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1 # A Comprehensive Roadmap Towards Generation of Influenza B Reporter Assay Using a
2 Single DNA Polymerase Based Cloning of Reporter RNA Construct.

3 Nandita Kedia[1], Saptarshi Banerjee[1], Arindam Mondal[1*]

4 [1]School of Bioscience, Indian Institute of Technology Kharagpur, Kharagpur -721302, India
5
6 *Corresponding author
7

8 **Abstract:**

9 Mini-genome reporter assay is a key tool for conducting RNA virus research. But, procedural
10 complications and lack of adequate literature pose major challenge towards developing these
11 assay systems. Here we present a novel yet generic and simple cloning strategy for construction
12 of influenza B virus reporter RNA template and describe extensive standardization of the reporter
13 RNP/ polymerase activity assay for monitoring viral RNA synthesis in infection free setting. Using
14 this assay system, we, for the first time showed the effect of viral protein NS1 and host protein
15 PKC-Delta upon influenza B virus RNA synthesis. Additionally, the assay system showed
16 promising results in evaluating efficacy of antiviral drugs targeting viral RNA synthesis and virus
17 propagation. Together, this work offers a detailed protocol for standardization of influenza virus
18 mini-genome assay and an excellent tool for screening of host factors and antivirals in a fast, user
19 friendly and high throughput manner.

20 **Keywords:**

21 Influenza B virus, engineering reporter construct, ribonucleoprotein particles, reporter based
22 activity assay, screening of host factors and antivirals.

23 **Introduction:**

24 First discovered in 1940[1], the influenza B virus has since been causing significant morbidity and
25 mortality in the global population[2]. As per the recent surveillance (seasons 2010–2018)
26 influenza B viruses are responsible for 15-30% of the total influenza like illness, with a number of
27 complications like fevers, body ache, fatigue and even life threatening acute respiratory distress
28 syndrome for patients having pre-existing lung diseases[3, 4]. There are two different lineages of
29 influenza B virus, Victoria and Yamagata, which circulate in the human population with various
30 degrees of predominance in different influenza seasons[5, 6]. Due to constant increase in the
31 influenza B virus related infections and limited cross protection offered by influenza B vaccine
32 against both of these lineages, there is a gradual transition from trivalent (against two subtypes
33 of FluA and one lineage of FluB) to quadrivalent (against two subtypes of FluA and two lineages
34 of FluB) flu shots offered across the world[7]. In spite of its immense importance in the context of
35 global healthcare ecosystem, influenza B virus research has drawn significantly lesser attention
36 in comparison to the closely related influenza A viruses, largely due to the scarcity of the tools
37 required to study virus replication cycle. This also severely restricts antiviral drug discovery
38 directed towards influenza B virus therapy[8].

39 Influenza viruses are segmented negative sense RNA viruses of the *Orthomyxoviridae* family.
40 Amongst the four types A, B, C and D, only influenza A and B cause human epidemics[9]. Viral

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41 genome consists of eight different segments each of which remains enwrapped with multiple
42 copies of nucleoprotein (NP) in their oligomeric form and associates with a single copy of RNA
43 dependent RNA polymerase (RdRp) to form the ribonucleoprotein complexs or RNPs[10]. RNPs
44 are the self-sufficient machinery for driving different modes of RNA dependent RNA synthesis
45 events including viral gene expression and genome replication, hence reside at the center of virus
46 replication cycle[11]. This is why reporter RNP based assay systems remain one of the invaluable
47 tools for studying virus replication, host-pathogen interaction and high-throughput screening of
48 antivirals without handling infectious virus particles and hence avoiding biosafety associated
49 procedural complications[12, 13].
50
51 Influenza virus genomic segments are single stranded RNA that are devoid of the 5'-Cap and 3'-
52 Poly(A) tail structures[14]. Different segments harbor conserved untranslated regions of variable
53 lengths both at the 5' and 3' ends, which bracket single or multiple open reading frames (in the
54 antisense orientation) encoding viral proteins[14, 15]. Terminal regions of the 5' and 3' UTRs
55 contain complementarity resulting in a partial duplex structure (also known as "panhandle RNA"
56 or "cork-screw RNA") that serves as the promoter for RdRp[16]. Additionally, the UTRs contain
57 cis-acting elements, necessary and sufficient for transcription and replication of viral[17–19]and
58 non-viral reporter genes[12, 16, 20, 21]. Several groups have established reporter RNA based
59 assay systems where viral open reading frames have been replaced with reporter genes of
60 fluorescence or chemiluminescent proteins[12, 13, 16, 22–25]. These reporter RNA templates,
61 when expressed inside the cells in combination with NP and RdRp proteins, reconstitute reporter
62 RNPs. RNA synthesis activity of these RNPs could be measured by quantifying the extent of
63 reporter gene expression. Although appears to be straightforward, successful establishment of
64 reporter assay system requires (i) complicated cloning strategies to synthesize the reporter RNA
65 construct, (ii) construction of plasmids for expression of viral NP and RdRp subunits (PB1, PB2
66 and PA), and (iii) optimized expression of the reporter RNA and viral proteins in required
67 stoichiometric amounts that leads to the reconstruction of reporter RNPs with maximum efficiency.
68
69 So far, different strategies have been used to construct plasmids expressing influenza A and B
70 virus reporter RNA template (reporter plasmids)[12, 13, 24–26]. In few studies, reporter luciferase
71 gene was amplified using primers containing long overhangs corresponding to 3' and 5' UTR
72 regions of influenza A or B viruses; resulting PCR fragment harboring luciferase ORF flanked by
73 the viral UTRs were then inserted into the target vector for RNA polymerase-I driven expression
74 of the same, using conventional restriction digestion and subsequent ligation method[12, 21, 27].
75 Alternatively, viral 5' and 3'-UTR containing vectors (amplified using inverse PCR from the cDNA
76 clone of the corresponding segment) were ligated with reporter gene insert predigested with
77 compatible restriction enzyme sites[28]. In an independent cloning strategy, a double-stranded
78 DNA linker encompassing 5' and 3'-UTRs was inserted into the vector in-between the Pol-I
79 promoter and terminator sequences with the help of compatible restriction enzyme sites. The
80 reporter gene was then inserted between the UTRs using a second restriction enzyme site[24].
81 These restriction enzyme based cloning strategies are laborious and often introduce additional
82 nucleotides between the UTRs and the reporter gene which may interfere with the activity of the
83 cis/trans acting elements[24]. In order to avoid these constraints, restriction enzyme free cloning
84 methods utilizing vectors and inserts containing overlapping sequences have also been

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85 implemented. For example, inserts containing reporter genes flanked by the 5' and 3'-UTRs were
86 created using long overhang primers (containing the UTR regions) which was then stitched to the
87 vector through the use of specialized proprietary enzymes/kits[13, 29]. With the inherent
88 limitations of the aforesaid cloning techniques, scarcity of information about the extensive
89 experimental protocol makes it difficult to establish and standardize the reporter based RNP
90 activity assay for influenza viruses. This situation gets further complicated for influenza B viruses
91 due to the larger size of the UTRs compared to the same for influenza A viruses.
92
93 Here we present a novel yet fairly simple cloning strategy, independent of any restriction enzyme
94 or specialized reagents or kits, to construct a firefly luciferase based reporter plasmid capable of
95 generating reporter genome template for influenza B/Brisbane/60/2008 virus. Additionally, we
96 present extensive standardization of this reporter plasmid based RNP activity assay through
97 optimization of various parameters regulating viral RNA synthesis. Using the reporter assay
98 system, we showed for the first time the effect of viral non-structural protein-1 (NS1) and host
99 protein kinase C delta (PKCD) upon influenza B virus RNA synthesis. We also demonstrated the
100 ability of this assay system to be used as a high throughput screening platform for the identification
101 of antiviral drugs specifically inhibiting RNA polymerase activity of the virus. Together this work
102 presents a great resource for cloning, standardization and implementation of reporter based RNP
103 activity assay for influenza and other related viruses.
104

105 **Materials and methods:**

106 **Cell lines and Viruses**

107 Human embryonic kidney 293T (HEK 293T) cells were maintained in Dulbecco's modified Eagle's
108 medium (DMEM; Invitrogen, Cat no. #12800017) supplemented with 10% (v/v) fetal bovine serum
109 (FBS; Invitrogen, Cat no. #10082147), 2mM GlutaMAX™ (Invitrogen, Cat no. #35050061), 1%
110 penicillin-streptomycin (Invitrogen, Cat no. #1514122) incubated at 37°C in a humidified 5% CO_2
111 incubator. Madin-Darby Canine Kidney (MDCK) cells were maintained in same conditions with
112 10% FBS (FBS; Invitrogen Cat no. #10270106). Influenza B/Brisbane/60/2008 virus was used in
113 this study.
114

115 **Virus amplification and RNA Extraction**

116 3 x 10^6 MDCK cells were seeded in 10 cm dishes, 24 hours before the infection. Prior to infection,
117 cell monolayer was washed with PBS twice and subsequently infected at an M.O.I. of 0.001. For
118 each 10cm plate, 1 mL virus inoculum was prepared in virus growth media (VGM; containing
119 DMEM, 0.2% bovine serum albumin (Sigma; Cat no. #A8412), 25 mM N-(2-hydroxyethyl)
120 piperazine-N′-ethanesulfonic acid (HEPES; Invitrogen, Cat no. #15630080) buffer, 2mM
121 GlutaMAX™, 1% penicillin-streptomycin and 0.5 µg/mL TPCK-trypsin (Thermo Scientific™, Cat
122 no. #20233)). Virus attachment was performed with 1ml of inoculum for 1 hour at 37°C in
123 humidified 5% CO_2 incubator with intermittent shaking at every 10 minutes to prevent drying of
124 cell monolayer and homogenous distribution of the inoculum. Post attachment, each 10 cm plate
125 was supplemented with 6 mL of VGM and incubated either at 37°C for Influenza A virus and 33°C
126 for Influenza B virus in humidified 5% CO_2 incubator. At 72 hours post infection, supernatant was
127 collected and centrifuged at 3200 g for 10 minutes at 4°C to remove the cell debris. The

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128 supernatants were collected and aliquots were stored at 80°C refrigerator for further
129 applications[30].
130

131 **Reverse transcription (RT)-PCR**

132 Viral RNA was extracted from amplified virus stock using Trizol reagent (Invitrogen; Cat no.
133 #15596018). Reverse transcription was carried out using primer 'Uni9', which is complementary
134 to all of the individual genomic RNA segments of Influenza B/Brisbane/60/2008 virus[31] with
135 Verso cDNA Synthesis Kit (Thermo Scientific™; #AB1453A) according to manufacturer's
136 instructions. After the RT reaction, segment 6 (NA-NB) of the viral genome was amplified using
137 the 'NA-NB_F' and 'NA-NB_R' primers with the help of Phusion High-Fidelity DNA Polymerase
138 (Thermo Scientific™, Cat no. #F530S). The PCR product was purified using PCR Purification Kit
139 (Invitrogen; Cat No. # K310001) and the yield and quality of the purified product was checked
140 measuring the absorbance at 260 and 280nm and subsequently by running it on agarose gel.
141

142 **PCR and Cloning**
143

144 i) **Amplification of 5' and 3' UTR:** The RT-PCR amplified DNA corresponding to
145 segment 6 of viral genome was used as a template for the amplification of 5'UTR and
146 3'UTR using Phusion High-Fidelity DNA Polymerase using 5' UTR_F, 5' UTR_R and 3'
147 UTR_F, 3' UTR_R primers pairs and the PCR product were purified using Quick gel
148 extraction kit (Invitrogen: Cat no. # K210012). The purified double-stranded 5'UTR
149 fragment and 3'UTR fragment were then used as primers for the amplification of
150 luciferase gene described in the next section.
151

152 ii) **Preparation of Insert:** The luciferase ORF was amplified using the pHH21-vNA-Luc
153 as a template, kindly provided by Dr. Andrew Mehle. The double-stranded 5'UTR and
154 3'UTR fragments, synthesized in the previous step, were used as primers (5uM final
155 concentration) for the PCR amplification of Luciferase ORF using Phusion high fidelity
156 DNA polymerase, following manufacturer's protocol. The PCR product was analyzed
157 on 0.8% agarose gel and purified using PCR purification kit.
158

159 iii) **Preparation of Vector:** The vector was amplified using the pHH21-vNA-Luc as a
160 template. PHH21_F' & 'PHH21_R' primers were used to amplify and linearize the
161 vector using Phusion high fidelity DNA polymerase using 5x Phusion GC rich buffer
162 following manufacturers' protocol. The PCR product was analyzed on 0.8% agarose
163 gel and purified using PCR purification kit.
164

165 iv) **Circular Polymerase Extension Cloning:** In the final CPEC assembly reaction, the
166 purified linearized vector and inserts are mixed together maintaining a vector to insert
167 molar ratio (V:I) = 1:1, 1:2 or 1:3, with the PCR reaction components, that consists of
168 0.4ul of 40mM dNTPs, 4ul of 5x Phusion HF Buffer, 0.25 µl of Phusion High-Fidelity
169 DNA Polymerase, 100ng of Vector and required amount of Insert to make the final
170 volume of 20ul. The composition of the CPEC reaction is identical to that of a standard
171 Phusion PCR mix, except that, there is a high concentration of dNTPs and no primers
172 are added. The thermal cycling conditions were as follows: 98 ◦C for 30 s, 25 cycles
173 of 98 ◦C for 10 s, 72 ◦C for 1 min 30 s, followed by 72 ◦C for 10 min. To assess if a
174 CPEC reaction is successful or not, 5ul of the product was analyzed by agarose gel

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175 electrophoresis. The V:I=1:3 reaction showing highest intensity of the high molecular
176 weight band corresponding to the total length of vector and Insert was selected for
177 transformation.
178

179 **Transformation**

180 *E. Coli.* DH5 alpha competent cells were prepared by modified rubidium chloride method as
181 described by Glover et al[32]. For transformation, 10ul of the CPEC reaction mixture were added
182 to the competent cells. Followed by an incubation of 30 minutes, cells were subjected to a brief
183 heat shock at 42^0C for 35 seconds followed by 5 minutes incubation in ice. Cells were then grown
184 in 400ul of Luria bertani broth (Himedia, Cat no. # M1245; 2.5% in double distilled water) at 37^0C
185 incubator for 1.5 hours with shaking at 220rpm and the entire culture volume was spread upon
186 LB-agar plates containing 100ug/ml of Ampicillin. Colony PCR was performed to identify positive
187 clones which were then verified through Sanger sequencing.
188

189 **Generation of polymerase protein expressing plasmids**

190 The PB2, PB1 and PA ORFs were cloned into the pCDNA-3X-FLAG vector (generously provided
191 by Dr. Andrew Mehle) which is a modified version of pcDNA3.1 (addgene) vector expressing
192 proteins under CMV promoter. This vector contains three FLAG epitopes joined in tandem (3X-
193 FLAG) after the NotI site at its MCS followed by a Cytosine. This results in the expression of a
194 protein having tri-alanine linker in between the individual ORFs and the C terminal 3X-FLAG tag.
195 For the expression of untagged version of each RdRp subunit, the stop codon has been kept
196 intact at the end of ORF. For the expression of V5 tagged NP, pcDNA3 vector has been modified
197 in order to have glycine-glycine-serine-glycine linker in between the ORF and the C terminal V5
198 epitope tag. Briefly, two primers of 58 nucleotide length were annealed to create double-stranded
199 piece of DNA having sticky ends on both sides (BamHI restriction site at the beginning of the
200 sequence and the NotI site at the end of the sequence). The thermal protocol for ramp down
201 annealing was as follows: 95 ◦C for 5 minutes, 70 cycles of 95^0 C (-1^0C/cycle) each for 1 minute,
202 followed by hold at 4◦C. The V5 linker was phosphorylated at 5' end by treatment with T4
203 Polynucleotide Kinase (PNK, Cat no. # M0201S). The pcDNA3.1 (addgene) vector was digested
204 with BamHI and NotI, treated with Calf alkaline phosphatase (CIP; NEB, Cat no. #M0290S) and
205 ligated with the V5 linker in V:I = 1:20 ratio.

206 Each individual insert fragment have been amplified using the cDNA template with the primers
207 containing restriction enzyme overhangs. The PB2, PB1 and PA have been amplified with EcoRI
208 and NotI overhang in two different PCR sets, one omitting the stop codon and the other including
209 the stop codon in the reverse primer. The NP have been amplified using primers with KpnI and
210 BamHI overhangs. For each amplification, 50 µl of PCR reaction consisted of 10 µl of 5x Phusion
211 HF buffer, 5 µl each of the forward and reverse 5 µM primers, 5 µl of 2mM dNTPs, 5 µl of cDNA
212 template, 19.5 µl sterile nuclease free water and 0.5 µl of Phusion High-Fidelity DNA
213 Polymerase. The modified pcDNA3-3X-FLAG have been digested with EcoRI & NotI (NEB) and
214 the pcDNA3-V5 vector has been digested with KpnI & BamHI, followed by treatment with CIP.
215 The digested vectors as well as insert fragments were gel excised and ligated in a vector to insert
216 ratio of 1:3 using T4 DNA ligase (Thermo Scientific™, Cat no. # EL0011) as per manufacturer's
217 protocol. 10 ul of ligation mixture was transformed into DH5 alpha competent cells. Sequences of
218 all the clones have been verified by sequencing.

219 **Transfection**

220 For examining protein expression level of each plasmid, HEK 293T cells were transfected using
221 lipofectamine3000 (Invitrogen: Cat no. # L3000015) as per manufacturer's instructions. All the
222 plasmids were prepared using plasmid DNA isolation kit (Promega, Cat No. # A1222). The

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223 pcDNA3.1 blank vector was used in the control cells. The media was changed 12 hours post
224 transfection and incubated for 36 (or stated otherwise) hours following transfection.
225
226 **Western Blot**
227 Protein levels for transiently transfected cells were assessed by Western blotting. Transfected
228 cells were lysed for 20 minutes in pre-chilled Co-Immunoprecipitation buffer (50mM Tris-HCl pH
229 7.4, 150mM NaCl, 0.53% NP-40) containing protease inhibitors (Roche-Sigma, Cat no. #
230 11873580001) and phosphatase inhibitor and subsequently total protein samples were separated
231 via 8% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF, Bio-Rad, Cat no.
232 #1620177) membrane using transfer buffer (25 mM Tris, 191 mM glycine, 0.025% SDS & 10%
233 methanol (vol/vol) in Trans-Blot Turbo Transfer System (Bio-Rad). After incubation with 5% nonfat
234 milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min, the membrane was
235 washed once with TBST and incubated with antibodies against 3xFLAG (1:5000, Sigma, Cat no.#
236 F3165), V5 (1:5000, CST, Cat no. # D3H8Q), HA (1:5000, Cat no. # C29F4), BNP (1:5000,
237 generated in collaboration with BioBharati LifeScience Pvt. Ltd, India), at 4 °C for 12 h.
238 Membranes were washed with TBST three times for 5 minutes and incubated with a 1:25,000
239 dilution of horseradish peroxidase-conjugated anti-mouse (Sigma, Cat no. # A9044-2ML6) or anti-
240 rabbit (Sigma, Cat no. # A0545-1ML) antibodies for 1 h. Blots were washed with TBST three times
241 for 5 minutes and developed with the ECL system (ThermoFisher, Cat No. #34095) according to
242 the manufacturer's protocols.
243
244 **Polymerase activity assay**
245 HEK293T cells were co-transfected with 94.11 ng of each of the pcDNA3-PB2-FLAG, pcDNA-
246 PB1, 11.76 ng of pcDNA-PA, 100 ng of pcDNA3-BNP and 100 ng of pHH21-vNA-Luc plasmids.
247 The additional plasmids were used in various amount upto 150 ng and is topped up by blank
248 vector in order to keep the amount of DNA same in all the sets. In absence of additional plasmid,
249 the total amount of RNP reconstitution plasmids were increased upto 500ng keeping the ratio of
250 each RNP component same (1/4th BNP, 1/4th pHH21-vRNA-Luc, and the half of the total amount
251 of DNA will be divided as 8:8:1 ratio for PB2, PB1 and PA). The transfection mix were prepared
252 with Lipofectamine 3000 as per manufacturer's protocol using optiMEM (Thermo Scientific™, Cat
253 no. #31985-070). At 12 hours, the media was changed very carefully without dislodging any cell
254 that may result in error in further signal. The cells were harvested at 36 hours (or as mentioned)
255 post transfection and luciferase activity assay was performed using Promega Luciferase Assay
256 System (Promega, #E1500 & #E1910), as per manufacturer's protocol using luminometer
257 (Promega Glomax 20/20).
258
259 **Ribavirin and Favipiravir dose-response assays in HEK 293T cells**
260 Ribavirin (Sigma-Aldrich, Cat no. # R9644) was dissolved in water to prepare 80 mM stock and
261 Favipiravir (MedChemExpress, Cat no. #HY-14768) was dissolved in DMSO to prepare a 200
262 mM stock which were aliquoted and stored at – 80^{0}C until used. 0.2 x 10^6 HEK 293T cells were
263 seeded in 24-well plates and post 24 hours were treated with specified concentrations of ribavirin
264 or favipiravir for 2.5 h at 37^0 C with 5% CO2. Subsequently, cells were transfected with
265 lipofectamine 3000 as per manufacturers protocol and incubated in fresh media containing
266 specified concentrations of drugs were for 36 hours. Polymerase activity was then assayed as
267 described above. The IC50 value was calculate by fitting the data to four parameter nonlinear
268 equation.
269
270 **3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay**
271 30,000 HEK293T cells were seeded in 96-well plate. After 24 hours of seeding, the cells were
272 treated with different concentrations of drugs in triplicates for 36 hours. Post-treatment, 100 μL of
273 MTT reagent (5 mg/mL, SRL) dissolved in phosphate-buffered saline (PBS) was added to the

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274 cells and incubated for 3 h at 37 °C. Subsequently, the reagent was removed and the formazan
275 crystals were dissolved by adding 100 µL of dimethyl sulfoxide (DMSO) (Sigma) in each well. The
276 absorbance of the suspension was measured at 595 nm using an Epoch 2 microplate reader
277 (BioTek Instruments). The percentages of metabolically active cells were compared with the
278 percentage of control cells treated with vehicle control.
279
280 **Transfection-Infection assay**
281 0.1 x 10^6 HEK 293T cells were seeded in 48-well plates. After 20 hours of seeding, the cells were
282 transfected with 0.25 ug of reporter plasmid using lipofectamine 3000 and 22 hours post
283 transfection, the cells were pre-treated with half-maximal inhibitory concentrations (IC50) of the
284 drugs (Ribavirin:18.54 uM; Favipiravir:25.46 uM) for 2.5 h at 37^0 C with 5% CO2. Following 2.5
285 hours of treatment, the cells were infected with Influenza B virus at an MOI of 0.1 in presence of
286 drugs and the polymerase activity was assayed at 16 hours of post infection.
287
288 **Statistical analysis**
289 The arithmetic mean and standard deviation of the firefly luciferase signal were calculated from
290 three biological replicates for each experiment. The data were plotted in bar diagram with
291 standard deviations as error bars. For dual luciferase assay, the firefly luciferase signal was
292 normalized with the *Renilla* luciferase signal for each replicate. In firefly luciferase assays
293 involving the host factors, viral factors, and antiviral molecules and dual luciferase assays;
294 normalized mean and standard deviation were calculated against the control. The normalized
295 mean was calculated by dividing the arithmetic mean of the experimental sets by the mean of the
296 control set and converting it to a percentage value. Normalized standard deviation was calculated
297 by normalizing the coefficient of variations against the control and augmenting it with the normal
298 mean. A two-tailed Student's T-test was performed for comparison of the data sets.
299
300 **Results:**

301 **Generation of influenza B virus reporter plasmid for expression of viral reporter RNA in**
302 **mammalian cells**

303 In order to establish a reporter based RNP activity assay, a template RNA harboring the reporter
304 gene flanked by the viral UTRs needs to be expressed under the control of RNA polymerase I
305 promoter. This ensures that the reporter RNAs are devoid of any 5'- or 3'- terminal modifications,
306 hence mimicking authentic viral genomic RNA. To achieve this, firstly we have constituted the
307 "insert" harboring the firefly luciferase gene in reverse orientation flanked by viral 5'- and 3'- UTRs.
308 Subsequently, this cassette was introduced into the pHH21 vector in between the RNA
309 polymerase I promoter and terminator. The entire process of constituting authentic viral UTRs,
310 assembling them with the reporter gene and introducing this cassette into the pHH21 vector
311 utilized a single DNA polymerase enzyme without the need of any restriction enzyme or
312 specialized kits (Figure 1).

313 Viral genomic RNA, purified from influenza B/Brisbane/60/2008 virus particles (Figure 1A), was
314 used as a source for the amplification of the 144nt 5'- and 95nt 3'-UTRs, using sequence specific
315 primers with 15-20 nucleotide overhanging sequences corresponding to the vector and the
316 reporter gene (Figure 1B, 2A). The resulting PCR products thus contain (i) viral 5'-UTR flanked
317 by the overlapping sequences with the Pol-I promoter and 3'-termini of the reporter gene and (ii)
318 viral 3' UTR region flanked by the overlapping sequence with 5'-termini of reporter gene and Pol-
319 I terminator. These double-stranded PCR products were then used as primers to amplify the

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320 firefly luciferase gene from the pHH21-vNA-Luc plasmid, kindly provided by Prof. Andrew Mehle,
321 University of Wisconsin Madison (Figure1C, 2B). The final PCR product, constitutes reporter gene
322 flanked by viral 5'- and 3'- UTR regions along with partial sequences from the Pol-I promoter and
323 terminator regions at the extreme 5'- and 3'- termini respectively. In order to synthesize the final
324 reporter plasmid construct, named as pHH21-BNA-Luc, this cassette was inserted into the pHH21
325 vector (amplified in a separate PCR reaction; Figure 2C) using the Circular Polymerase Extension
326 Cloning (CPEC), as originally described by Quan et al[33, 34] (outlined in the Figure 1D). A vector
327 to insert molar ratio of 1:3 generated maximum amount of assembled product (Figure 2D).
328 Reaction product was transformed in chemically competent *E. coli* and successful incorporation
329 of the insert was confirmed by the colony PCR screening method. All the PCR amplifications were
330 performed using a single Phusion High-Fidelity DNA polymerase as described in further detail in
331 the methods section.

332 To reconstitute functional reporter RNPs inside the cells, reporter RNA template needs to be co-
333 expressed with NP and the RdRp subunits, PB1, PB2 and PA (Figure 1E, F). The RdRp subunits
334 were cloned into the pCDNA-3X-FLAG vector (generously provided by Dr. Andrew Mehle) under
335 the control of CMV promoter with the help of EcoRI and NotI restriction enzymes, which results in
336 incorporation of a tri-alanine linker in between the individual ORFs and the three FLAG epitopes
337 joined in tandem (3X-FLAG). For expression of untagged proteins, ORFs with the stop codon
338 were cloned using the same strategy. The NP gene was cloned into a modified pcDNA3 vector
339 harboring V5 epitope tag (mentioned in the methods section) with the help of the KpnI and BamHI
340 sites, with a glycine-glycine-serine-glycine linker in between the ORF and the V5 epitope tag.

341 **Standardization of influenza B virus RNP activity assay**

342 Influenza B virus reporter RNPs were reconstituted in HEK293T cells through transient
343 transfection of the reporter plasmid (pHH21-BNA-Luc) either in the absence (negative control) or
344 the presence of the plasmids expressing PB1, PB2, PA and NP proteins (Figure 3A). Cells were
345 harvested at 24 hours of post-transfection and luciferase activity was measured to quantitate the
346 Influenza B RNP activity. To our surprise, the positive control set showed only 10^4 signal
347 (luciferase light unit or RLU) which is only two log higher than the negative control set (10^2 RLU),
348 suggesting suboptimal activity of the reconstituted RNPs. This could be due to the poor
349 expression levels of the RdRp subunits, PB1 and PB2, in comparison to the PA and NP proteins,
350 as observed from the western blot analysis using specific antibodies, hence prohibiting the
351 successful assembly of reporter RNPs inside the cell. To investigate this further, we examined
352 the sequence of the constructs carefully and noticed that all of the protein expression plasmids
353 lack the Kozak sequence which may result in their suboptimal translation. The PA gene have a
354 Guanosine after the ATG sequence which is a part of Kozak sequence. PB1 and PB2 do not have
355 this Guanosine after the start codon ATG. Thus half Kozak sequence is conserved in PA gene
356 which makes its expression better than PB2 and PB1.

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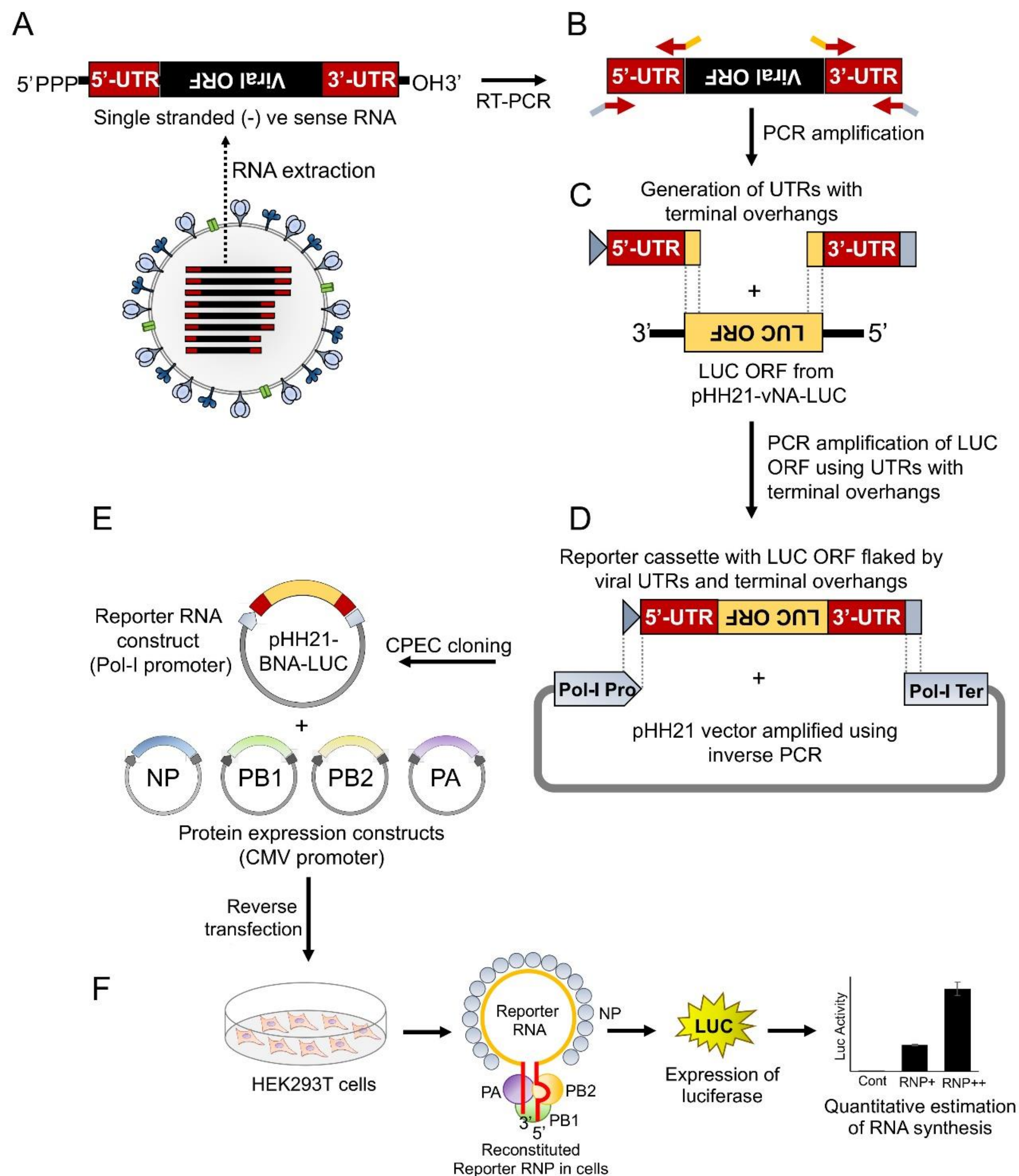

357

358 **Figure 1:** An illustrative overview of cloning strategy of influenza B reporter plasmid and reporter assay (A) Total RNA
359 was isolated from amplified stocks of Influenza B/Brisbane/60/2008 virus (B) total RNA was converted to cDNA by
360 performing RT-PCR and 5' & 3' UTRs were amplified using specific primers containing overhangs. (C) The double-
361 stranded 5' and 3' UTRs containing overlapping regions were used as primers to amplify the luciferase ORF. (D) The
362 resulting PCR product was used as an insert for CPEC assembly with the PCR amplified vector fragment. (E-F) The
363 generated reporter and other protein expressing plasmids upon co-transfection in HEK293T cells reconstitute the
364 luciferase RNP's that express luciferase enzyme under the control of the viral promoter. The quantification of the
365 luciferase signal gives the measure of viral polymerase activity.

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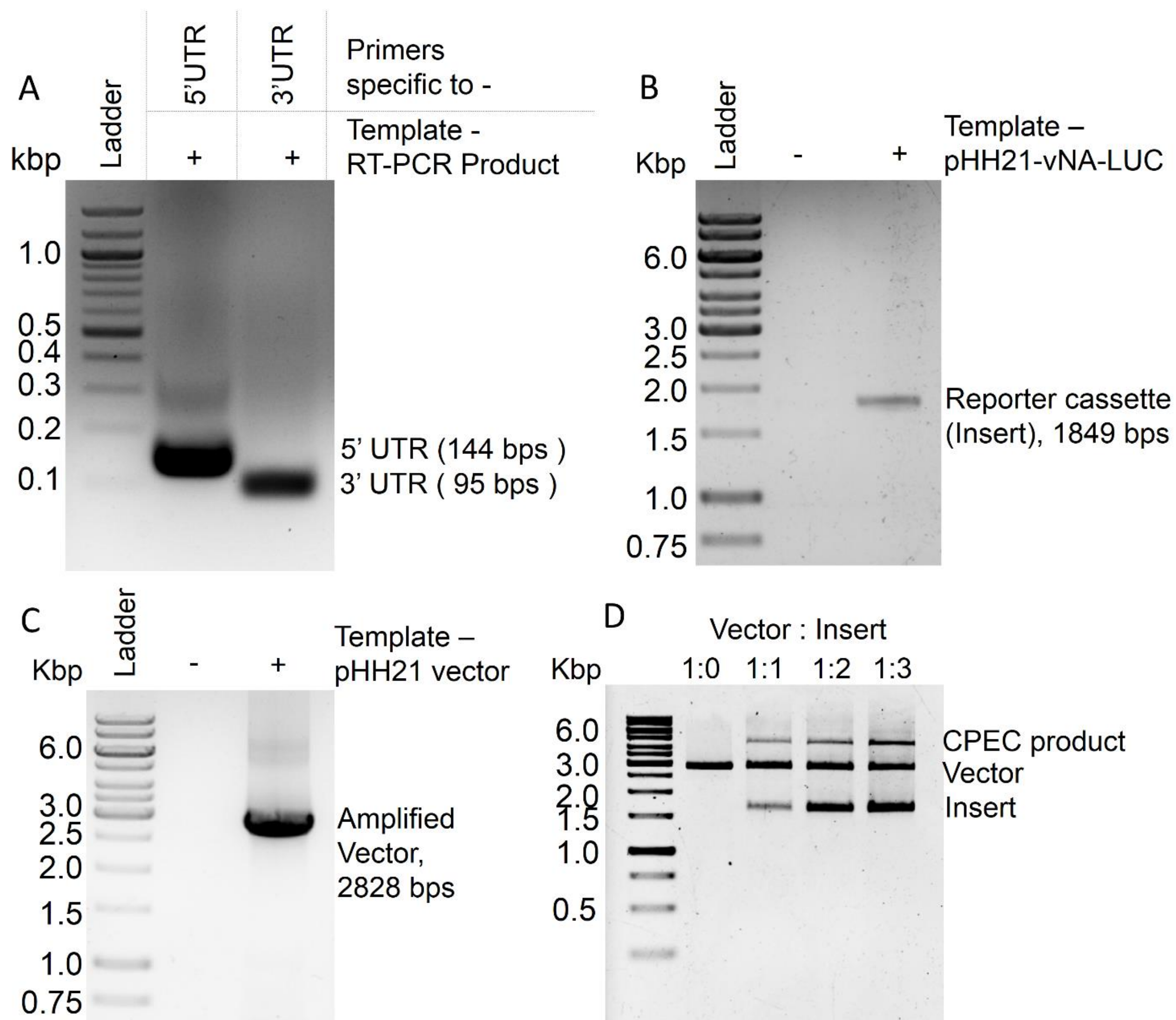
366 To address this, we performed site-directed mutagenesis to introduce partial Kozak sequences in
367 each of these plasmids without any alteration in the ORF and repeated the polymerase activity
368 assay with them. As evident from figure 3A, the introduction of the Kozak sequence significantly
369 boosted the expression of all of the RNP proteins which together resulted in reporter activity of
370 10^6 RLU, four logs higher than the negative control set. Interestingly, the expression levels of
371 the PA subunit still remained severalfold higher than the other two subunits of RdRp, PB1 and
372 PB2 (Figure 3A).



373
374 **Figure 2:** PCR amplification and CPEC reaction for construction of reporter construct: Agarose gel electrophoresis
375 images of (A) PCR amplification products corresponding to the 5'UTR and 3'UTR of NA-NB segment. (B) PCR
376 amplification product of luciferase ORF using double-stranded PCR products corresponding to 5' and 3' UTRs as
377 primers. (C) PCR amplification of pHH21 vector. (D) CPEC products with different ratio of vector to insert.
378

379 Precise abundance of the PB1, PB2 and PA subunits in equimolar amounts is a pre-requisite for
380 the successful assembly of the heterotrimeric RdRp complex and hence, reconstitution of reporter
381 RNPs to optimum levels. Therefore, we tried to optimize the amount of the plasmids to be
382 transfected in order to have a comparable expression of the RdRp subunits. Reporter RNPs were

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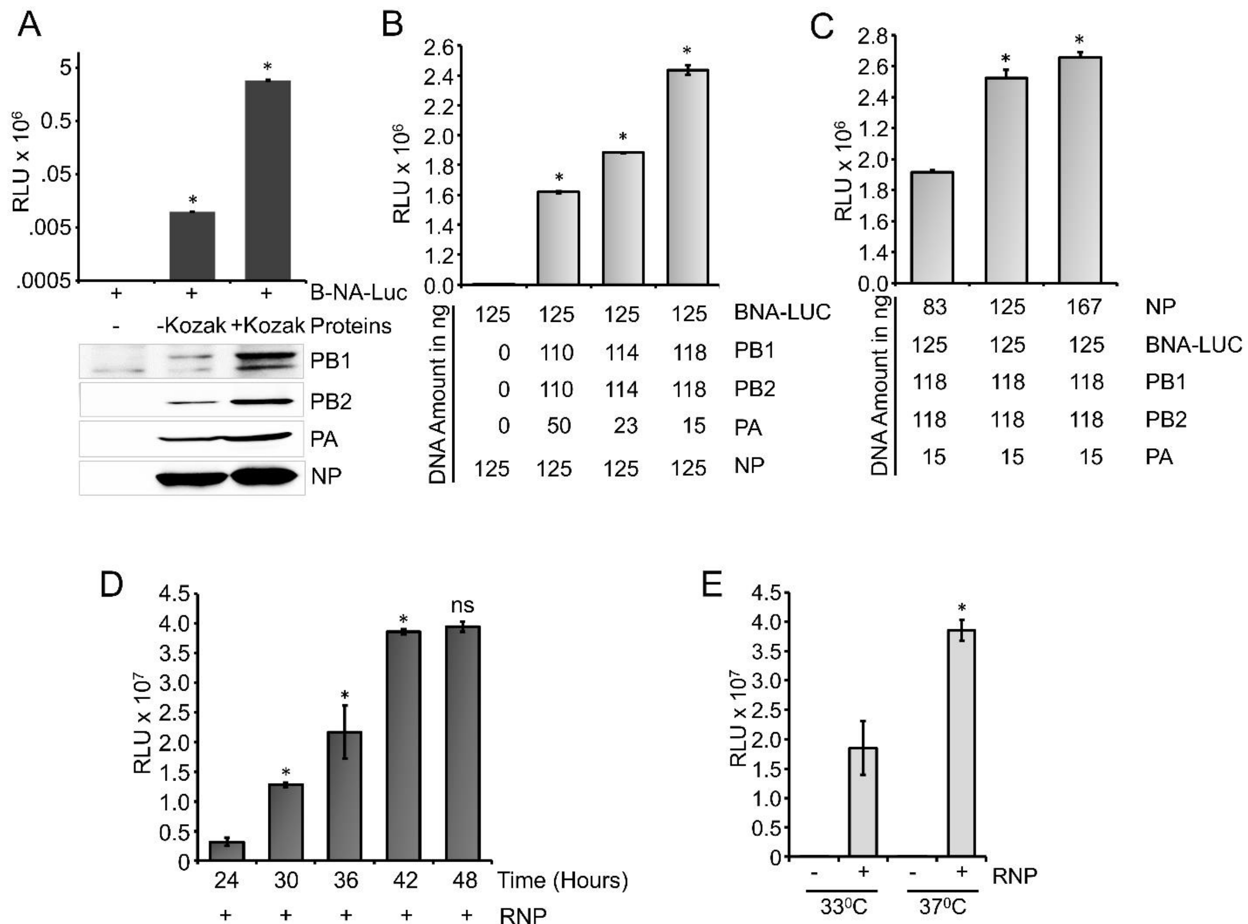
383 reconstituted using different ratios of RdRp subunit plasmids, while keeping the amount of the
384 reporter RNA and NP plasmid constant. As shown in Figure 3B, increasing the amount of PB1
385 and PB2 expressing plasmids compared to the PA led to a gradual increase in reporter activity
386 and a ratio of 8:8:1 for PB1: PB2: PA resulted in comparable expression of all three polymerase
387 subunits and maximum reporter activity. Subsequently, keeping the ratio of the polymerase
388 subunit plasmids constant, we increased the amount of the NP expressing plasmid, which
389 resulted in increase in the reporter activity, hence stretching the sensitivity of this reporter assay
390 to the maximum level (Figure 3C). The NP to polymerase proportion up to 1:2 results in increase
391 in polymerase activity. Further increase in the amount of NP does not result in substantial increase
392 in polymerase activity. Hence for our further experiments, we have used this ratio of RNP
393 reconstituting plasmids. Once we optimize the amounts of various plasmids reconstituting reporter
394 RNPs, we have performed a time kinetics experiment in order to assess the optimum time
395 required to obtain a signal of 10^6 RLU or more (Figure 3D).



396

397 **Figure 3:** Optimization of the reporter system: (A) Effect of Kozak sequence on expression of viral polymerase proteins
398 and influenza B RNP activity assay (B) Reporter RNP activity assay with different ratio of PA protein expression plasmid
399 with respect to PB1 and PB2. (C) Reporter RNP activity assay with various amount of NP expression plasmid. (D)
400 Optimization of time for reporter activity assay (E) Optimization of incubation temperature for influenza B RNP activity

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401 assay (n=3 ± standard deviation, *p<0.05 one-way ANOVA with post hoc Student's t-test when compared to the
402 preceding set, for Figure E, comparison was performed in between two RNP positive sets, ns = not significant).

403 A time dependent increase in the reporter activity was observed which reaches a plateau by 42
404 hours of post transfection. Additionally, influenza B polymerase activity was assessed at different
405 temperatures (33^0C and 37^0C) by reconstituting the polymerase through transient transfection at
406 37^0C for 12 hours followed by an additional incubation of 30 hours at respective temperatures
407 (Figure 3E). As observed at 37^0C, reporter activity was almost two fold higher than the activity at
408 33^0C, a data corroborated perfectly with the previous results obtained by Santos et al[35].
409 Together, we present a fast sensitive and high throughput reporter assay for monitoring influenza
410 B virus RNA synthesis in an infection free setting.

411 All of the assays were performed in 24, 48 and 96 well plates, in triplicates for each of the
412 biological sets (data presented in this manuscript is from 24 well plates), hence confirming that
413 this assay system is high-throughput compatible. Additionally, five log difference between the
414 signal and background readouts provides a wide dynamic range for this reporter based assay
415 system. Together, we have been able to establish a fast, reliable and high-throughput compatible
416 assay system for monitoring influenza B virus RNP/ polymerase activity, which is suitable for
417 assessing the effect of various viral or cellular factors in modulating RNP activity and hence viral
418 RNA synthesis.

419 **The influenza B RNP activity assay is suitable for evaluating the efficacy of viral or host**
420 **factors in regulating viral RNA synthesis.**

421 To this end, we set out to evaluate the efficacy of the newly developed polymerase activity assay
422 in identifying novel viral and host factors that may regulate viral RNA synthesis. Influenza virus
423 Nonstructural Protein 1 (NS1) is a multifunctional protein participating mainly in the suppression
424 of antiviral defense mechanisms exerted by a wide variety of host factors [36, 37]. Additionally,
425 influenza A virus NS1 protein has been shown to boost viral RNA synthesis[38–40], possibly
426 through interfering with antiviral activity DDX21 and RAP55 [41, 42]. While the immune
427 suppression activity of influenza B NS1 was well studied[37, 43, 44], little is known about the role
428 of NS1 in regulating influenza B virus RNA synthesis. Hence, we evaluated the ability of influenza
429 B virus NS1 protein to promote viral RNA synthesis with the newly developed reporter RNP activity
430 assay. Influenza B virus NS1 ORF was cloned into the pCDNA-3X-FLAG vector that resulted in
431 the expression of the C-terminally FLAG tagged NS1 protein. Influenza B reporter RNPs were
432 reconstituted in HEK293T cells either in the absence or presence of increasing amounts of NS1
433 protein and reporter activity was monitored to assess the extent of viral RNA synthesis. Increasing
434 amount of NS1 resulted in 1.5 to 2 folds increase in reporter activity (Figure 4A) establishing it as
435 a positive regulator of viral RNA synthesis. Furthermore, reconstituting reporter RNPs in the
436 presence of NS1 presents an assay system that closely resembles RNA synthesis, which occurs
437 during the course of infection.

438 Subsequently, we tested the ability of a host factor to regulate influenza B virus RNA synthesis
439 using our reporter RNP activity assay. Host Protein Kinase C, specifically the delta isoform, has
440 been shown to positively influence influenza A virus RNA synthesis by regulating the
441 phosphorylation and subsequent assembly of viral nucleoproteins into RNPs. Interestingly, the
442 constitutively active catalytic domain of PKC delta (PKCD), when overexpressed, negatively

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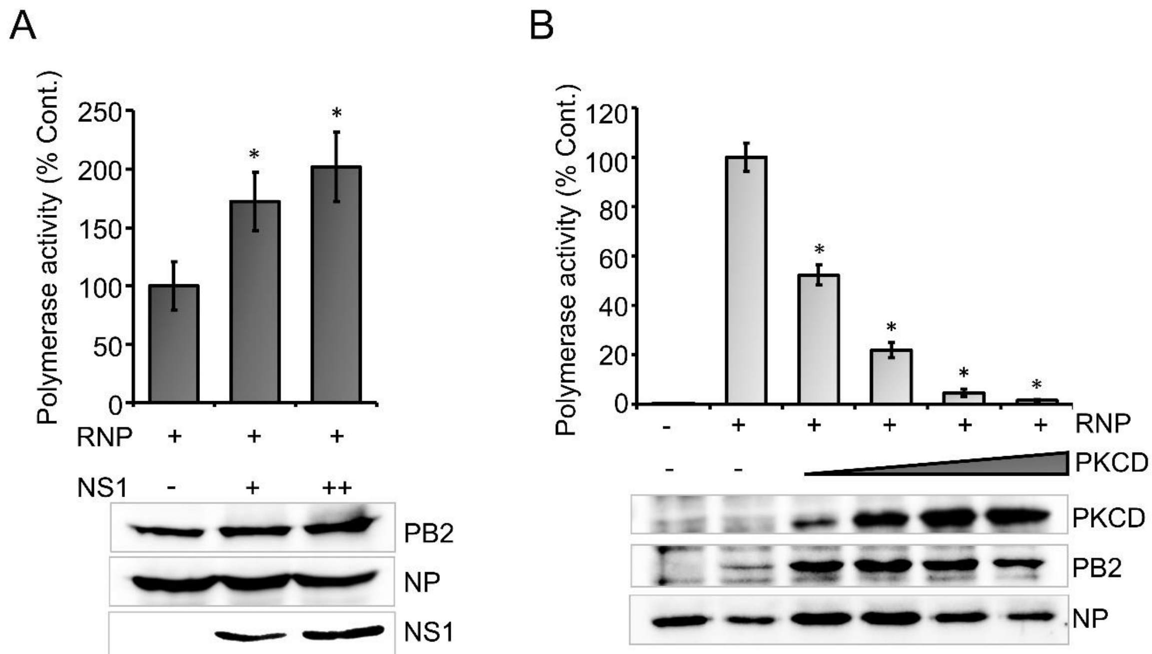
443 regulates influenza A virus RNA synthesis [45]. To determine the role of PKCD in regulating
444 influenza B virus RNA synthesis we employed the newly developed reporter RNP activity assay.
445 As evident from figure 4B, increasing amounts of PKCD resulted in a gradual decrease in RNP
446 activity and hence viral RNA synthesis without any severe impact upon the translation of viral
447 proteins. These data not only substantiates the role of PKCD in regulating influenza B virus RNP
448 activity, but also validates the efficacy of our assay system in studying the effect of pro- or antiviral
449 factors regulating viral RNA synthesis.



450

451 **Figure 4:** Effect of host & viral factors upon viral RNA synthesis: (A) Effect of an increasing amount of viral NS1 protein
452 on Influenza B RNP activity assay (B) Effect of an increasing amount of constitutively active host protein kinase c delta
453 (PKCD) protein on B RNP activity assay. (n=3± standard deviation. *p<0.05 one-way ANOVA with *post hoc* student's
454 t-test when compared to the preceding set.

455 **Reporter based RNP activity assay as high throughput screening platform of antiviral**
456 **drugs.**

457 Finally, we intend to establish the suitability of the RNP activity assay as a high throughput
458 screening platform of antiviral drugs that can inhibit viral RNA synthesis and hence virus
459 replication. Ribavirin and Favipiravir are nucleoside (purine) analogues, which inhibit the
460 replication of a wide variety of RNA viruses by acting as an alternative substrate for viral RNA
461 polymerase[46, 47]. Additionally, Ribavirin also inhibits inosine monophosphate

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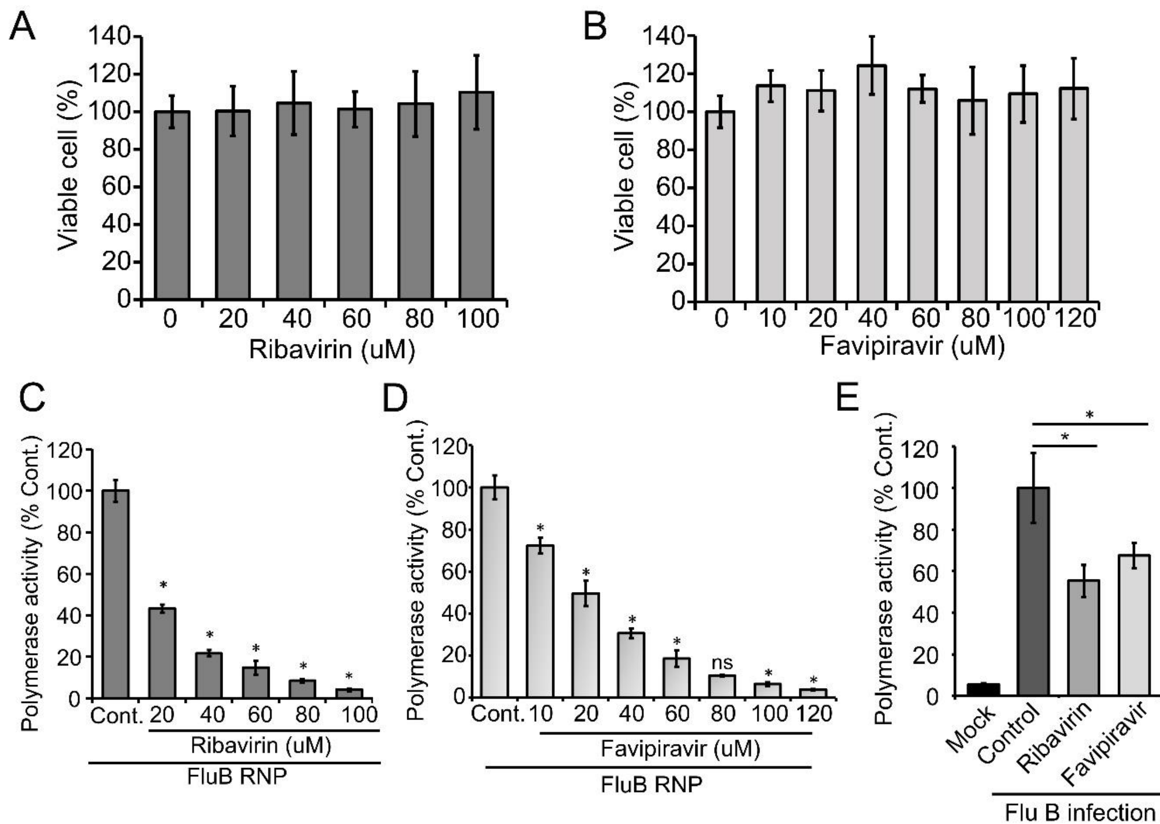

462
463 **Figure 5:** Effect of antiviral drugs upon viral RNA synthesis in infection-free and infection setting: (A, B) MTT assay to
464 determine the cytotoxicity of Ribavirin and Favipiravir on HEK293T cells. (C, D & E) Effect of Ribavirin and Favipiravir
465 on influenza B virus RNP activity. Viral polymerase proteins in HEK293T cells are expressed either by transient
466 transfection (C, D) or by infecting the cells with Influenza B virus (E). (n=3 ± standard deviation, *p<0.05 one-way
467 ANOVA with post hoc Student's t-test when compared to the preceding set, for Figure E, comparison was performed
468 with control set, ns = not significant).

469 dehydrogenase thereby depleting the GTP and creating an imbalance in the nucleotide pool
470 inside the cell[48]. Both Ribavirin and Favipiravir has been approved as chemoprophylaxis as well
471 as therapy against influenza A and B viruses[49–51]. Hence, we used these two drugs as positive
472 controls to test the efficacy of our assay system for antiviral screening. MTT assay was performed
473 in HEK293T cells (Figure 4A, B), where neither of the drugs show any cytotoxicity. HEK293T cells
474 were pretreated with different concentrations of the drugs followed by forward transfection to
475 reconstitute the influenza B reporter RNPs and subsequent incubation with the drugs for 36 hours.
476 Reporter activities were measured and expressed as relative percentages with respect to the
477 vehicle control. Data presented in Figure 5 C, D, shows a dose-dependent decrease in the
478 reporter activity and hence viral RNA synthesis with increasing amounts of the drugs with IC50
479 values of 18.54 uM and 25.46 uM for Ribavirin and Favipiravir respectively.

480 To further extend the scope of the assay system, we sought to check if this system is capable of
481 assessing the effect of host factors or antivirals upon the overall progress of infection. For this
482 purpose, we transfected HEK293T cells with the reporter construct and subsequently infected

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483 them with influenza B virus at 20 hours post transfection. It is expected that in infected cells
484 reporter RNA template will get transcribed with the help of RdRp and NP proteins expressed from
485 viral genomic RNA segments. As evident from Figure 5E, infected cells supported successful
486 generation of reporter RNPs and hence showed high reporter activity, while the uninfected cells
487 showed no such effect. Interestingly, when parallel set of cells were treated with Ribavirin and
488 Favipiravir prior to infection with Influenza B virus, significant reduction in reporter activity were
489 observed, hence suggesting an overall reduction in viral gene expression and hence virus
490 replication in presence of the drugs. Together our data reconfirms the activity of the two well
491 established antiviral drugs against influenza B virus RNA synthesis machinery and also
492 establishes the newly developed reporter based influenza B virus RNP activity assay as a high
493 throughput screening platform of antivirals specifically inhibiting viral RNA synthesis.

494

495 Discussion:

496 Luciferase based reporter assay systems remain a key tool for analyzing gene expression in a
497 wide variety of organisms; viruses are not exceptions. While, for positive sense RNA viruses,
498 introduction of the single sub-genomic reporter RNA template in cells is sufficient for expression
499 of reporter genes; for negative sense RNA viruses, RNP associated viral proteins needs to be
500 synthesized along with the reporter RNA in order to reconstitute complete RNPs, which then leads
501 to the expression of reporter enzyme as a proxy of viral genes[12, 52, 53]. This is why, successful
502 reconstruction of reporter viral RNPs require extensive cloning of multiple RNA and protein
503 expressing constructs, standardization of their expression in right stoichiometric ratios and
504 optimization of other crucial parameters like time, temperatures etc. Although, several groups
505 have reported reporter assay systems for monitoring influenza A and B virus RNA synthesis, non-
506 availability of detailed methodical description makes the process of establishing the assay system
507 non-trivial[12, 13, 24–26]. In this work, we have established a firefly luciferase-based influenza B
508 virus RNP activity assay and presented the detailed methodology of the entire procedure which
509 could be easily followed for the development of such viral and non-viral reporter assay systems.

510 We have introduced a unique cloning strategy for the construction of the influenza B virus reporter
511 RNA construct that is devoid of restriction enzymes or any other specialized enzymes. This
512 cloning strategy utilizes a single DNA polymerase, which is widely used for regular molecular
513 biology work and hence easily available. Using this polymerase, two consecutive PCR
514 amplification reactions led to the generation of the reporter RNA cassette encompassing the
515 reporter ORF flanked by viral 5'- and 3'-UTR regions which were then inserted into the vector
516 using CPEC cloning method. While the vector and the insert used for CPEC, are also compatible
517 for Gibson assembly based cloning method, we intentionally avoided use of any specialized
518 enzymes to make the overall procedure simple and user-friendly that could be adapted for cloning
519 of any other reporter RNA constructs. In addition to reporter RNA construct, we also cloned ORFs
520 corresponding to viral PB1, PB2, PA and NP proteins and optimized their expression to
521 reconstitute reporter RNPs at maximum levels. The robustness of this assay system was
522 substantiated by testing the efficacy of antiviral drugs, Ribavirin and Favipiravir, to inhibit influenza
523 B virus RNA synthesis either in the context of reconstituted RNPs (through transfection) or during
524 the course of infection. The fact that the reporter RNA template can be preferentially recognized

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525 by viral NP and RdRp subunits to reconstitute reporter RNPs during the course of infection,
526 confirms that the reporter RNA mimics viral genomic RNA segments and hence validates its
527 suitability to be used for the study of viral RNA synthesis and effect of various viral and host
528 factors upon the same. In fact, using the newly developed reporter RNP system, we for the first
529 time showed that viral NS1 protein can boost influenza B virus RNA synthesis and constitutively
530 active form of host PKCD can downregulate the same. While effect of NS1 and PKCD proteins
531 has been previously characterized in case of influenza A virus[38–40, 45], our results
532 substantiates that these proteins participate similarly regulate influenza B virus RNA synthesis as
533 well.

534 Altogether, we present a comprehensive roadmap for development, characterization and
535 validation of a reporter-based Influenza B virus polymerase/ RNP activity assay and made it
536 generic enough to be followed by others who intend to develop similar assay systems for influenza
537 and other negative sense RNA viruses. We also made all the resources publicly available (upon
538 request) to enrich the armoury for combating influenza B viruses and hope that it will be widely
539 utilized to identify new therapeutic strategies against this deadly human pathogen.

540 **Table 1.** Primers used in this study.

Name of the primer	Sequence (5'->3')
Uni 9	AGCAGAAGC
NA-NB_F	AGTAGTAACAAGAGCATTTTTCAG
NA-NB_R	AGCAGAAGCAGAGCATC
5' UTR_F	CATTTTGGGCCGCCGGGTTATTAGTAGTAACAAGAGCATTTTTCAG
5' UTR_R	CGGAAAGATCGCCGTGTAATGGAGGAATGGTTGAGTC
3' UTR_F	CTTTATGTTTTTGGCGTCTTCCATTGTTCATTTTTGGCCTATTTG
3' UTR_R	CCTCCGAAGTTGGGGGGGAGCAGAAGCAGAGCATCTTC
PHH21_F	CCCCCCCAACTTCGGAGG
PHH21_R	AATAACCCGGCGGCCCAAAATG
PHH21 SEQ2	AAAACGCTGGGCGTTAATCAAAGAGGCG
PHH21 SEQ1	GGGGGACACTTTCGGACATCTGGTC
pcDNA3-V5 For	GATCCGGAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGTAGTAAGC
pcDNA3-V5 Rev	GGCCGCTTACTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCTCC G
NP_For	ATTCGGGGTACCGCCACCATGTCCAACATGGATATTGACG
NP_Rev_V5	ATTCGCGGATCCACCATAATCGAGGTCATCATAATCCTC
NP_Rev_Stop	ATTCGCGGATCCTTAATAATCGAGGTCATCATAATCCTC
PB1_For	TAAGCGGAATTCACCATGAATATAAATCCTTATTTTCTCTTC
PB1_Rev_FLAG	ATTGAGGCGGCCGCTATGTACCCAATCTCACCAAG
PB1_Rev_Stop	ATTGAGGCGGCCGCTTATATGTACCCAATCTCACC
PB2_For	TAAGCGGAATTCACCATGACATTGGCCAAAATTGAATTG
PB2_Rev_FLAG	ATTGAGGCGGCCGCGCTCAAGGCCCACCCC
PB2_Rev_Stop	ATTGAGGCGGCCGCTTAGCTCAAGGCCCACCC
PA_For	TAAGCGGAATTCACCATGGATACTTTTATTACAAGAAACT
PA_Rev_FLAG	ATTGAGGCGGCCGCTTCGTCCATAATCTCGTC
PA_Rev_Stop	ATTGAGGCGGCCGCTTATTCGTCCATAATCTCGTC

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542 **Table 2**: PCR conditions used in the study for amplification of individual inserts.

PCR amplification	Forward Primer	Reverse Primer	Denaturation (temp./duration)	Cyclic denaturation, annealing and elongation (temp./duration)	No. of cycles	Final elongation
NA-NB fragment	NA-NB_F	NA-NB_R	98^{0}C/30 seconds	98^{0}C/10 seconds 60^{0}C/30 seconds 72^{0}C/95 seconds	35	72^{0}C/5 minutes
5' UTR	5' UTR_F	5' UTR_R	98^{0}C/30 seconds	98^{0}C/10 seconds 60^{0}C/25 seconds 72^{0}C/5 seconds	35	72^{0}C/5 minutes
3' UTR	3' UTR_F	3' UTR_R	98^{0}C/30 seconds	98^{0}C/10 seconds 59^{0}C/25 seconds 72^{0}C/5 seconds	35	72^{0}C/5 minutes
Insert for Reporter plasmid	5' UTR double-stranded PCR product	3' UTR double-stranded PCR product	98^{0}C/30 seconds	98^{0}C/10 seconds 72^{0}C/80 seconds	35	72^{0}C/5 minutes
Vector for Reporter plasmid	PHH21_F	PHH21_R	98^{0}C/30 seconds	98^{0}C/10 seconds 67^{0}C/30 seconds 72^{0}C/90 seconds	35	72^{0}C/5 minutes
PB1-FLAG	PB1_For	PB1_Rev_FLAG	98^{0}C/30 seconds	98^{0}C/10 seconds 60^{0}C/30 seconds 72^{0}C/90 seconds	35	72^{0}C/10 minutes
PB1-STOP	PB1_For	PB1_Rev_Stop	98^{0}C/30 seconds	98^{0}C/10 seconds 58^{0}C/30 seconds 72^{0}C/90 seconds	35	72^{0}C/10 minutes
PB2-FLAG	PB2_For	PB2_Rev_FLAG	98^{0}C/30 seconds	98^{0}C/10 seconds 69^{0}C/30 seconds 72^{0}C/90 seconds	35	72^{0}C/10 minutes
PB2-STOP	PB2_For	PB2_Rev_Stop	98^{0}C/30 seconds	98^{0}C/10 seconds 69^{0}C/30 seconds 72^{0}C/90 seconds	35	72^{0}C/10 minutes
PA-FLAG	PA_For	PA_Rev_FLAG	98^{0}C/30 seconds	98^{0}C/10 seconds 60^{0}C/30 seconds 72^{0}C/90 seconds	35	72^{0}C/10 minutes
PA-STOP	PA_For	PA_Rev_Stop	98^{0}C/30 seconds	98^{0}C/10 seconds 58^{0}C/30 seconds 72^{0}C/90 seconds	35	72^{0}C/10 minutes
NP-FLAG	NP_For	NP_Rev_V5	98^{0}C/30 seconds	98^{0}C/10 seconds 64^{0}C/30 seconds 72^{0}C/90 seconds	35	72^{0}C/10 minutes
NP-STOP	NP_For	NP_Rev_Stop	98^{0}C/30 seconds	98^{0}C/10 seconds 66^{0}C/30 seconds 72^{0}C/90 seconds	35	72^{0}C/10 minutes

543 **Declaration of competing interest:**

544 The authors declare that they have no known competing financial interests or personal
545 relationships that could have appeared to influence the work reported in this paper.

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555 **Address of correspondence:**

556 Dr. Arindam Mondal, Assistant Professor, School of Bioscience, Indian Institute of Technology
557 Kharagpur, Kharagpur-721302, Dist- Paschim Medinipur, West Bengal, India. E-mail address:
558 arindam.mondal@iitkgp.ac.in.

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