

1 **Determining the Mutation Bias of Favipiravir in Influenza Using Next-**
2 **generation Sequencing**

3 Daniel H. Goldhill^{1,2}, Pinky Langat², Hongyao Xie², Monica Galiano¹, Shahjahan Miah¹, Paul
4 Kellam², Maria Zambon¹, Angie Lackenby¹ and Wendy Barclay²#.

5 ¹Public Health England, London, UK; ² Department of Virology, Faculty of Medicine, Imperial
6 College, London, UK.

7 #Corresponding Author: w.barclay@imperial.ac.uk

8 Running Title- Mutation Bias of Favipiravir in Flu using NGS

9

10

11 **Abstract**

12 Favipiravir is a broad spectrum antiviral drug that may be used to treat influenza. Previous
13 research has identified that favipiravir likely acts as a mutagen but the precise mutation bias
14 that favipiravir induces in influenza virus RNAs has not been described. Here, we use next-
15 generation sequencing (NGS) with barcoding of individual RNA molecules to accurately and
16 quantitatively detect favipiravir-induced mutations and to sample orders of magnitude
17 more mutations than would be possible through Sanger sequencing. We demonstrate that
18 favipiravir causes mutations and show that favipiravir primarily acts as a guanine analogue
19 and secondarily as an adenine analogue resulting in the accumulation of transition
20 mutations. We also use a standard NGS pipeline to show that the mutagenic effect of
21 favipiravir can be measured by whole genome sequencing of virus.

22 **Importance**

23 New antiviral drugs are needed as a first line of defence in the event of a novel influenza
24 pandemic. Favipiravir is a broad-spectrum antiviral which is effective against influenza. The
25 exact mechanism of how favipiravir works to inhibit influenza is still unclear. We used next-
26 generation sequencing (NGS) to demonstrate that favipiravir causes mutations in influenza
27 RNA. The greater depth of NGS sequence information over traditional sequencing methods
28 allowed us to precisely determine the bias of particular mutations caused by favipiravir. NGS
29 can also be used in a standard diagnostic pipeline to show that favipiravir is acting on the
30 virus by revealing the mutation bias pattern typical to the drug. Our work will aid in testing
31 whether viruses are resistant to favipiravir and may help demonstrate the effect of

32 favipiravir on viruses in a clinical setting. This will be important if favipiravir is used during a
33 future influenza pandemic.

34 **Keywords:** Influenza, Favipiravir, Mutation bias, Next-generation sequencing, Primer ID.

35

36 Introduction

37 Influenza virus is responsible for the deaths of between 290,000-650,000 people
38 globally each year¹. The emergence of a novel strain of influenza in humans could lead to an
39 influenza pandemic with significant mortality worldwide². Whilst vaccination provides good
40 levels of protection against seasonal influenza, at the start of a pandemic, antiviral drugs
41 would be the frontline of defence during a period of development of a specific vaccine³.
42 Historically, there have been only two licensed classes of antiviral drug for influenza:
43 adamantanes and Neuraminidase inhibitors (NAIs). Adamantanes are no longer in clinical
44 use as almost all circulating viruses are resistant^{4,5}. Furthermore, some previous seasonal
45 viruses have shown high levels of resistance to the most commonly administered NAI,
46 oseltamivir⁶ and oseltamivir resistant A(H7N9) viruses with pandemic potential have
47 emerged and are transmissible between ferrets⁷⁻⁹. New drugs are needed for treatment of
48 seasonal influenza as well as for pandemic preparedness and a number of drug classes are
49 under development including compounds that target the viral RNA dependent RNA
50 polymerase (RdRP)¹⁰. In 2014, Favipiravir, an antiviral drug developed by Toyama, was
51 licensed for use in Japan against emerging influenza viruses that exhibit resistance to other
52 antivirals¹¹. However, the exact mechanism through which favipiravir exerts an antiviral
53 effect on influenza is unclear. An increased knowledge of the mechanism of action of
54 favipiravir could be useful in determining whether specific viruses are less susceptible and
55 evaluating the potential for emergence and transmission of resistant viruses.

56 Favipiravir is a nucleoside analogue that is active against all subtypes of influenza
57 and has shown a potent antiviral effect both *in vitro* and *in vivo*¹²⁻¹⁷. Favipiravir has
58 completed a phase III clinical trial in Japan and has undergone a phase III trial in the USA¹⁸.

59 Favipiravir has also been shown to be active *in vitro* and in animal models against a wide
60 range of RNA viruses, some for which there are no licensed drugs as a treatment option¹⁸⁻²⁵.
61 There is strong evidence that favipiravir acts as a mutagen by incorporating into both
62 positive and negative stranded RNA and being aberrantly copied as multiple bases^{15,26-30}.
63 This is thought to be a different mechanism of action from ribavirin, another broadly acting
64 nucleoside analogue that has been used previously to treat influenza^{26,31}. Studies have
65 shown that favipiravir competes against guanine and adenine to be incorporated into RNA
66 and is non-competitive against cytosine and uracil^{30,32-34}. This would suggest that favipiravir
67 acts as a purine analogue and should cause mostly transition mutations. Studies measuring
68 the mutation bias of favipiravir in influenza have had mixed results. Baranovich *et al.* used
69 Sanger sequencing of virus passaged in presence of drug to show a C->U and a G->A
70 mutation bias as expected but also saw a G->U mutation bias after 48hrs of exposure to
71 favipiravir²⁷. Vanderlinden *et al.* also used Sanger sequencing to show a C->U and G->A bias
72 following a passaging experiment and showed an increase in Shannon entropy using next-
73 generation sequencing (NGS)³⁵. However, in contrast to studies using Sanger sequencing,
74 Marathe *et al.* reported a slight bias towards transversions in influenza infected mice
75 treated with favipiravir using NGS³⁶. Studies with other viruses have given mutation patterns
76 which suggest that favipiravir acts as a purine analogue^{28,29,37,38}. Interestingly, several
77 studies with favipiravir and influenza have suggested that favipiravir acts not as a mutagen
78 but as a chain terminator preventing the extension of the RNA strand following
79 incorporation^{32,33}. A primer extension study suggested that the block could occur with a
80 single molecule of favipiravir³² but other studies have suggested that chain termination
81 occurs following the incorporation of two molecules of favipiravir^{30,33,34}.

82 In this study, we used next generation sequencing to determine the mutation bias of
83 favipiravir on influenza virus RNAs. We employed two methods of analysis: the first method
84 uses Primer ID which is a technique for labelling each individual RNA molecule with a
85 barcode to account for PCR and sequencing errors³⁹⁻⁴¹. This technique can very precisely
86 uncover the mutation bias by analysing small, targeted areas of the genome. The second
87 method developed a novel analysis of data obtained from a standard sequencing pipeline as
88 would be found in many National Influenza Centres or public health laboratories. This
89 showed the mutation bias induced by drug treatment over the whole genome was similar to
90 that detected using the precise Primer ID methodology and confirmed that the effect of
91 favipiravir could be readily measured using NGS from a standard sequencing pipeline.

92

93 **Methods**

94 **Reagents, Cells and Viruses**

95 Favipiravir was kindly provided by Toyama Chemical Company under an MTA and
96 reconstituted in DMSO and frozen into aliquots. MDCK and 293-T cells were grown in
97 Dulbecco's modified Eagle's medium (DMEM; Gibco) with the addition of 10% Fetal Bovine
98 Serum (labtech.com), 1% non-essential amino acids (Gibco) and 1% penicillin/streptomycin
99 (Sigma-Aldrich). A/England/195/2009 (Eng195) is an early isolate from the 2009 A(H1N1)
100 pandemic provided by Public Health England (PHE).

101 **Minigenome Assay**

102 Four pCAGGS plasmids encoding the polymerase (PA, PB1 and PB2) and NP from influenza
103 A/England/195/2009 A(H1N1)pdm09 virus, were transfected using Lipofectamine 3000

104 (Invitrogen) into 293T cells in 24 well plates. In addition, we transfected plasmids directing
105 expression from a Poll promoter of either a Firefly luciferase gene in negative sense flanked
106 with influenza A non-coding sequence from the NS segment or the HA gene segment from
107 influenza A/Victoria/3/75 H3N2 virus (Vic75), and a Poll Renilla luciferase plasmid as a
108 transfection control. Cells were lysed with 200µl of passive lysis buffer (Promega) and
109 polymerase activity was measured using Dual-Luciferase Reporter Assay (Promega) on the
110 FLUOstar Omega plate reader (BMG Labtech). Polymerase activity is reported as Firefly
111 luciferase activity normalized by Renilla activity.

112 **Next-Generation Sequencing with Primer ID**

113 At 24 hours after transfection, 293T cells from the minigenome assay were lysed and RNA
114 was extracted using the RNA mini kit (Qiagen). The reverse transcription primer for primer
115 ID (5'-TGCGTTGATAACCACTGCTTTNNNNTNNNNTNNNCCCAGTCCAAGTGAAACCCTC-3')
116 consisted of a PCR tag, random barcode of the form NNNNTNNNNTNNNN and sequence
117 specific to the H3 HA. Reverse transcription was performed with Superscript III (Thermo
118 Fisher). qPCR using SYBR green (Thermo Fisher) was used to calculate the number of cDNA
119 molecules to use for each PCR reaction. 20,000-40,000 molecules were used for each
120 reaction. The PCR primers were 5'-CGGGGAAAATATGCAACAATCCT-3' and 5'-
121 TGCGTTGATAACCACTGCTTT. The PCR product was designed to be 279 bases to avoid any
122 fragmentation step during sample preparation ensuring the barcode was not sheared from
123 the sample. Sample preparation was performed using the NEBNext Ultra kit (NEB). Samples
124 were sequenced giving 150bp paired end reads on an Illumina MiSeq. Sequencing data for
125 the samples were processed and analysed using custom scripts in Python and R. Reads were
126 first paired to form a single sequence and subjected to quality control using QUASR v7.01⁴²

127 to retain reads with a median phred score of 20 and minimum read length of 250bp. Intact
128 barcode sequences were extracted from the read pairs; any sequences without a fully
129 formed barcode or with errors in the internal Ts of the barcode were discarded. Consensus
130 sequences were generated for each barcode that had more than three reads with the
131 consensus taken as the majority of the reads. Samples for which there was no majority read
132 were discarded as potentially this could be an example of two RNA sequences having the
133 same barcode⁴³. The consensus sequences were mapped and compared to the Vic75
134 reference and any variants were extracted. We subsequently decided to use a more
135 stringent cut-off of four reads per barcode to minimize errors caused by barcodes with a low
136 number of reads. We present all our sequencing results as mutations in positive orientation
137 as would have been seen in the mRNA.

138

139 **qPCR**

140 RNA was extracted from the mini-genome assay. Specific primers were used to reverse
141 transcribe mRNA from the firefly luciferase as previously described⁴⁴. qPCR was performed
142 with SYBR Green using 18S RNA as a control. $\Delta\Delta C_t$ was calculated and the results are shown
143 normalized to the drug free control.

144

145 **Next-Generation Viral Sequencing with Primer ID**

146 1.2×10^6 cells were inoculated with Eng195 at a MOI of 1.5 and incubated at 37°C for 18
147 hours in serum free media with added 1 μ g/ml trypsin (Worthington) and with different
148 concentrations of favipiravir diluted in DMSO. Control wells contained DMSO without
149 favipiravir. After 18 hours, samples were taken from the supernatant and plaqued on MDCK

150 cells to determine final viral titre. RNA was extracted from the cells using RNEasy kit
151 (Qiagen). Sequencing was performed as described above with the exception that the Primer
152 ID RT primer contained sequence specific for PB1 vRNA (5'-
153 TGTCCAGCACGCTTCAGGCTNNNNTNNNNTNNNNAGAAGATGGTCACGCAAAGAA-3') and the
154 PCR product was 302 bases long including the PCR primers (5'-TCACAACATTTGCCAGTTTGG-
155 3', 5'-TGTCCAGCACGCTTCAGGCT-3'). On analysing the sequencing data, a site which varied
156 considerably in all samples was detected which was likely a polymorphism in the initial
157 population. This site was removed from all analyses.

158 **Next-Generation Sequencing without primer ID**

159 1.2×10^6 cells were inoculated with England 195 at a MOI of 1 and incubated at 37°C for 24
160 hours as described above. Control wells contained DMSO but no favipiravir. After 24 hours,
161 samples were taken from the media and titred on MDCK cells by plaque assay. Whole
162 genome next generation sequencing was performed using a pipeline at Public Health
163 England. RNA was extracted from viral lysate using easyMAG (bioMérieux). One step
164 Reverse-Transcription-PCR was performed with Superscript III (Invitrogen), Platinum Taq
165 HiFi Polymerase (Thermo Fisher) and influenza specific primers⁴⁵. Samples were prepared
166 for NGS using Nextera library preparation kit (Illumina). Samples were sequenced on an
167 Illumina MiSeq generating a 150-bp paired end reads. Reads were mapped with BWA v0.7.5
168 and converted to BAM files using SAMTools (1.1.2). Variants were called using QuasiBAM,
169 an in-house script at Public Health England. Samples were compared using a permutation
170 analysis to calculate the probability of a magnitude of mutation bias as great as observed
171 given the mutations in the samples. Permutation analyses were performed in R with 10,000
172 iterations for each analysis. Mutations were randomised between two samples maintaining

173 the number of mutations found within each sample. The magnitude of the mutation bias
174 was then calculated as the sum of the absolute value of the difference in the relative
175 proportions of each mutation type. The p value was then calculated as the number of
176 iterations/10000 with a value greater than the observed value. A further permutation
177 analysis calculated the probability of a bias of guanine analogue mutations (e.g. C->U and G-
178 >A). This analysis was performed as above but only used the sum of the absolute value of
179 the difference in the relative proportions of C->U and G->A.

180 **Results**

181 **Primer ID allows calculation of mutation bias and relative mutation rate**

182 In order to determine the mutagenic effect of favipiravir, we employed next generation
183 sequencing using Primer ID to analyse the products of a minigenome assay⁴⁶, which allowed
184 for the unbiased measurement of mutations (Figure 1). When sequencing virus, particularly
185 over several rounds of replication, a proportion of possible mutations will not be measured
186 as they would cause too large a fitness cost to the virus and thus will not be amplified. To
187 avoid this scenario, we sequenced the reporter gene from the minigenome assay as the
188 reporter protein has no effect on further RNA accumulation. Thus, this strategy should
189 reveal the complete spectrum of mutations caused by replication in the presence of
190 favipiravir. Primer ID is a method which labels each molecule of RNA with a unique barcode
191 (Figure 1). This method allowed us to examine a large number of independent mutational
192 events as each mutation could be associated with an individual RNA molecule. In addition,
193 by comparing multiple sequencing reads with the same barcode, we could remove
194 sequencing errors as these would not appear in the majority of the reads. The sample
195 without favipiravir provides a baseline mutation rate consisting of the background mutation

196 rate of the influenza virus polymerase plus mutations caused by the reverse transcriptase
197 during reverse transcription. Drug-treated samples can be compared to this sample to
198 measure how favipiravir increased the mutation rate.

199 We reconstituted influenza RdRP in situ by expressing the polymerase proteins and
200 nucleoprotein from transfected plasmids. We introduced two viral-like RNA templates, one
201 in which the authentic open reading frame was replaced by the firefly luciferase gene, and
202 one that represented RNA segment 4 and encoded H3 haemagglutinin (HA). The transfected
203 cells were incubated in the presence of favipiravir. Increasing concentrations of favipiravir
204 from 1 to 100 μ M caused a reduction in the activity of the luciferase reporter (Figure 2A).
205 However, qRT-PCR analysis of the amount of H3 HA mRNA accumulated revealed no
206 decrease in mRNA levels that would account for the loss of luciferase activity at least up to
207 50 μ M drug (Figure 2B). At 100 μ M favipiravir, there was a significant reduction in mRNA
208 ($P < 0.0001$). This suggested that at doses up to 50 μ M, the inhibitory effect of favipiravir in
209 the minigenome assay was caused by mutagenesis and not through chain termination,
210 which could have played a role at the highest dose of drug.

211 In order to test how favipiravir affected the mutation rate of the reconstituted viral
212 polymerase, we sequenced the positive stranded H3 HA RNAs. As each individual barcode
213 represents a single RNA molecule, we calculated consensus sequences for each barcode.
214 Mutations which did not appear in a majority of reads were ascribed to PCR or sequencing
215 error and removed from further analyses. In total, we analysed 6,623 substitutions in
216 ~6,900,000 bases of sequencing data. Figure 2C shows the number of mutations per 10,000
217 nucleotides above the baseline (0 μ M favipiravir) for each sample. As the concentration of
218 favipiravir increased, the number of mutations increased. At the highest concentration of

219 favipiravir tested (100 μ M), there would be an additional 15 errors per 10,000 nucleotides
220 on average compared to the control. We varied the cut-off for the number of sequencing
221 reads needed to include a barcode (Supplemental figure 1). The choice of cut-off did not
222 significantly alter the results for values <10 reads. We chose a cut-off of 4 reads per barcode
223 as this removed some errors associated with low numbers of reads per barcode whilst
224 including the majority of the data.

225 We next categorised the mutations identified by sequencing as transitions or transversions,
226 or as the individual base-pair mutations (Figure 2D, E). Our results confirmed that the main
227 cause of the increase in mutation rate was transition mutations (Figure 2D). There was no
228 increase in the rate of transversion mutations as the concentration of favipiravir increased
229 (F-test, $F = 0.4593$, d.f. 1,4, $p = 0.5351$). Figure 2E shows the increase in the likelihood of
230 different categories of mutations compared to the control. The most common transitions
231 were C->U and G->A mutations that would be induced when favipiravir is acting as a
232 guanine analogue. However, there was also a smaller increase in the reverse transitions
233 from U->C and A->G where favipiravir acts as an adenine analogue. On average, there was
234 an approximately 3.5-fold increase in the rate of C->U or G->A mutations compared to a U-
235 >C or A->G mutations.

236 **Primer ID sequencing of viruses confirms that favipiravir causes mutations**

237 We next tested whether we could use Primer ID to measure the increase in mutation rate of
238 RNAs generated during virus infection. To minimize the loss of viral RNAs that contained
239 mutations rendering the virus nonviable, we infected cells at a high MOI so that there was
240 only a single replication cycle. We first confirmed that favipiravir inhibited influenza under
241 these conditions (Figure 3A). There was a greater than 1000-fold reduction in infectious titre

242 of influenza A/Eng195/2009 A(H1N1)pdm09 virus (Eng 195) after 24 hours infection at high
243 concentrations of favipiravir and a 10-fold reduction at 1 μ M drug. We extracted RNA from
244 the cells and sequenced the vRNA of RNA segment 2 with appropriate barcoded primers. In
245 total, we analysed \sim 56,000,000 bases and found 25,441 substitutions. All concentrations of
246 favipiravir showed an increase in mutation rate compared to the no drug control (Figure
247 3B). The mutation rate caused by favipiravir was \sim 3 fold higher at 10 μ M than at 1 μ M, but
248 surprisingly, the mutation rate at 100 μ M favipiravir was lower than at 10 μ M. The increase
249 in mutation rate at all concentrations of favipiravir was almost entirely due to transitions
250 (Figure 3C). The mutation bias measured was subtly different than that seen using the
251 minigenome assay with C \rightarrow U occurring most often but G \rightarrow A and U \rightarrow C mutations occurring
252 at comparable rates (Figure 3D). This suggests that there was a higher rate of incorporation
253 of favipiravir during negative strand synthesis compared to positive strand synthesis in virus
254 infected cells (see Figure 5).

255 **Next generation sequencing can reveal mutation bias**

256 The experiments with Primer ID showed the mutation rate and bias for a small targeted
257 portion of influenza genome. Next, we wanted to test whether we could measure the
258 mutagenic effect of favipiravir using a standard NGS pipeline typical of those in public health
259 laboratories (Supplemental figure 2). Eng195 virus was propagated at a high MOI for 24
260 hours in the presence of 10 or 100 μ M favipiravir. The supernatant was plaqued to confirm
261 that favipiravir had an inhibitory effect on the virus and there was >2 log inhibition at 10 μ M
262 and >4 log inhibition at 100 μ M. We extracted RNA from virus particles in the supernatant
263 and used next generation sequencing to obtain sequence data from the population of
264 surviving viruses. In order to analyse mutation bias using next generation data, it is

265 necessary to ensure that the mutations used for the analysis are independent so that the
266 same mutation occurring on multiple reads is not counted as multiple mutational events but
267 as a single mutational event. Therefore, we treated each base in the influenza genome
268 independently and recorded only the most common mutation (if any) for each site
269 (Supplemental figure 2). Taking these sites in aggregate will give a combination of true
270 mutations as well as other sources of error, most notably sequencing error. Figure 4 shows
271 the sum of mutations over the whole genome for viruses propagated in 10 μ M or 100 μ M
272 favipiravir or for control viruses which were not exposed to favipiravir. Comparing the
273 pattern of mutations between the control viruses and the viruses exposed to drug allowed
274 us to control for sequencing errors. The pattern of mutations seen in both samples exposed
275 to favipiravir were significantly different to the control (Permutation analysis, $p < 1 * 10^{-4}$;
276 Supplemental figure 3A, 3C.) The mutation bias was caused by an excess of C->U and G->A
277 transitions compared with the control viruses (Permutation analysis, $p < 1 * 10^{-4}$,
278 Supplemental figure 3B, 3D). There was no significant difference between the mutation bias
279 at the two different concentrations of favipiravir tested (Permutation Analysis, $p = 0.26$,
280 Supplemental figure 3E, 3F). To demonstrate further that this method measures a true
281 mutational signal, we took the 500 sites with the highest degree of polymorphism and
282 repeated the analysis (Supplemental figure 4). The new analysis showed an increased effect
283 size strongly suggesting that mutations caused by favipiravir lead to a signal in the
284 sequencing data that is not masked by sequencing error. We chose to use the relative
285 proportion of the mutation types to compare between samples as opposed to the absolute
286 number of polymorphisms. This was a conservative choice as there may be biases between
287 samples which could affect the absolute number of polymorphism due to the number of
288 viruses in the sample.

289

290 **Discussion**

291 In this study, we used two different methods of analysing next-generation sequencing data
292 in order to show that favipiravir acts as a mutagen with a distinct bias to induce transitions
293 in influenza virus RNAs. The first method used Primer ID to measure precisely the increase in
294 mutation rate and the mutation bias of the influenza polymerase caused by favipiravir in an
295 *in vitro* system. We confirmed that favipiravir has a bias for transition mutations and acts as
296 a purine analogue^{17,26,32,33}. We were able to demonstrate that favipiravir competed
297 primarily with guanine and secondarily with adenine resulting in an increase in C->U and G-
298 >A mutations at higher concentrations of drug and a lower rate of increase in U->C and A->G
299 mutations (Figure 5). The second method used data from whole-genome sequencing of
300 viruses that had been exposed to favipiravir during single cycle replication and showed that
301 viral populations exposed to favipiravir had a distinct bias for transition mutations,
302 specifically C->U and G->A mutations.

303 Previous methods of sequence analysis for determining mutation bias in influenza RNAs
304 induced by favipiravir have relied on Sanger sequencing of individual viral clones^{27,31}. This
305 technique is laborious and results in the detection of relatively few mutations: on the order
306 of 100 mutations for an entire experiment^{27,31}. Furthermore, the technique can be biased
307 due to selection of beneficial mutations which may appear in multiple clones or to
308 accidentally counting an initial polymorphism in the population as a mutational event that
309 occurred in multiple clones. Sequencing a small region of the genome across many clones is
310 especially prone to this error. Next-generation sequencing with Primer ID is a powerful

311 technique which allowed us to examine orders of magnitude more mutations than Sanger
312 sequencing and was less prone to biases present in examining a small number of mutations.
313 Primer ID allowed us to remove sequencing error from next-generation sequencing data and
314 to detect changes in mutation rate and mutation bias^{39,40}. Primer ID identified thousands of
315 mutations in a single sample exposed to favipiravir, a number which would be impractical
316 using Sanger sequencing. We were able to show that favipiravir acts as both a guanine and
317 an adenine analogue whereas Sanger sequencing was not sensitive enough to measure the
318 lower rate of adenine mutations²⁷.

319 The use of the minigenome assay allowed us to see all mutations generated by polymerase
320 and not just those that would allow viable viruses. Pauly *et al.* have recently shown that the
321 mutation rate for influenza has been significantly underestimated by only counting
322 mutations which occur in plaque forming viruses⁴⁷. Sequencing only viruses which have
323 exited the cell ignores mutations that cause defects in packaging or cellular exit. By contrast,
324 as the mRNA from the reporter in the minigenome assay is not translated to a protein that
325 can impact on viral fitness, the full spectrum of drug-induced mutations can be seen.

326 Allowing for multiple rounds of virus replication makes it difficult to see strongly deleterious
327 mutations, which make up a significant proportion of the mutations for influenza, because
328 they are selected against⁴⁸. The minigenome assay has no selection on mutations and does
329 not suffer from this bias. However, when we used a Primer ID approach to sequence a small
330 portion of the viral genome from PB1 amplified during virus infection rather than in the
331 minigenome assay, we found, contrary to the minigenome sequencing, that there was no
332 increase in the mutation rate at the highest concentrations of favipiravir. This is likely due to
333 selection against deleterious mutations that occurs even in a single cycle of replication.

334 Favipiravir causes mutations randomly and therefore there will be a distribution in the
335 number of mutations during each strand replication. Some RNAs will have many mutations
336 whereas others will have fewer. The majority of the RNA that was sequenced will come
337 from viruses which have suffered few mutations, as viral RNAs with more mutations will
338 interfere with ongoing replication. Therefore, the more successful favipiravir is at causing
339 mutations, the greater the bias to sequencing the small number of viruses with fewer
340 mutations. This most likely explains why the mutation rate we measured appeared lower at
341 100 μ M favipiravir than at 10 μ M.

342 Although Primer ID can remove sequencing error, it is still impossible to distinguish between
343 errors due to the flu polymerase and the reverse transcriptase used during the Primer ID
344 reaction. A recent paper has suggested that care must be taken as these two error rates are
345 the same order of magnitude⁴⁷. For this reason, we have not reported an absolute error rate
346 but a relative error rate compared to the drug-free baseline sample. However, for our
347 experiments, the mutation rate caused by favipiravir was much higher than the calculated
348 baseline mutation rate caused by reverse transcription errors plus errors naturally caused by
349 the influenza polymerase. Furthermore, as all samples underwent identical processing,
350 there is no reason to believe that the error rate during reverse transcription differed
351 between samples and therefore, this is unlikely to bias our data. Care would need to be
352 taken before comparing samples which have not been prepared concurrently especially if
353 using different reverse transcription enzymes.

354

355 One disadvantage to Primer ID is that it sequences only a small part of the genome. This
356 potentially could lead to mutation biases if that part of the genome was under strong

357 selection or due to local sequence structure. As we sampled only one region of the HA, we
358 could not test whether there were specific structural differences between the HA sequence
359 and other flu segments leading to mutational hotspots. However, the similarity between our
360 analysis of RNAs from primer ID vs whole genome sequencing suggests we did not
361 inadvertently sample a mutational hotspot. The precision and ease with which Primer ID
362 was able to distinguish mutation bias and observe changes in mutation rate leads us to
363 suggest that it could become a standard method for analysing the effects of nucleoside
364 analogues and other mutagenic drugs.

365 Our second method of analysis sequenced the whole flu genome in populations of viruses
366 that had been exposed to favipiravir and a control population that was not exposed to the
367 drug as might be found in a clinical setting. The main disadvantage of this technique is that it
368 is unable to distinguish between sequencing error and 'true' errors caused by the flu
369 polymerase. Therefore, it is not possible to quantify the actual number of errors due to
370 polymerase nor was the method sensitive enough to demonstrate any increase in the rate
371 of U->C and A->G mutations. Despite these limitations, there are several advantages to this
372 method that may prove to be of use in clinical settings. This method is extremely simple to
373 use as the viruses can be entered into the standard influenza sequencing pipeline without
374 any additional processing steps and could also be used to reanalyse data that had been
375 previously collected. The analysis also encompasses the whole genome and so is resistant to
376 any biases caused by local RNA structure nor is it biased by single polymorphisms that may
377 have been present in the initial populations. If favipiravir is used in a clinical setting, this
378 method may be a simple way to show that favipiravir is having a measurable effect by
379 comparing viral mutations in pre-treatment and post-treatment samples.

380 In contrast to our finding that favipiravir acts as a purine analogue, a previous study that
381 used NGS to determine the mutation bias of favipiravir *in vivo* found an excess of
382 transversion mutations³⁶. The analysis in Marathe *et al.* counted each individual NGS read as
383 a separate mutational event, which may have led to a bias, as mutations from pre-existing
384 polymorphisms or mutations that are positively selected will be counted multiple times. By
385 contrast, our method of analysing NGS data ensured that mutations were independent by
386 only counting one mutation at each site in the genome (Supplemental figure 2). Many
387 recent papers that analyse NGS data use a cut off e.g. 5% or 1% of reads below which
388 variants are not counted^{31,36,38}. However, using a cut-off discards a large amount of the
389 sequence data as only a small proportion of sites are included. Our analysis (Figure 4,
390 Supplemental figure 2) used all the sequencing data without imposing a cut-off and this led
391 to increased noise in the data but ensured that there was no bias towards pre-existing
392 polymorphisms or variations in sequencing depth. We also tested the mutational bias by
393 only counting the 500 sites with the largest degree of polymorphism (Supplemental figure 4)
394 which showed similar results to our main analysis though potentially with less noise. This
395 suggests that imposing a cut-off on variants will not bias the results if the sequencing
396 contains enough variants that positive selection and pre-existing polymorphisms are unlikely
397 to influence the results.

398 Our data showed that favipiravir acts as a mutagen with a bias towards transitions in
399 agreement with most other studies of this drug's effect on RNA viruses^{27,28,35}. We found that
400 at lower concentrations of favipiravir, there was no evidence that the drug was acting as a
401 chain terminator as there was no reduction in the amount of mRNA despite a reduction in
402 reporter gene activity (Figure 2A, B). At the highest concentration tested (100 μ M), there

403 was a reduction in mRNA which could have been caused by chain termination or through
404 introduced mutations preventing RNA replication. The lack of evidence for chain
405 termination at lower concentrations of favipiravir suggests that favipiravir is primarily acting
406 as a mutagen. Biochemically, favipiravir acts as a purine analogue binding to either C or U in
407 place of G or A respectively. The most common mutations caused by favipiravir were C->U
408 and G->A. These mutations were caused by favipiravir binding to C in place of a G on the
409 positive or negative strand synthesis and subsequently pairing with a U in the next synthesis
410 cycle (Figure 5). The reverse transitions caused by favipiravir binding to U happened at a
411 ~3.5-fold lower rate. This confirms that favipiravir is most competitive against G as had been
412 previously seen in primer extension assays^{32,33}.

413 Next-generation Sequencing is a powerful technique for analysing mutational data and
414 determining mutational biases. Care must be taken to perform analyses which minimize
415 potential biases by ensuring that mutations are only counted when they occur
416 independently of each other. We used NGS to show that favipiravir is acting as a mutagen
417 causing multiple additional mutations per influenza genome on average at higher
418 concentrations of favipiravir. Lethal mutagenesis of influenza is a viable antiviral strategy
419 and may be difficult to evolve resistance against clinically⁴⁹. Our increased knowledge of the
420 precise mechanism of favipiravir means that we are better placed to test whether the drug
421 is having a clinical effect as well as to see whether viruses are becoming resistant to
422 favipiravir. This will be important when this drug is used in a pandemic situation.

423 **Acknowledgments**

424 This work was supported by the National Institute for Health Research Health
425 Protection Research Unit (NIHR HPRU) in Respiratory Infections at Imperial College in

426 partnership with Public Health England (PHE). PL, PK and WB were supported by Wellcome
427 Trust Grant 200187/Z/15/Z. The views expressed are those of the authors and not necessarily
428 those of the NHS, the NIHR, the Department of Health or PHE.

429

430 References

- 431 1 WHO. *Influenza (Seasonal) Factsheet.*, <[http://www.who.int/en/news-room/fact-](http://www.who.int/en/news-room/fact-sheets/detail/influenza-(seasonal))
432 [sheets/detail/influenza-\(seasonal\)](http://www.who.int/en/news-room/fact-sheets/detail/influenza-(seasonal))> (2018).
- 433 2 Ferguson, N. M. *et al.* Strategies for mitigating an influenza pandemic. *Nature* **442**, 448
434 (2006).
- 435 3 Fauci, A. S. Seasonal and pandemic influenza preparedness: science and countermeasures.
436 *Journal of Infectious Diseases* **194**, S73-S76 (2006).
- 437 4 Deyde, V. M. *et al.* Surveillance of resistance to adamantanes among influenza A (H3N2) and
438 A (H1N1) viruses isolated worldwide. *Journal of Infectious Diseases* **196**, 249-257 (2007).
- 439 5 Hurt, A. C. The epidemiology and spread of drug resistant human influenza viruses. *Curr Opin*
440 *Virology* **8**, 22-29 (2014).
- 441 6 Moscona, A. Global transmission of oseltamivir-resistant influenza. *New England Journal of*
442 *Medicine* **360**, 953-956 (2009).
- 443 7 Watanabe, T. *et al.* Characterization of H7N9 influenza A viruses isolated from humans.
444 *Nature* **501**, 551 (2013).
- 445 8 Zhu, H. *et al.* Infectivity, Transmission, and Pathology of Human-Isolated H7N9 Influenza
446 Virus in Ferrets and Pigs. *Science* **341**, 183-186, doi:10.1126/science.1239844 (2013).
- 447 9 Imai, M. *et al.* A highly pathogenic avian H7N9 influenza virus isolated from a human is lethal
448 in some ferrets infected via respiratory droplets. *Cell host & microbe* **22**, 615-626. e618
449 (2017).
- 450 10 Koszalka, P., Tilmanis, D. & Hurt, A. C. Influenza antivirals currently in late-phase clinical trial.
451 *Influenza and other respiratory viruses* **11**, 240-246 (2017).
- 452 11 De Clercq, E. & Li, G. Approved Antiviral Drugs over the Past 50 Years. *Clinical Microbiology*
453 *Reviews* **29**, 695-747 (2016).
- 454 12 Furuta, Y. *et al.* In vitro and in vivo activities of anti-influenza virus compound T-705.
455 *Antimicrob Agents Ch* **46**, 977-981 (2002).
- 456 13 Sidwell, R. W. *et al.* Efficacy of orally administered T-705 on lethal avian influenza A (H5N1)
457 virus infections in mice. *Antimicrob Agents Ch* **51**, 845-851 (2007).
- 458 14 Kiso, M. *et al.* T-705 (favipiravir) activity against lethal H5N1 influenza A viruses. *Proceedings*
459 *of the National Academy of Sciences* **107**, 882-887 (2010).
- 460 15 Furuta, Y. *et al.* T-705 (favipiravir) and related compounds: Novel broad-spectrum inhibitors
461 of RNA viral infections. *Antiviral research* **82**, 95-102 (2009).
- 462 16 Sleeman, K. *et al.* In vitro antiviral activity of favipiravir (T-705) against drug-resistant
463 influenza and 2009 A (H1N1) viruses. *Antimicrob Agents Ch* **54**, 2517-2524 (2010).
- 464 17 Furuta, Y. *et al.* Favipiravir (T-705), a novel viral RNA polymerase inhibitor. *Antiviral research*
465 **100**, 446-454 (2013).
- 466 18 Furuta, Y., Komeno, T. & Nakamura, T. Favipiravir (T-705), a broad spectrum inhibitor of viral
467 RNA polymerase. *Proceedings of the Japan Academy, Series B* **93**, 449-463,
468 doi:10.2183/pjab.93.027 (2017).

- 469 19 Mendenhall, M. *et al.* T-705 (favipiravir) inhibition of arenavirus replication in cell culture.
470 *Antimicrob Agents Ch* **55**, 782-787 (2011).
- 471 20 Mendenhall, M. *et al.* Effective oral favipiravir (T-705) therapy initiated after the onset of
472 clinical disease in a model of arenavirus hemorrhagic Fever. *PLoS neglected tropical diseases*
473 **5**, e1342 (2011).
- 474 21 Oestereich, L. *et al.* Successful treatment of advanced Ebola virus infection with T-705
475 (favipiravir) in a small animal model. *Antiviral research* **105**, 17-21 (2014).
- 476 22 Safronetz, D. *et al.* The broad-spectrum antiviral favipiravir protects guinea pigs from lethal
477 Lassa virus infection post-disease onset. *Scientific reports* **5**, 14775 (2015).
- 478 23 Oestereich, L. *et al.* Efficacy of favipiravir alone and in combination with ribavirin in a lethal,
479 immunocompetent mouse model of Lassa fever. *The Journal of infectious diseases* **213**, 934-
480 938 (2015).
- 481 24 Tani, H. *et al.* Efficacy of T-705 (Favipiravir) in the treatment of infections with lethal severe
482 fever with thrombocytopenia syndrome virus. *mSphere* **1**, e00061-00015 (2016).
- 483 25 Delang, L., Abdelnabi, R. & Neyts, J. Favipiravir as a potential countermeasure against
484 neglected and emerging RNA viruses. *Antiviral research* (2018).
- 485 26 Furuta, Y. *et al.* Mechanism of action of T-705 against influenza virus. *Antimicrob Agents Ch*
486 **49**, 981-986 (2005).
- 487 27 Baranovich, T. *et al.* T-705 (favipiravir) induces lethal mutagenesis in influenza A H1N1
488 viruses in vitro. *J Virol* **87**, 3741-3751 (2013).
- 489 28 Arias, A., Thorne, L. & Goodfellow, I. Favipiravir elicits antiviral mutagenesis during virus
490 replication in vivo. *Elife* **3**, e03679 (2014).
- 491 29 de Ávila, A. I. *et al.* Lethal Mutagenesis of Hepatitis C Virus Induced by Favipiravir. *PLOS ONE*
492 **11**, e0164691, doi:10.1371/journal.pone.0164691 (2016).
- 493 30 Barauskas, O. *et al.* Biochemical characterization of recombinant influenza A polymerase
494 heterotrimer complex: Polymerase activity and mechanisms of action of nucleotide analogs.
495 *PloS one* **12**, e0185998 (2017).
- 496 31 Vanderlinden, E. *et al.* Distinct effects of T-705 (favipiravir) and ribavirin on influenza virus
497 replication and viral RNA synthesis. *Antimicrob Agents Ch* **60**, 6679-6691 (2016).
- 498 32 Sangawa, H. *et al.* Mechanism of action of T-705 ribosyl triphosphate against influenza virus
499 RNA polymerase. *Antimicrob Agents Ch* **57**, 5202-5208 (2013).
- 500 33 Jin, Z., Smith, L. K., Rajwanshi, V. K., Kim, B. & Deval, J. The ambiguous base-pairing and high
501 substrate efficiency of T-705 (favipiravir) ribofuranosyl 5'-triphosphate towards influenza A
502 virus polymerase. *PloS one* **8**, e68347 (2013).
- 503 34 Jin, Z. *et al.* Structure-activity relationship analysis of mitochondrial toxicity caused by
504 antiviral ribonucleoside analogs. *Antiviral research* **143**, 151-161 (2017).
- 505 35 Vanderlinden, E. *et al.* Distinct Effects of T-705 (Favipiravir) and Ribavirin on Influenza Virus
506 Replication and Viral RNA Synthesis. *Antimicrob Agents Chemother* **60**, 6679-6691,
507 doi:10.1128/AAC.01156-16 (2016).
- 508 36 Marathe, B. M. *et al.* Combinations of oseltamivir and T-705 extend the treatment window
509 for highly pathogenic influenza A (H5N1) virus infection in mice. *Scientific reports* **6** (2016).
- 510 37 Delang, L. *et al.* Mutations in the chikungunya virus non-structural proteins cause resistance
511 to favipiravir (T-705), a broad-spectrum antiviral. *Journal of Antimicrobial Chemotherapy* **69**,
512 2770-2784 (2014).
- 513 38 Guedj, J. *et al.* Antiviral efficacy of favipiravir against Ebola virus: A translational study in
514 cynomolgus macaques. *PLOS Medicine* **15**, e1002535, doi:10.1371/journal.pmed.1002535
515 (2018).
- 516 39 Jabara, C. B., Jones, C. D., Roach, J., Anderson, J. A. & Swanstrom, R. Accurate sampling and
517 deep sequencing of the HIV-1 protease gene using a Primer ID. *Proceedings of the National*
518 *Academy of Sciences* **108**, 20166-20171 (2011).

- 519 40 Zhou, S., Jones, C., Mieczkowski, P. & Swanstrom, R. Primer ID Validates Template Sampling
520 Depth and Greatly Reduces the Error Rate of Next-Generation Sequencing of HIV-1 Genomic
521 RNA Populations. *J Virol* **89**, 8540-8555, doi:10.1128/JVI.00522-15 (2015).
- 522 41 Kosik, I. *et al.* Influenza A virus hemagglutinin glycosylation compensates for antibody
523 escape fitness costs. *PLoS pathogens* **14**, e1006796 (2018).
- 524 42 Watson, S. J. *et al.* Viral population analysis and minority-variant detection using short read
525 next-generation sequencing. *Phil. Trans. R. Soc. B* **368**, 20120205 (2013).
- 526 43 Sheward, D. J., Murrell, B. & Williamson, C. Degenerate Primer IDs and the Birthday
527 Problem. *Proceedings of the National Academy of Sciences* **109**, E1330-E1330,
528 doi:10.1073/pnas.1203613109 (2012).
- 529 44 Long, J. S. *et al.* Species difference in ANP32A underlies influenza A virus polymerase host
530 restriction. *Nature* **529**, 101, doi:10.1038/nature16474 (2016).
- 531 45 Zhou, B. *et al.* Single-reaction genomic amplification accelerates sequencing and vaccine
532 production for classical and Swine origin human influenza A viruses. *J Virol* **83**, 10309-10313
533 (2009).
- 534 46 Jabara, C. B., Jones, C. D., Roach, J., Anderson, J. A. & Swanstrom, R. Accurate sampling and
535 deep sequencing of the HIV-1 protease gene using a Primer ID. *Proc Natl Acad Sci U S A* **108**,
536 20166-20171, doi:10.1073/pnas.1110064108 (2011).
- 537 47 Pauly, M. D., Procario, M. & Luring, A. S. The mutation rates and mutational bias of
538 influenza A virus. *bioRxiv*, doi:10.1101/110197 (2017).
- 539 48 Visher, E., Whitefield, S. E., McCrone, J. T., Fitzsimmons, W. & Luring, A. S. The Mutational
540 Robustness of Influenza A Virus. *Plos Pathogens* **12**, doi:10.1371/journal.ppat.1005856
541 (2016).
- 542 49 Pauly, M. D. & Luring, A. S. Effective Lethal Mutagenesis of Influenza Virus by Three
543 Nucleoside Analogs. *J Virol* **89**, 3584-3597, doi:10.1128/Jvi.03483-14 (2015).

544

545 Figure Legends

546 **Figure 1- Primer ID Method for determining mutation bias.** RNA was extracted and a
547 unique barcode of the form NNNNTNNNNTNNNN added during reverse transcription. qPCR
548 was used to standardize the number of barcodes for NGS. Samples were sequenced and
549 barcodes matched to allow removal of PCR and sequencing errors.

550 **Figure 2- Favipiravir causes transition mutations which reduces polymerase activity in a**
551 **minigenome assay. A)** Minigenome Assay. Plasmids were transfected into 293-T cells and
552 favipiravir was added. At ~21hrs the cells were lysed and luciferase activity was measured.
553 The relative polymerase activity is calculated as firefly activity / renilla activity. **B)** A reporter
554 plasmid (HA pol1) from the minigenome above was sequenced using Primer ID and NGS.
555 The mutations were tallied as described in Methods. Two independent biological samples
556 were sequenced for each concentration of drug in the same sequencing reaction. The
557 number of mutations per 10,000 nucleotides above the average of the two control samples
558 were compared for each sample. **C)** The number of mutations per 10,000 nucleotides above
559 the control for each sample was calculated for transitions and transversions. **D)** As 1C
560 calculated for each class of transition mutation. The values are calculated as the mutation
561 rate for an individual base. The average for the two samples is plotted. **E)** qPCR was
562 performed on the luciferase reporter mRNA from a minigenome assay. $\Delta\Delta C_t$ was calculated
563 using 18sRNA and results are shown normalized to the drug-free control. N=6. *** p<0.001.

564 **Supplemental figure 1- Determination of the optimal cut-off for number of reads per**
565 **barcode.** The cut-off for the number of reads necessary to include a barcode was
566 systematically varied and the number of mutations per 10,000 nucleotides above the
567 control plotted for each sample.

568 **Figure 3- Favipiravir causes transition mutations reducing viral fitness. A)** Virus was added
569 to MDCK cells at a high MOI of 1.3 and favipiravir was added at an appropriate
570 concentration diluted in DMSO. The supernatant was plaqued after 20 hours and the titre
571 calculated in plaque forming units/ml. N=3. **B)** After 18 hours the cells were lysed and the
572 RNA extracted for sequencing using Primer ID. The number of mutations per 10,000
573 nucleotides above the control was plotted for each sample. **C)** The number of mutations per
574 10,000 nucleotides above the control for each sample was calculated for transitions and
575 transversions. **D)** The number of mutations per 10,000 nucleotides above the control for
576 each sample was calculated for each class of transition mutation. The values are calculated
577 as the mutation rate for an individual base.

578 **Supplemental figure 2- A method to analyse mutation bias from whole genome NGS data.**

579 Whole genome sequencing data from a standard pipeline was aligned to a reference. The
580 most common polymorphism for each site in the genome was calculated. These
581 polymorphisms were summed up and the mutation bias of different samples can be
582 compared.

583 **Figure 4- Next-generation sequencing data shows that favipiravir acts as a guanine**

584 **analogue.** Virus was added to MDCK cells at a high MOI of 1 and drug was added as
585 previously described. Supernatant was taken and was sequenced and analysed as described
586 in *Methods*. The most common polymorphism for each base is shown for virus exposed to
587 drug and to a drug free control. The comparison shows the difference in percentage for
588 each class of mutations revealing mutation bias.

589 **Supplemental figure 3-** A permutation analysis was performed on the mutation data. The

590 substitutions were randomized between the treatment and control and either the total

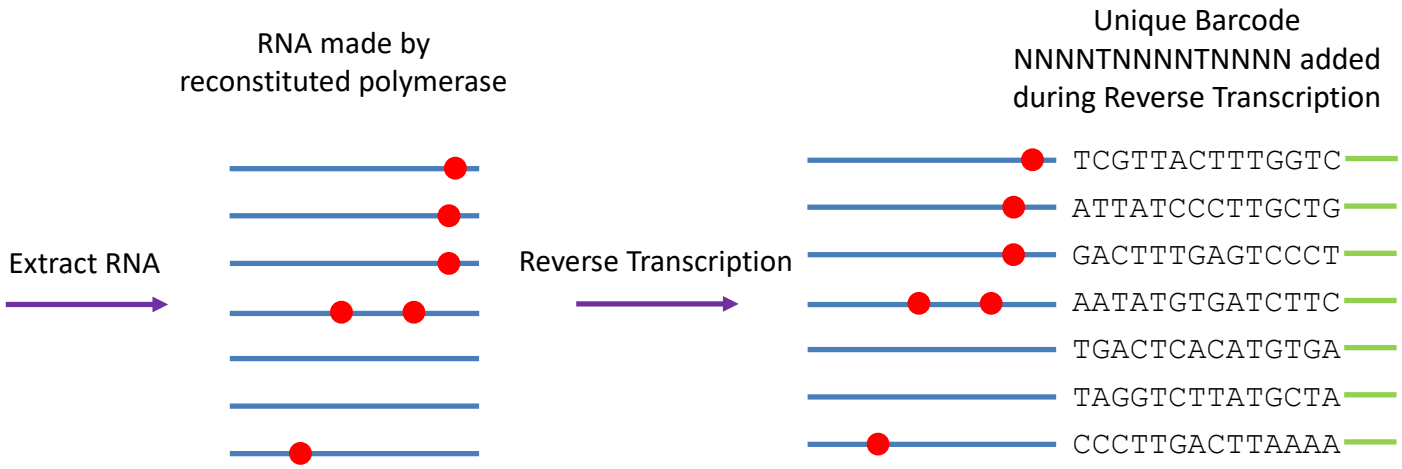
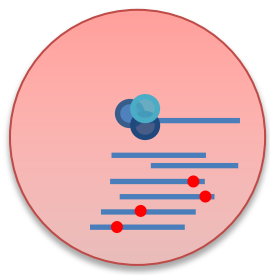
591 difference in mutation bias was calculated (A, C, E) or the bias for acting as a guanine
592 analogue (B, D, F.) 10,000 permutations were performed for each analysis. The red bars
593 show the observed value where it occurs within the values generated by the permutations.
594 **A)** The mutation bias for 10 μ M favipiravir was compared to the control (observed value =
595 0.39; $p < 1 \cdot 10^{-4}$). **B)** The difference in bias for guanine mutations (observed value = 0.19;
596 $p < 1 \cdot 10^{-4}$). **C)** The mutation bias for 100 μ M favipiravir was compared to the control
597 (observed value = 0.37; $p < 1 \cdot 10^{-4}$). **D)** The difference in bias for guanine mutations
598 (observed value = 0.19; $p < 1 \cdot 10^{-4}$). **E)** The mutation bias for 10 μ M favipiravir was
599 compared to 100 μ M favipiravir (observed value = 0.03; $p = 0.26$). **F)** The difference in bias for
600 guanine mutations (observed value = 0.007; $p = 0.34$).

601 **Supplemental figure 4-** The same data from Figure 3 was reanalysed using only the 500 sites
602 with the largest degree of polymorphism.

603 **Figure 5-** A schematic showing how favipiravir causes mutations during +ve and -ve strand
604 synthesis.

605

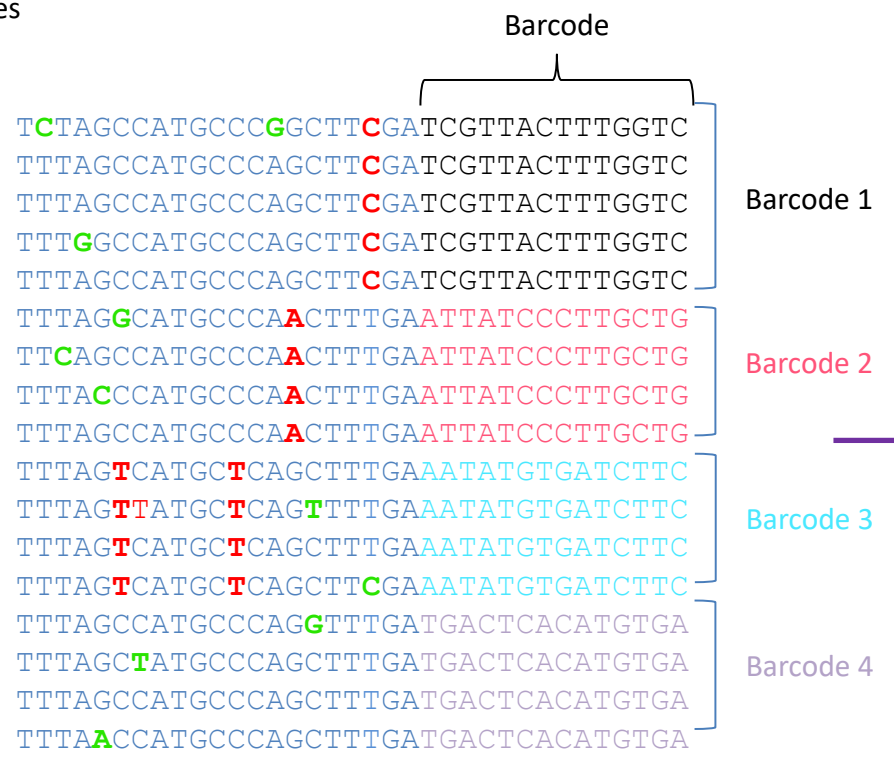
Figure 1
Minigenome Assay



qPCR to standardize number of barcodes

PCR and NGS sample prep

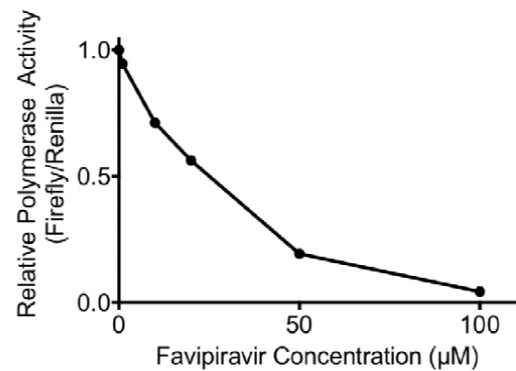
Next generation sequencing



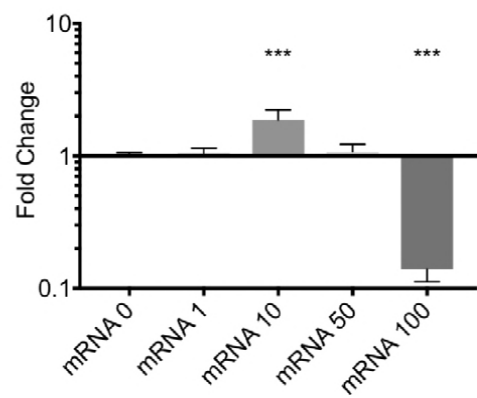
Match barcodes to remove sequencing errors and identify errors caused by the viral polymerase

Figure 2

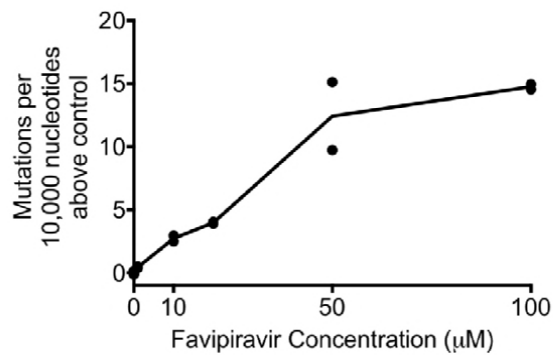
A



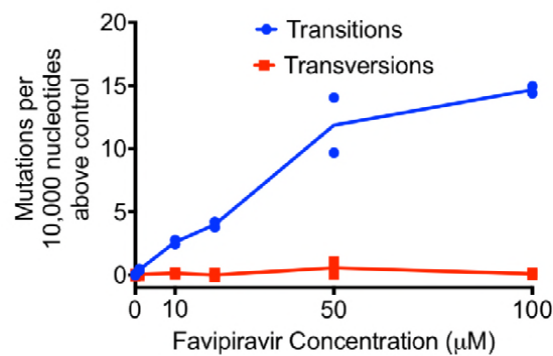
B



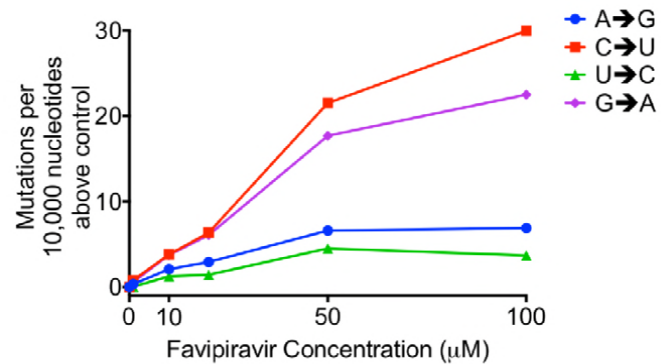
C



D



E



Supplementary Figure 1

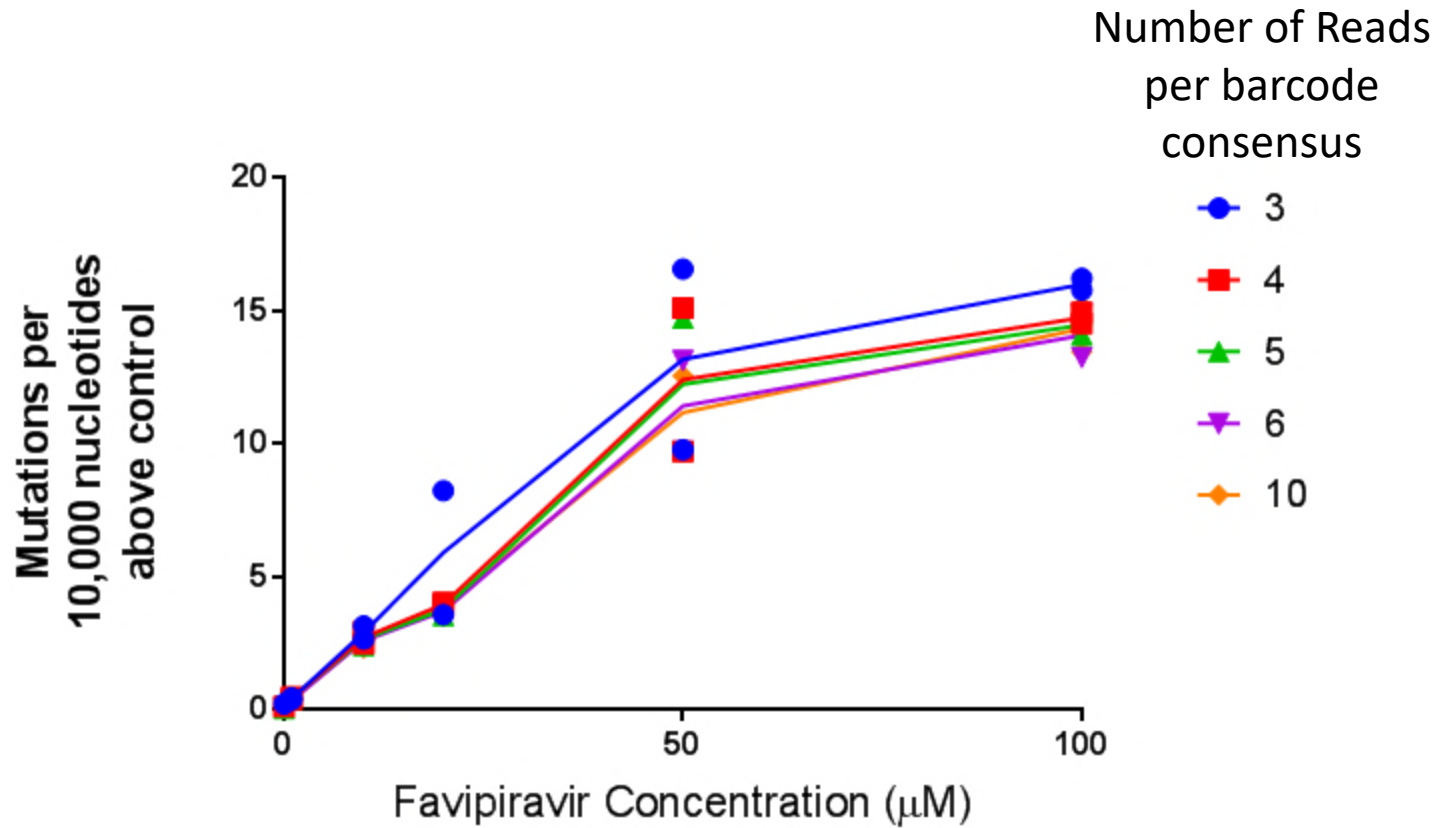
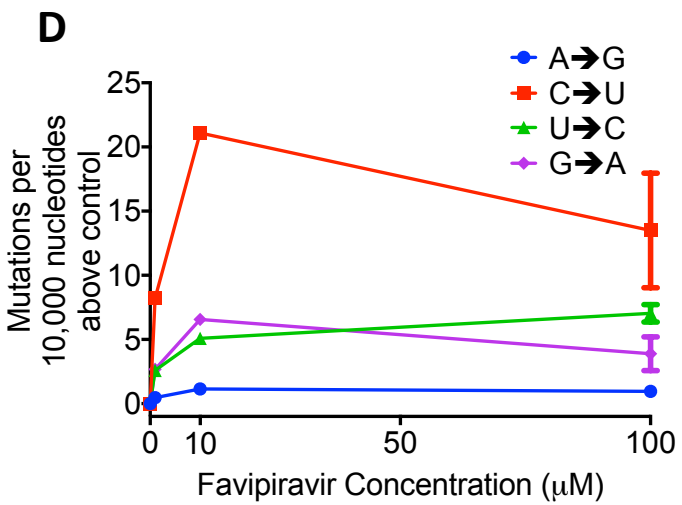
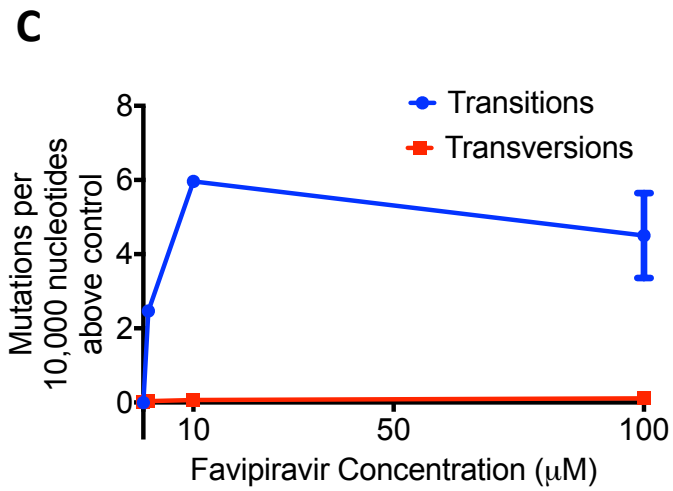
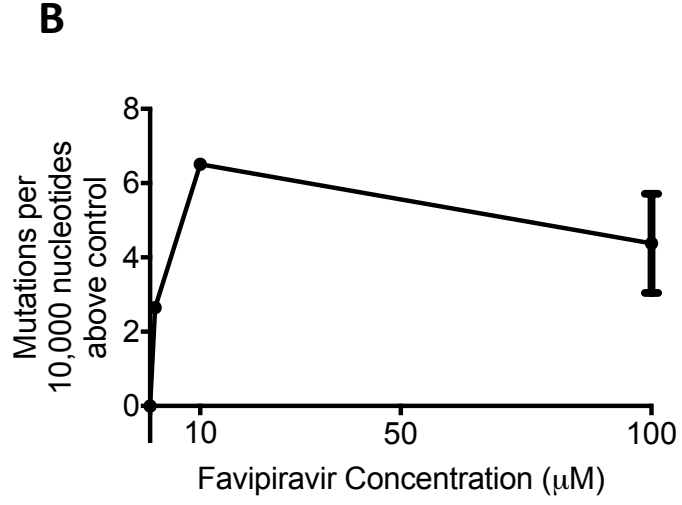
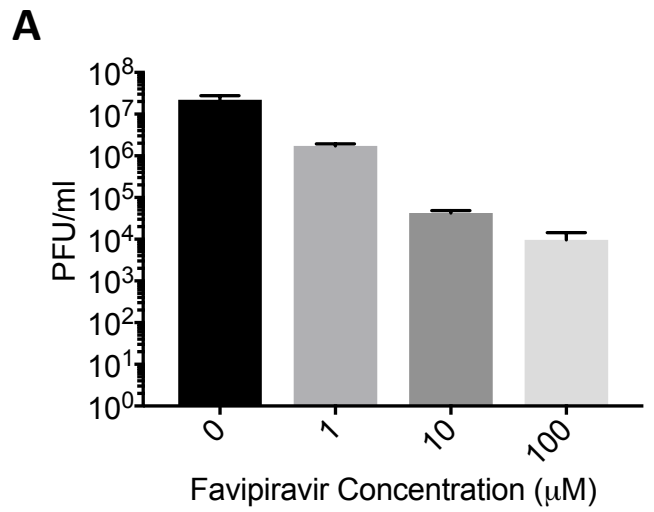


Figure 3



Supplemental Figure 2



NGS Reads

```

    CATATACGACACTGCGCGCCGGCATAACGCCTCCGCTA
    CATATACGACACTGCGCGCCGGCATAACGCTTCCGCTAGAGCTA
    CATATAGGACACTGCGCGCCGGCATAACGCTTCCGCTAGAGCTATTCG
    CATATACGACACTGCGGGCCGGCATAACGCTTCCGCTAGGGCTATTCGTACATG
    CATATACGACACTGCGCGCCGGCATAACGCTTCCGCTAGGGCTATTCGTACATG
    CATATACGACACTGCGCGCCGGCATAACGCTTCTGCTAGGGCTATTCATACATG
    CATTTACGACACTGCGTGCCGGCATAACGCTTCCGCTAGGGCTATTCGTACATG
    CATATAGGACACTGCGCGCCGGCGTACGCTTCCGCTAGGGCTATTCGTACATG
    CATATACGACACTGCGCGCCGGCATAACGCTTCCGCTAGAGCTATTCGTACATG
    CATATACGACACTGCGTGCCGGCATAACGCTTCCGCTAGGGCTATTCGTACATA
    CATATACGACACTGCGCGCCGGCATAACGCCTCCGCTAGGGCTATTCGTACATG
    TATACGACATTGCGCGCCGGCATAACGCTTCCGCTAGGGCTATTCGTATATG
    TACGACACTGCGCGCCGGTATACGCTTCCGCTAGAGCTATTCGTACATG
    GACACTGCGCGCTGGCATAACGCTTCCGCTAGAGCTATTCGTACATG
  
```

Most Common Polymorphism

```

    ---T--G----T----T--T--TG-----C--T-----A-----A--T--A
  
```

| | Treatment | | | | Control | | | | Comparison to control | | | | | | |
|--|-----------|------|------|------|---------|---|------|------|-----------------------|------|---|-------|-------|-------|-------|
| | → | A | C | U | G | → | A | C | U | G | → | A | C | U | G |
| Sum up polymorphisms across the genome | A | * | 93 | 50 | 2597 | A | * | 129 | 119 | 2213 | A | * | -0.01 | -0.01 | -0.02 |
| | C | 231 | * | 1713 | 8 | C | 579 | * | 708 | 27 | C | -0.05 | * | 0.09 | 0.00 |
| | U | 73 | 1801 | * | 33 | U | 130 | 1543 | * | 72 | U | -0.01 | -0.02 | * | -0.01 |
| | G | 2349 | 9 | 183 | * | G | 1098 | 47 | 553 | * | G | 0.10 | -0.01 | -0.06 | * |

Figure 4

Number of polymorphisms

Comparison to control

Control

| → | A | C | U | G |
|---|------|------|-----|------|
| A | * | 129 | 119 | 2213 |
| C | 579 | * | 708 | 27 |
| U | 130 | 1543 | * | 72 |
| G | 1098 | 47 | 553 | * |

10uM Favipiravir

| → | A | C | U | G |
|---|------|------|------|------|
| A | * | 93 | 50 | 2597 |
| C | 231 | * | 1713 | 8 |
| U | 73 | 1801 | * | 33 |
| G | 2349 | 9 | 183 | * |

| → | A | C | U | G |
|---|-------|-------|-------|-------|
| A | * | -0.01 | -0.01 | -0.02 |
| C | -0.05 | * | 0.09 | 0.00 |
| U | -0.01 | -0.02 | * | -0.01 |
| G | 0.10 | -0.01 | -0.06 | * |

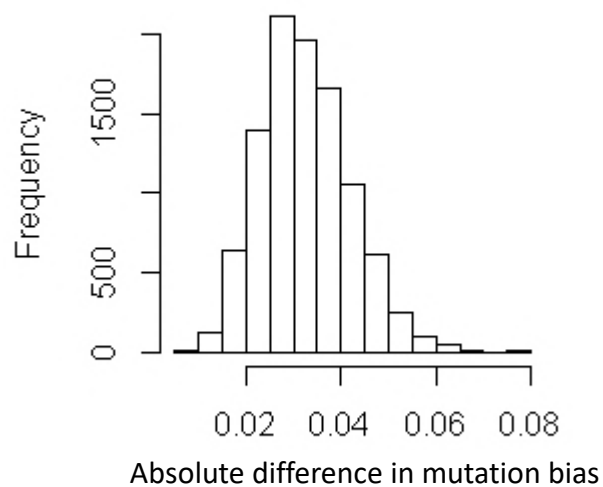
100uM Favipiravir

| → | A | C | U | G |
|---|------|------|------|------|
| A | * | 128 | 47 | 2452 |
| C | 230 | * | 1572 | 16 |
| U | 64 | 1791 | * | 47 |
| G | 2273 | 12 | 157 | * |

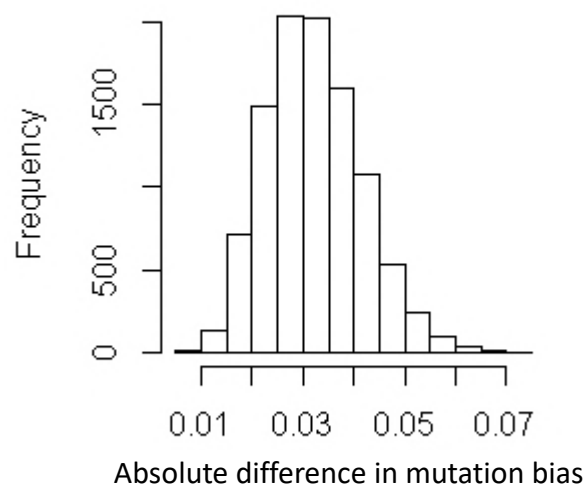
| → | A | C | U | G |
|---|-------|-------|-------|-------|
| A | * | 0.00 | -0.01 | -0.03 |
| C | -0.05 | * | 0.08 | 0.00 |
| U | -0.01 | -0.01 | * | 0.00 |
| G | 0.11 | -0.01 | -0.06 | * |

Supplemental Figure 3

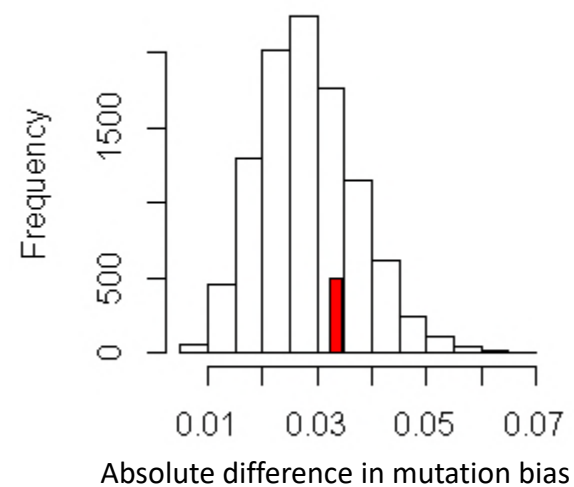
A
10 μ M Favipiravir vs. Control



C
100 μ M Favipiravir vs. Control



E
10 μ M vs 100 μ M Favipiravir



B

Frequency

Guanine mutation bias

Detailed description: Histogram B shows the distribution of Guanine mutation bias. The x-axis ranges from 0.000 to 0.030 with major ticks at 0.000, 0.010, 0.020, and 0.030. The y-axis represents frequency, with major ticks at 0, 400, and 800. The distribution is unimodal and roughly bell-shaped, peaking at approximately 0.005 with a frequency of about 1000.

D

Frequency

Guanine mutation bias

Detailed description: Histogram D shows the distribution of Guanine mutation bias. The x-axis ranges from 0.000 to 0.030 with major ticks at 0.000, 0.010, 0.020, and 0.030. The y-axis represents frequency, with major ticks at 0, 200, 600, and 1000. The distribution is unimodal and roughly bell-shaped, peaking at approximately 0.005 with a frequency of about 1000.

F

Frequency

Guanine mutation bias

Detailed description: Histogram F shows the distribution of Guanine mutation bias. The x-axis ranges from 0.000 to 0.020 with major ticks at 0.000, 0.010, and 0.020. The y-axis represents frequency, with major ticks at 0, 400, and 800. The distribution is unimodal and roughly bell-shaped, peaking at approximately 0.005 with a frequency of about 1000. A single bar at approximately 0.007 is highlighted in red.

Supplemental Figure 4

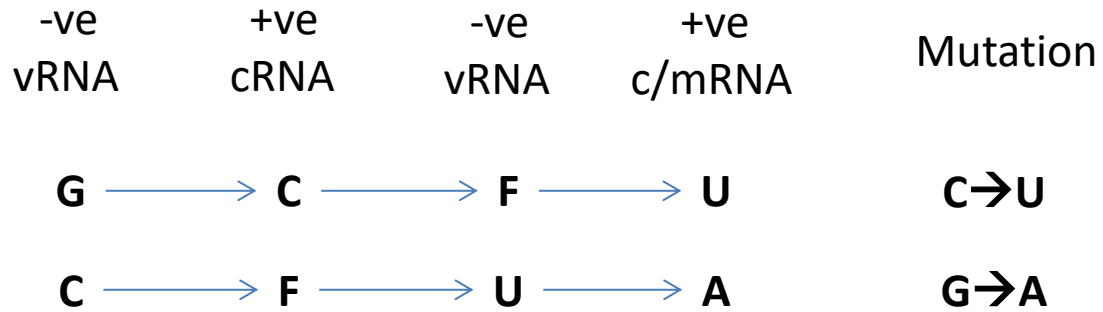
| | <u>Control</u> | | | | | <u>Drug - 10μM Favipiravir</u> | | | |
|---------------|----------------|-----|----|-----|---------------|---|----|-----|----|
| \rightarrow | A | C | U | G | \rightarrow | A | C | U | G |
| A | * | 4 | 2 | 209 | A | * | 0 | 1 | 66 |
| C | 22 | * | 41 | 1 | C | 5 | * | 133 | 0 |
| U | 4 | 144 | * | 3 | U | 1 | 53 | * | 0 |
| G | 52 | 1 | 17 | * | G | 237 | 0 | 4 | * |

Comparison between Drug and Control

| \rightarrow | A | C | U | G |
|---------------|-------|-------|-------|-------|
| A | * | -0.01 | 0.00 | -0.29 |
| C | -0.03 | * | 0.18 | 0.00 |
| U | -0.01 | -0.18 | * | -0.01 |
| G | 0.37 | 0.00 | -0.03 | * |

Figure 5

Guanine Analogue



Adenine Analogue

