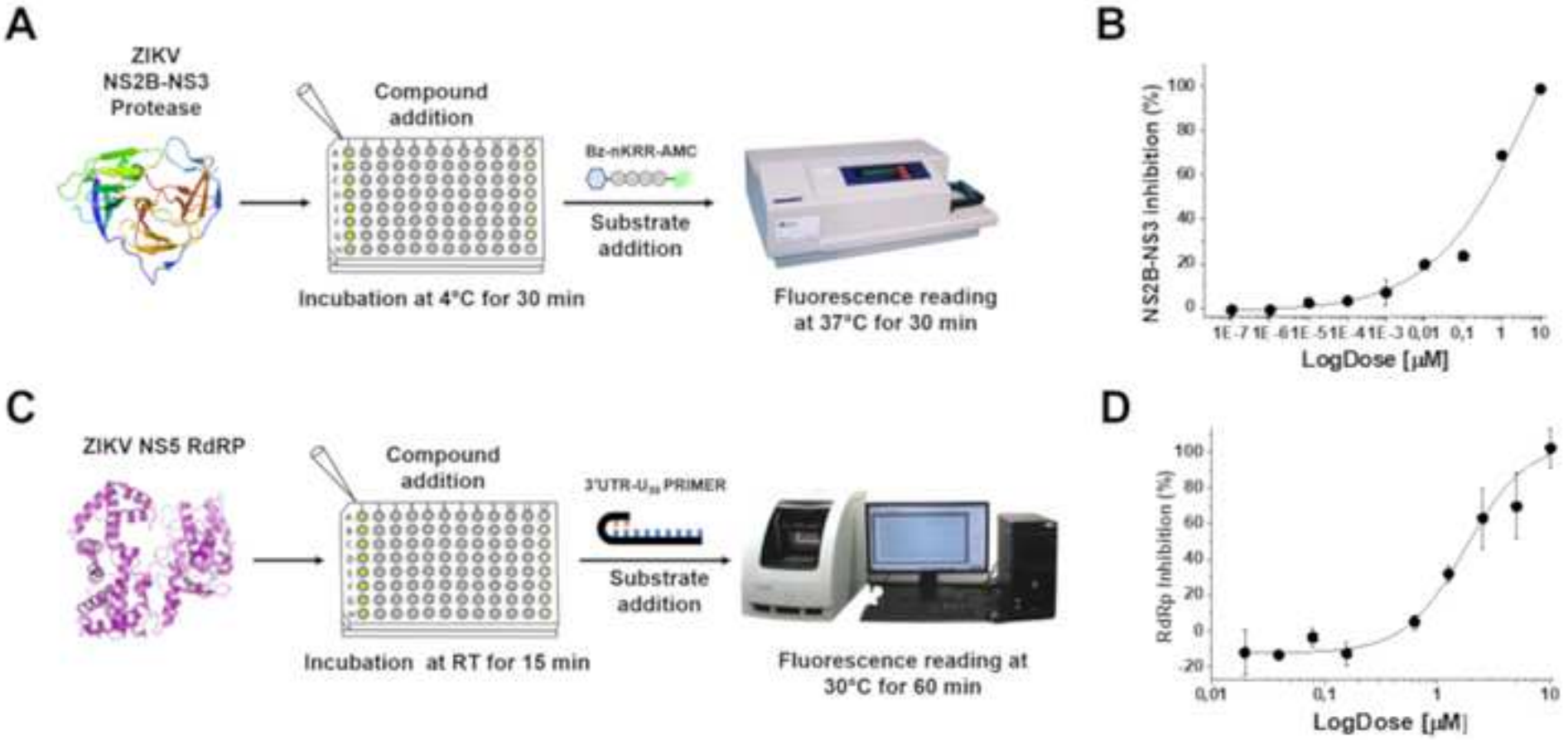


Figure2

Click here to access/download;Figure;J2.tif



Point-by-point reply

Editorial and production comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done.

2. We cannot have paragraphs of text in the protocol section. Please consider moving lines 116-130 to the introduction section in the paragraph style.

Lines 116-130 were moved to the introduction, as requested.

3. Please revise the following lines to avoid overlap with previously published work: 317-323

Lines were revised.

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

The numbering was adjusted, as requested.

5. What kind of compounds are used in this study?

The tested compounds are molecules with antibacterial, antifungal and antiviral activities from the MMV/DNDi Pandemic Response Box. This information was included in the manuscript.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

All the text in the protocol section were modified to the imperative tense.

7. For each step, please ensure you answer the “how” question, i.e., how is the step performed?

Checked.

8. Please include a single line space between each step in the protocol section.

A single line space was added between each step.

9. Please ensure each section of the protocol has associated representative results.

Checked.

10. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

No figures were reused from a previous publication.

11. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

All checked.

12. We cannot have in preparation/to be published manuscripts as citations. Only accepted or in press articles can be cited.

We corrected the citation. The article was recently published.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.

All images in the video appears in the written manuscript.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.

We revised the narration and made modifications to be more homogenous with the written manuscript. However, it was not possible to do a word by word reading of the manuscript because of the limited time duration of the video (15 min).

3. Please ensure protocol subheadings are same both in the test and the video. Please ensure each section of the protocol has associated representative result.

Done.

4. Please add on-screen text introducing/identifying the speaker (name and affiliated institution) during interview segments.

Done.

5. Consider reducing background noise if software allows.

Done.

6. 0:00-1:50, 9:49-end - We recommend editing the interview statements to excise mistakes and long pauses to maintain a consistent pace for viewers. Overlaying relevant footage can help make these edits seem more natural.

Done.

7. Consider a quicker fade to white between title cards and footage or graphics (0:16, 1:52, 5:03, 5:06, 5:40, 5:43, 6:48, 6:50, 8:14, 8:17)

Done.

8. Consider editing down Chapter 1, "Luciferase activity assay" (1:50-4:50). We generally recommend no more than 3 seconds of video without narration. Try to match the pacing of the other chapters.

We shortened the intervals without narration, as possible.

9. There are many instances where significant camera movement or a zoom is visible during the crossfade between one shot and the next. Unexpected and unnecessary camera movement during these transitions can look messy and be disorienting for the viewer. Consider trimming the clips a bit more to ensure that such footage is not included in the edit. examples: 1:59, 2:15, 2:55, 3:41, 4:20, 5:19, 5:23, 5:34, 5:53, 6:04, 6:17, 6:43, 7:03, 7:17, 7:47, 8:14

Points indicated were trimmed, as possible.

10. 2:28 - A "jump cut" is a type of edit where the camera does not move but the action changes instantly, like at this time point. This type of edit is distracting and jarring for the viewers, and we suggest using a quick dissolve (or fade) to move quickly between similar shots

Done.

11. 5:03 - consider extending this narration clip so that it ends less abruptly and is more clearly understood.

Done.

12. 9:49 - Please insert a title card for "Conclusion" to separate the results from the concluding interview statements

Done.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors described protocols for cell based ZIKV replicon and biochemical assay using NS3 and NS5 for high-throughput antiviral screening. The manuscript is quite straightforward and is good for publication. Here are some minor comments for improvement:

Major Concerns:

No

Minor Concerns:
Give the name and models of the machine used for experiment. For example: plate reader

As suggested by the reviewer, we added the name and models of machines in the Table of Materials.

Give reference paper for ZIKV-replicon cell line.

The reference paper was added to the manuscript, as suggested by the reviewer.

How to discard the supernatant for every step from 96-well plate?

We thank the reviewer for this question. We modified the text, for clarification.

Give an example for how to calculate the Z factor value?

We added this information in item 1 step 1.15.

2.3. How to vortex?

To address this question, we added "until complete solubilization of MTT" to the sentence on item 2.3.

Briefly explained how to measure RdRP activity using primer 3'UR-U30? Give a reference paper for this assay. What is the template for elongation activity? Through 3'UR-U30 self-annealing?

We have added a few sentences in introduction, representative results and discussion to explain how the activity is measured and also to emphasize that the template used for the elongation assay (3'UTR-U30) is a self-priming RNA. We have modified line 347 to clarify the papers that were used as reference to adapt the NS5 RdRp elongation assay.

In what buffer used for annealing?

We modified item 4.2. and added the concentration and buffer used for the 3`UTR-U30 stock solution.

In what buffer for dilution of the ATP and the annealed RNA?

We have modified item 4.5 to clarify that the dilutions should be done in 3 mL of assay buffer.

What is the stock concentration of 3UTR-U30?

We added the 3UTR-U_{30} stock concentration in item 4.2.

4.9. What is substrate solution?

We modified item 4.5 to define the substrate solution.

Reviewer #2:

Manuscript Summary:

Fernandes et al. report on the development of different assays to measure either the replication of Zika virus (in the form of a synthetic replicon) or the enzymatic activity (by fluorescence-based assays) of the two most studied non-structural proteins of ZIKV: The NS3 and NS5. They claim that they can be easily implemented as Hight-throughput screening (HTS) platforms to assess different compounds with antiviral activity against ZIKV and might be adapted, with slight modifications, to monitor for inhibitors of other flavivirus.

Major Concerns:

The results interesting and important, and in principle, with high potential usefulness. However, the authors do not provide very important information regarding control experiments that I consider necessary to evaluate properly the feasibility of the two fluorescence-based assays explained in the manuscript. Therefore, this information should be added prior to publication. Furthermore, the article is hastily and untidily written, and the protocols are difficult to follow in some cases due to steps are not well structured

1. In the manuscript, is described the protocol of a replicon-based assay recently developed (Fernandes et al. to be published). To assess the suitability of this method, as a positive control of antiviral effect, they use the compound NITD008 as a representative antiviral, which inhibitory effect against Zika virus has been already proven (Yin Z, Proc Natl Acad Sci U S A. 2009. 106(48):20435-9). However, to assess the suitability of NS3 and NS5 enzymatic fluorescence-based assays, authors use two compounds (MMV1634402 and MMV1782220, respectively, that belong to MMV/DNDi Pandemic Response Box). The possible antiviral effect of both compounds, as far as I

know, has been not described previously (If it is the case, they should provide the corresponding references). Therefore, these compounds cannot be used as standards of ZIKV protease or polymerase, respectively, inhibitors. In this regard, as the own authors remark wisely in the manuscript discussion:

"The major drawback (of fluorescence-based method) is the possible quenching between tested compounds and the fluorophore that can lead to false-positive hits. However, this issue could be addressed by an additional fluorescence measurement in the presence of AMC. Also, compounds showing emission or absorption in the same wavelength of the fluorophore cannot be evaluated by this method" (Lines 332-336)

and

"The main disadvantage of this method (fluorescence-based) is the high number of false-positive hits that interact with the dye, either by interfering with the fluorescence or by decreasing the dsRNA intercalation" (lines 340-342).

So, it could be the case of either MMV1634402 or/and MMV1782220, respectively.

Authors should provide suitable experimental controls for both fluorescence-based assays. The following enumeration is not intended to be comprehensive, but rather to show some of the controls that might warrant the appropriateness of these assays to be used in drug screening platforms: experimental positive control in the absence of inhibitory compounds, experimental negative controls in the absence of Mn or NaCl for NS5 and NS3, respectively; experimental negative controls in the presence of catalytically inactive NS5 and NS3, experimental positive control of enzymatic activity inhibition in the presence of well-known broad-spectrum protease and RNA-dependent RNA polymerase inhibitors, respectively (e.g. 3'dATP for RdRPs), etc.

The authors thank the reviewer for these relevant observations. Indeed, the RdRp fluorescence-based assay lacks an experimental positive control in the manuscript, however, for the NS3 protease, Aprotinin was used for this purpose (line 290). The text was modified to clarify the use of aprotinin as an experimental control for the ZIKV protease assay (item 3, steps 3.6, 3.8 and 3.13). Since there are already published papers that validate RdRp fluorescence-based assays with templates like 3'UTR-U_{30} (Niyomrattanakit et al. 2011; Kocabas, Turan, and Aslan 2015) *or with similar*

intercalating dyes such as PicoGreen (Eltahla et al. 2013; Tarantino et al. 2016) *and SYTO9* (Sáez-Álvarez et al. 2019)*, we didn't consider to validate the assay with a positive control. However, recently, we identified one compound, Clofazimine, a commercial antibiotic, from the MMV/DNDi Pandemic Response Box* (Fernandes et al. 2021) *that specifically inhibits the viral RdRp without interfering directly with the fluorescence, so this compound could be used in next assays as an experimental positive control. We added this information to the manuscript, lines 364-369.*

Regarding the results, both MMV compounds were used only as examples of inhibitors found in screening campaigns and not as experimental positive controls. Also, their antiviral activities have not been previously published. For compound MMV1634402, we performed an additional fluorescence measurement in the presence of AMC and found that this compound did not interfere with the fluorescence but with the activity of NS3. Likewise, for compound MMV178220, we measured the fluorescence of SYBR Green I with 3'UTR-U_{30} (which is a dsRNA in the self-priming region) and without NS5, and found that the compound didn't interfere in the fluorescence or intercalation of the dye to the 3'UTR-U_{30}.

2. For replicon-based (line 168) and both fluorescence-based screening method (lines 224 and 257), authors ask for calculation of Z-factor value. They do not give the reason or importance of that specific step and how this calculation might affect the trustability of the corresponding assay outcome. In the Representative Results section, using their standards inhibitors, authors give Z-factor values using the word "usually" (lines 274 and 283) for replicon-based and protease-based assays, and the word "reliable" (line 288) in the case of polymerase-based assay. I think that authors should recalculate these values using broad-spectrum protease and polymerase inhibitors to obtain a standard Z-factor values for each assay that can warrants the HTS format of the assays.
To support the HTS format claims of both replicon-based and fluorescence-based assays, the Z-value got with any compound screened should always show reliable values above 0.5 (or 0.7 for enzymatic assays). Otherwise, I reckon they should withdraw the HTS format claim of these assays from the manuscript (including the title).

We thank the reviewer for the observation. We added two sentences on lines 381-384 to explain the importance of the Z' factor calculation. As defined in (Zhang, Chung, and Oldenburg 1999)*, Z' factor values between 0.5 and 1.0 are considered good quality. Those values vary between plates even if we use the same positive control for each one, that is why we used "usually" to describe the Z' factor values. However, we only consider*

the results from the plates that gave a value of ≥ 0.5 for replicon-based screenings or ≥ 0.7 for enzymatic assays. For NS3, we used aprotinin as positive control of the assays, as explained on lines 353-355. Regarding NS5, as explained above, we did not have a positive experimental control for the enzymatic assay. However, Clofazimine, a commercial antibiotic that specifically inhibits the viral RdRp (Fernandes et al. 2021) *could be used in next assays as an experimental positive control.*

Minor Concerns:

3. Line 170: In order to perform step 17., A new plate with cells should be produced (steps 1. to 8.). Please, rewrite this step to clearly indicate this fact.

The sentence in line 170 was corrected, as suggested by the reviewer.

4. Line 190: I reckon that Instead of "[…] to the cells in step 7 […]" you should say "[…] to the cells after step 1 […]" or "[…] to the cells in step 7 from item 1 […]", or something like that. Also check the point 3. of this review.

The sentences in item 2 step 8 and step 3 were corrected, according to the reviewer's suggestions.

5. Line 240: You use a biotinylated RNA template. Do you really need this specific kind of 5'-tagged RNA for a fluorescence-based assay? Could you use and untagged RNA instead?

We thank the reviewer for these questions. This template was used because we were first developing a fluorescence-coupled assay adapted from (Niyomrattanakit et al. 2011)*, that uses a biotinylated 3'UTR-U_{30} template. However, the authors don't explain the reason for a biotinylated RNA. Since there are several papers that use poliU templates to measure the RdRp activity, using similar intercalating dyes such as PicoGreen* (Eydoux et al. 2021; Eltahla et al. 2013; Tarantino et al. 2016) *and SYTO9* (Sáez-Álvarez et al. 2019)*, there is no need to use this specific self-priming biotinylated template. We have modified the Discussion section to add the information that different and simpler RNA templates can be used as well.*

6.The figures are pretty clear. The authors provide a comprehensive list of reagents.

However, they do not provide the name of any equipment (i.e. Fluorimeter, Real time PCR, 96-well plates, biosafety cabinet and so on).

The authors thank the reviewer for this observation. We added the names of equipments at the Table of Materials.

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Eltahla, Auda A., Kurt Lackovic, Christopher Marquis, John Sebastian Eden, and Peter A. White. 2013. “A Fluorescence-Based High-Throughput Screen to Identify Small Compound Inhibitors of the Genotype 3a Hepatitis c Virus RNA Polymerase.” *Journal of Biomolecular Screening* 18 (9): 1027–34. https://doi.org/10.1177/1087057113489883.

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