Fluorometric RdRp assay with self-priming RNA

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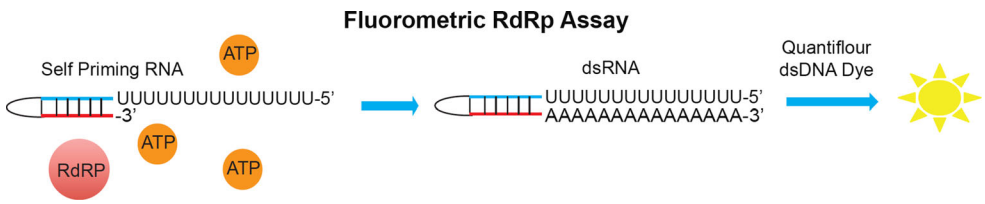
Abstract There is an outmost need for the identification of specific antiviral compounds. Current antivirals lack specificity, making them susceptible to off-target effects, and highlighting importance of development of assays to discover antivirals targeting viral specific proteins. Previous studies for identification of inhibitors of RNA-dependent RNA polymerase (RdRp) mostly relied on radioactive methods. This study describes a fluorometric approach to assess in vitro activity of viral RdRp for drug screening. Using readily available DNA- and RNA-specific fluorophores, we determined an optimum fluorometric approach that could be used in antiviral discovery specifically for RNA viruses by targeting RdRp. Here, we show that double-stranded RNA could be successfully distinguished from single-stranded RNA. In addition, we provide a strategy based on self-priming RNA to assess RdRp activity.

Graphical abstract



Keywords Viral RdRp · RNA-dependent RNA polymerase · Fluorometric RdRp assays · dsRNA and ssRNA

Introduction

RNA-Dependent RNA polymerase (RdRp) catalyzes the synthesis of RNA from RNA template [1–5]. It is an essential enzyme found in RNA-containing viruses including but not limited to Ebola virus, polio virus, Hepatitis C virus, influenza virus, measles virus, and Crimean Congo hemorrhagic fever virus (CCHFV) [6, 7]. Life cycle of the RNA viruses depends on the function of the RdRp to replicate and transcribe messenger RNA from RNA genome. Thus, understanding of RdRp activity and development of RdRp inhibitors are crucial for treatment of human diseases associated with RNA-containing virus infections. Toward this end, various RdRp proteins or subunits have been crystalized such as HCV RdRp protein known as NS5B, norovirus RdRp, dengue RdRp, Japanese

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encephalitis virus RdRp, bacteriophage phi6 RdRp, and others [5, 8, 9].

Correct initiation and completion of RNA synthesis by RdRps are essential for invasion of RNA-containing virus into host organism. Various viral RdRps have been found to utilize similar catalysis mechanism along with similar domain structure [9]. RdRps, however, are distinct from other polymerases by possessing a closed hand conformation compared to open hand conformation found in other polymerases. As an advantage of this closed hand conformation, initiation site is well recognized [10, 11]. The finger and thumb domains ensure the closed hand conformation and let to formation of specific channels in structure of RdRps. Positively charged tunnels close active site in RdRps support to bind negatively charged RNA template. In addition, C termini of RdRps accomplish to prevent exit of template and stabilize complex [11, 12]. Another shared mechanism in RdRps is sequentially binding of incoming NTPs. It is believed that positively charged amino acids in the tunnel led to interact with incoming NTP to direct them to active site. According to the study with bacteriophage Φ6 RdRp, RNA synthesis starts with entrance of RNA template followed by interaction of RNA template with S pocket (specificity pocket) on RdRp to stabilize the complex. Incoming NTPs occupy near the active site of RdRp and this site could be targeted for inhibition of RdRp activity thus may prevent infection of the RNA-containing viruses. However, identification of RdRp inhibitors is limited with availability of functional in vitro assays and access to BSL4 facility for deadly viruses.

Current approaches in identification of RdRp inhibitors largely rely on viral infection inhibition assays, and radioisotope-labeled RNA product-based RdRp assays [13, 14]. However, difficulties in working with deadly viruses like CCHFV often require use of BSL4 facilities, which limits number of laboratories to work on [15]. In addition, safety concerns regarding use of radioactively labeled RNA products urges development of safe and reliable RdRp assays. Thus, alkaline phosphatase-coupled polymerase assay based on cleavage of BBT-ATP [γ-(2′(2-benzothiazoyl)-6′-hydroxybenzothiazole)-adenosine-5′-triphosphate, sodium salt] has been recently utilized for development of Dengue RdRp inhibitor [16], albeit with several limitations. Fluorophores have been extensively used for both DNA and RNA detection. In vitro activity of RdRps could be measured by determining the double-stranded RNA (dsRNA) formation from single-stranded RNA (ssRNA) template. Thus, we aimed to determine fluorophores that can distinguish dsRNA from ssRNA and develop a reliable and steadfast fluorometric RdRp assay for drug discovery. By employing various in vitro assays and use of recombinant phi6 RdRp from bacteriophage Φ6, we show that dsRNA could be distinguished from ssRNA using various flourophores. Moreover, RdRp activity could be measured based on self-priming RNA followed by dsRNA quantification.

Materials and methods

Materials

The fluorophore systems QuantiFluor® dsDNA system (E2670) and QuantiFluor® RNA System (E3310) were obtained from Promega. The Quant-iT™ PicoGreen® dsDNA reagent was purchased from Life Technologies (P7581). Sense, antisense, and self-priming RNA (100 nmol RNA oligo) used in this study were purchased from Integrated DNA Technologies (IDT). The phi6 RdRp (aka RNA replicase) (1 U/μL, F611S) was purchased from Thermo Scientific. BBT-ATP (NU1700) was purchased from Jena Bioscience GmbH, and Adenosine 5′-triphosphate (ATP) was purchased from Sigma–Aldrich. Deoxyethanolamine (DEA) was purchased from The Dow Chemical Company. Alkaline phosphatase, Calf intestinal (CIP) was purchased from New England Biolabs.

Double-stranded RNA generation

We have used following complementary ssRNAs for dsRNA generation:

ssRNA Sense: 5′-UUUUUUUUUUUAACAGGUUCUA-3′
ssRNA Antisense: 5′-UAGAACCUGUUAAAAAAAAAA-3′

Sense and antisense RNA (100 nmol RNA oligo) were purchased from IDT. dsRNA has been produced by incubating equal concentrations of sense RNA and antisense RNA at 55 °C for 5 min, which is followed by cooling down at room temperature for 5 min. ssRNAs and dsRNA have been kept at −80 °C for long-term storage.

Characterization of fluorophores for RdRp assay

Ability of fluorophores to distinguish dsRNA from ssRNA has been analyzed for Quant-iT(tm) PicoGreen (Life Sciences), QuantiFluor® dsDNA System and QuantiFluor® RNA System (Promega). Varying concentrations of ssRNA and dsRNA have been incubated with Quant-iT™ PicoGreen® dsDNA reagent, QuantiFluor® dsDNA system, and QuantiFluor® RNA system at room temperature for 5 min, according to manufacturers recommendations. Briefly, QuantiFluor® dsDNA and QuantiFluor® RNA system, and Quant-iT™ PicoGreen® were diluted to 1:200 in 1X TE buffer and added to samples in black microplates.

Table 1 Excitation and emission wavelengths of corresponding fluorophore systems

Fluorophore system	Excitation wavelength (nm)	Emission wavelength (nm)
Quant-iT™ PicoGreen® dsDNA reagent	480	520
QuantiFluor® dsDNA system	504	531
QuantiFluor® RNA system	492	540

This is followed by incubation for 5 min at room temperature protected from light. Fluorescence intensity of samples was assessed using neo HTS multi-mode microplate reader (Biotek) at indicated excitation and emission wavelength for each fluorophore system (Table 1).

Development of fluorometric RdRp assay with self-priming RNA

RNA-dependent RNA polymerase activity of phi6 RdRp has been tested using quantiflour dsDNA system (E2670, Promega). RdRp RNA template with self-hairpin primer used for RdRp assays in this study as following [16]:

5′-bio-UUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUAACAGGUUCUAGAACCUGUU-3′. Self-priming regions are highlighted in red. Phi6 RdRp has been incubated in phi6 reaction buffer 50 mM Tris–HCl (pH 8.75 at 21 °C), 1.5 mM $MnCl_2$, 0.1 mM ATP, and 50 mM NH_4Ac along with various RNA contents (0, 250, and 500 μM) for 1 h at 32 °C. Formation of dsRNA is assessed by QuantiFluor® dsDNA system (Promega). This is followed by addition of 100 μL of QuantiFluor® dsDNA dye mix and incubation for 5 min at room temperature, according to manufactures recommendation. Increased fluorescence was assessed using neo HTS multi-mode microplate reader (Biotek) at excitation 504 nm and emission 531 nm. To make RdRp assay suitable for high-throughput assays, we optimized it for 96-well plates. Thus, we set up the reactions using 96-well plates as following (Table 2):

RdRp assay based on BBT fluorescence

RdRp assay based on release of BBT from BBT-ATP has been performed as described previously with modifications [16]. Briefly, we have used self-priming RNA dissolved in 50 mM Tris–Cl, pH 8.0, 150 mM NaCl, and 0.1 % DEPC following formation of hairpin with incubation at 55 °C for 5 min. We dispensed a mixture of 20 μl of self-priming RNA (100 nM final concentration) and phi6 RdRp into 96-well, half volume plates. RdRp activity was investigated with 1 U of phi6 RdRp with provided reaction buffer (50 mM Tris–HCl (pH 8.75 at 21 °C), 1.5 mM $MnCl_2$, 0.1 mM ATP, and 50 mM NH_4Ac) according to manufacturers recommendations. This is followed by dispensing 20 μl of BBT-ATP (4 μM final concentration) and incubation at 25 °C for 1 h. Later, we added 40 μl of stop solution (25 nM of CIP stop buffer containing 200 mM NaCl, 25 mM MgCl2, 1.5 M DEA), which also allowed hydrolysis of BBTPPi by CIP. This is followed by measurement of released BBT fluorescence intensity at excitation 422 nm and emission 566 nm for up to 4 h at 5 min intervals.

Statistical analysis

Statistical analysis was performed using the Student's *t* test. $p < 0.05$ were considered statistically significant.

Results

Quantification of dsRNA by fluorometric approaches

There is an urgent need for the development of in vitro assays that are reliable and easy for identification of RdRp inhibitors for treatment of infections of deadly viruses. Previous studies relied mostly on radioactive approaches for measurement of RdRp activities, which could be replaced with fluorometric approaches. This requires determination of fluorophores with ability to differentiate dsRNA from ssRNA in vitro following RdRp activity. Thus, we have tested ability of different fluorophores systems used in fluorometric quantification of nucleic acids in vitro and how they are effective in distinguishing dsRNA from ssRNA. We have tested readily available fluorophores namely Quantiflour RNA, Quantiflour dsDNA, and picoGreen Systems. We have used varying amounts of RNA for each system and determined degree of

Table 2 Phi6 RdRp reaction setup for 96-well plate

Components	Blank (μL)	ATP control (μL)	RNA control (μL)	RdRp assay (μL)
RNA (0–500 μM/Rxn)	–	–	5	5
RdRp buffer (1X)	10	10	10	10
ATP (0.1 mM/rxn)	–	1	1	1
Phi6 RdRp (1U/rxn)	–	–	–	1
ddH_2O (DEPC treated)	90	89	84	83

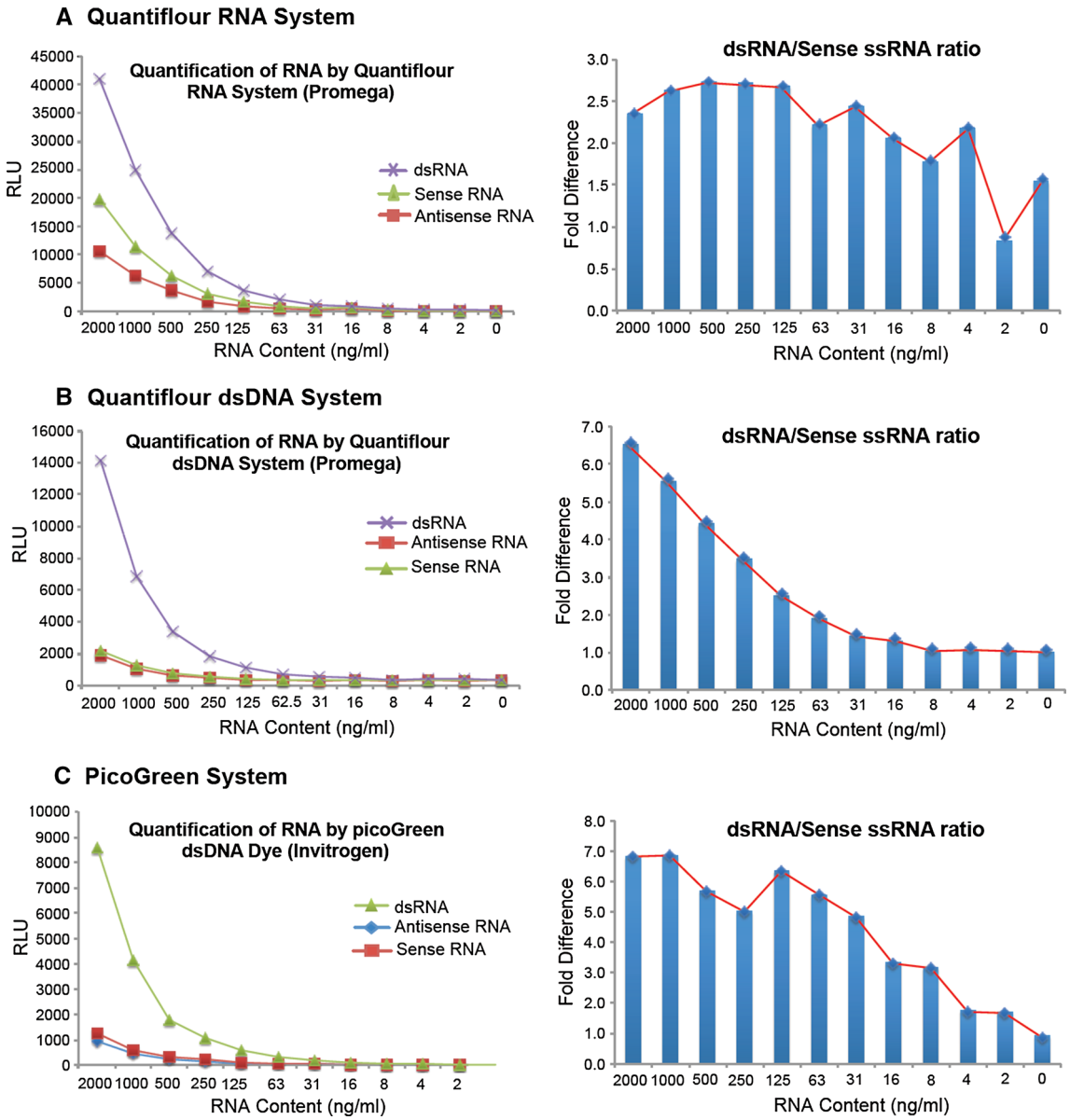

Fig. 1 Quantification of dsRNA by Quantiflour RNA, Quantiflour dsDNA, and picoGreen systems. **a** Quantiflour RNA system **b** Quantiflour dsDNA system, and **c** picoGreen system are tested for their ability to differentiate with low signal-to-noise ratio for dsRNA quantification. We have used varying amounts of RNA for each system and determined degree of difference for dsRNA/dense ssRNA signal

difference for dsRNA/sense ssRNA signal (Fig. 1). Each fluorophore has been tested with 22 nucleotide long sense RNA, antisense RNA, and dsRNA, which is made by combination of sense and antisense RNA. Each nucleotide dye has been tested with decreasing content of RNA for their ability to differentiate dsRNA from ssRNA at low RNA content. We have determined the fluorescence intensity of dsRNA and ssRNA at each corresponding RNA content to determine robustness of the dsRNA quantification versus ssRNA. Quantiflour RNA system allowed to differentiate dsRNA content as low as 4 ng/mL and demonstrated up to 2.5 dsRNA/ssRNA signal ratio albeit with variability (Fig. 1a). In addition, Quantiflour RNA system showed preference toward sense RNA, which is rich in uracil (U). This indicates the use of Quantiflour RNA system with U rich RNAs and other combinations of oligonucleotides should be taken into consideration. When we looked at the Quantiflour dsDNA and picoGreen systems, which are mainly used for dsDNA quantification, we have observed higher ratio of dsRNA/ssRNA up to sevenfold difference (Fig. 1b, c). Quantiflour dsDNA system showed a consistent curve of dsRNA/ssRNA ratio

indicating that this fluorophore has a consistent ratio of dsRNA/ssRNA and suitable for measurement of dsRNA formation from ssRNA following RdRp reaction. On the other hand, while picoGreen system provided at least twofold dsRNA/ssRNA fluorescence difference as low as 8 ng/mL, signal ratio varied at different RNA content. We conclude that Quantiflour dsDNA system (and to some extent picoGreen system) could provide an ideal fluorophore for quantification of dsRNA formed during RdRp reactions.

Fluorometric RdRp assay based on BBT-ATP cleavage is limited by inhibitory effect of BBT-ATP in RdRp reaction

It has been recently shown that cleavage of BBT-ATP from following incorporation into dsRNA oligonucleotides could be used for RdRp activity in vitro [16]. We have tested efficacy of this method along with dsRNA specific fluorophores that we have identified (Fig. 2). Thus, we have included various controls including reactions with ATP instead of BBT-ATP. We have found that phi6 RdRp reaction with BBT-ATP is about two times higher compared to no phi6 RdRp reaction (Fig. 2a). However, control group with only BBT-ATP was not different from the full RdRp reaction. This raised a question regarding if phi6 RdRp is functional in this set up. Therefore, we tested formation of dsRNA by Quantiflour dsDNA system. We found that BBT-ATP shows an inhibitory effect on phi6 RdRp activity while ATP doesn't (Fig. 2b). This was also suggested in Niyomrattanakit et al. (2011) as they mention that BBT-ATP inhibits the NS5 activity in 1–4 h (about 20–60 %, respectively) [16]. This indicates that use of BBT-ATP cleavage system in measurement of RdRp activity should be carefully controlled in terms of reaction length along with appropriate use of controls (like of BBT-ATP only reaction).

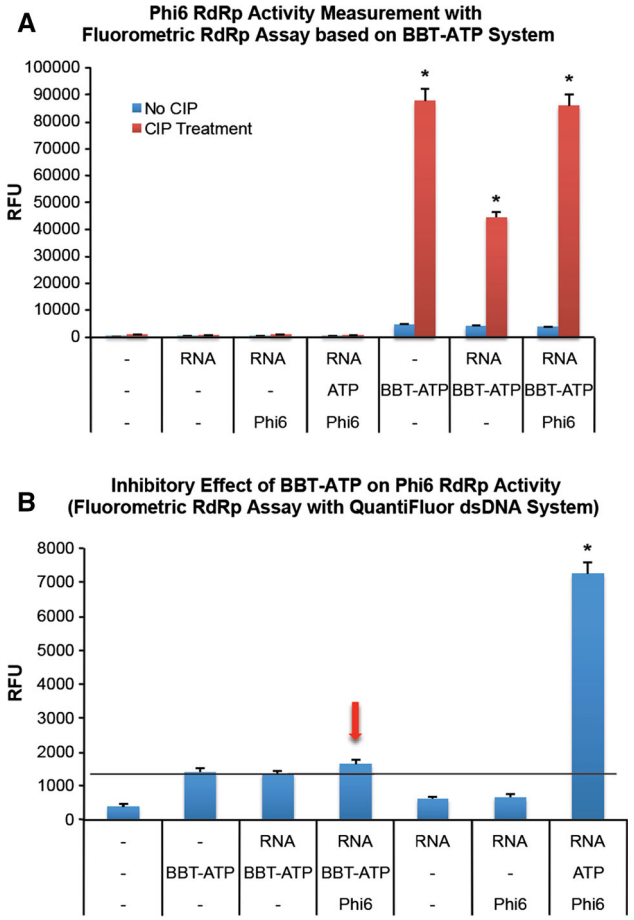

Fig. 2 Fluorometric RdRp assay based on BBT-ATP cleavage. **a** Measurement of phi6 RdRp activity based on fluorometric assay based on BBT-APT system. Note that control group with only BBT-ATP is not different from the full RdRp reaction **b** inhibitory effect of BBT-ATP on phi6 RdRp activity as measured by QuantiFluor dsDNA system (Promega), $*p < 0.05$

In vitro RdRp assay with self-priming RNA

We further characterized the RdRp assay using self-priming RNA followed by quantification of dsRNA by Quantiflour dsDNA fluorophore (Fig. 3a). We found that phi6 RdRp could successfully synthesize dsRNA from self-priming RNA at concentrations up to 500 μM of RNA (Fig. 3b, left panel). This provided up to 2.5-fold difference in dsRNA fluorescence compared to no RdRp reaction (Fig. 3b, right panel). In conclusion, these studies allowed us to identify most potent fluorophores suitable for measurement of in vitro RdRp activity with self-priming RNA.

Discussion

Viral infections could have unanticipated global pandemics with deaths up to half million in a year. Thus, any discovery in the war with viral diseases could have tremendous benefits worldwide. Given that various RNA-containing enzymes are among deadly viruses, antivirals targeting RNA-dependent machinery of viruses such as RdRp are highly valued. Therefore, we studied flouorometric approaches for discovery of RdRp-specific inhibitors. To this end, we used a highly characterized RdRp, the protein P2 from bacteriophage Φ6 as model RdRp. This RdRp could catalyze full-length complementary RNA following initiation at 3′ end of the ssRNA template. Phi6 RdRp has low template specificity, which allowed us to use with self-priming RNA in our RdRp assays.

We have restricted the flourophores tested for RdRp activity based on their readily availability and sensitivity toward nucleotide detections. In addition, flourophores that

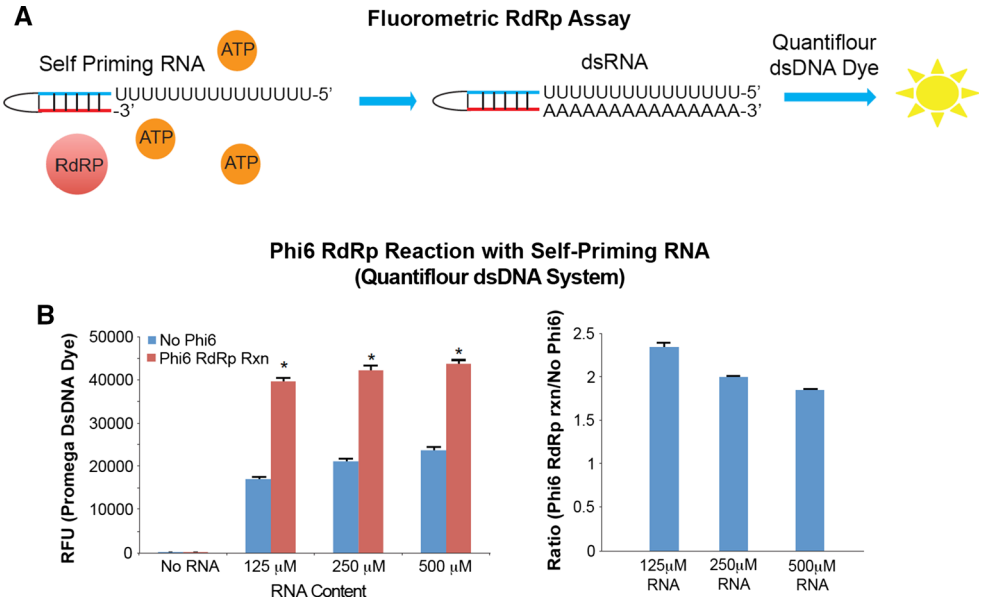

Fig. 3 In vitro RdRp Assay with self-priming RNA. **a** Schematic of fluorometric RdRp assay with self-priming RNA and Quantiflour dsDNA flourophore **b** validation of fluorometric RdRp assay with self-priming RNA, Quantiflour dsDNA system, and phi6 RdRp, $*p < 0.05$

we tested for RdRp assays were suitable for high-throughput assays in 96- or 384-well plate formats. Thus, we used the fluorophore from PicoGreen dsDNA system since its high selectivity (as low as 25 pg/ml of dsDNA) toward double-stranded nucleotides in the presence of free nucleotides, with preference to dsDNA. In addition, PicoGren dsDNA flourophore demonstrated no sequence dependence with self-priming RNA or sense/antisense RNAs that we used in our assays. Moreover, we have tried QuantiFluor® RNA system to find out if flourophore in this system was suitable for dsRNA detection. Although QuantiFluor RNA flourophore were sensitive for quantification of small amounts of RNA, it demonstrated sequence dependence (as seen with sense RNA measurements, rich in Uracil, Fig. 1a) and low signal ratio of dsRNA/ssRNA. QuantiFluor dsDNA system provided another flourophore specific for double-stranded nucleotides, with minimal binding to single-stranded nucleotides and sensitivity as low as 50 pg/ml of dsDNA. In addition to this, here we have shown that fluorophore from QuantiFluor dsDNA system allows robust and consistent quantification of dsRNA at different RNA concentrations and suitable for RdRp assays with self-priming RNA template.

Initial radioactive methods to measure RdRp activity relied on the incorporation of ^{32}P-labeled nucleotides or primer into nascent RNA strands. However, drawbacks of radioactive methods are due to safety concerns, potential harm to environment, and obstacles for high-throughput screening. Apart from radioactive methods, there were attempts to develop nonradioactive assays, depend on digestion of *p*-nitrophenyl moiety from ATP or GTP (PNP-NTPs) by alkaline phosphatase followed by generation of colorimetrically measurable chromophore *p*-nitrophenylate. However, it was resulted low rate affinity of RdRp for those modified NTPs [17]. Another nonradioactive method to measure RdRp activity utilized covalent binding of RNA template to covalink module on solid surface, and colorimetric detection of incorporated biotin-16-UTP to identify NSB5 polymerase inhibitors [18]. However, this study was not readily feasible for high-throughput screening due to its experimental setup. Moreover, a continuous nonradioactive RdRp assay, in which released PP_i reacts with APS by sulfurylase to generate ATP coupled to luciferin/luciferase reaction, was developed to screen NSB5 polymerase activity [19]. Yet, the signal generated could be obscured due to reaction of unmodified NTPs either with luciferase or sulfurylase and might be resulted in inaccurate readings.

RdRp assay based on cleavage of BBT-APT was also reported albeit with some downsides [16]. This assay was limited with inhibition RdRp activity in the presence of BBT-ATP. Moreover, when BBT-ATP content is not properly controlled, free BBT-ATP cleavage may mask the signal from dsRNA products. Intriguingly, although both PicoGreen and QuantiFluor dsDNA system were previously tested by manufactures for their ability to measure dsDNA in the presence of ssDNA, ssRNA, and free nucleotides, they have not been tested for their capacity toward dsRNA [20]. This raises a question whether presence of dsRNA during dsDNA measurements will interfere with the signal. From this study, we clearly show that presence or formation of dsRNAs could be measured using both PicoGreen and QuantiFluor® dsDNA systems, thus presence of dsRNA should be monitored as well when dsDNA measurement is intended.

In conclusion, we have shown that dsRNA could be distinguished from ssRNA based on fluorometric assays, of

which Quantiflour dsDNA system is being the most potent. This has provided in vitro assessment of RdRp activity with self-priming RNA, radioactivity free, and suited for high-throughput drug screening. These findings could be applied to newly purified RdRp proteins from various RNA-containing viruses such as CCHFV, Hazara virus, and others for their activity measurement as well as small molecule inhibitor discovery for respective viral RdRps.

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Conflict of interest All authors declare that they have no conflicts of interest concerning this work.

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