Improved plaque assay for human coronaviruses 229E and OC43

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In light of the COVID-19 pandemic, studies that work to understand SARS-CoV-2 are urgently needed. In turn, the less severe human coronaviruses such as HCoV-229E and OC43 are drawing newfound attention. These less severe coronaviruses can be used as a model to facilitate our understanding of the host immune response to coronavirus infection. SARS-CoV-2 must be handled under biosafety level 3 (BSL-3) conditions. Therefore, HCoV-229E and OC43, which can be handled at BSL-2 provide an alternative to SARS-CoV-2 for preclinical screening and designing of antivirals. However, to date, there is no published effective and efficient method to titrate HCoVs other than expensive indirect immunostaining. Here we present an improved approach using an agarose-based conventional plaque assay to titrate HCoV 229E and OC43 with mink lung epithelial cells, Mv1Lu. Our results indicate that titration of HCoV 229E and OC43 with Mv1Lu is consistent and reproducible. The titers produced are also comparable to those produced using human rhabdomyosarcoma (RD) cells. More importantly, Mv1Lu cells display a higher tolerance for cell-cell contact stress, decreased temperature sensitivity, and a faster growth rate. We believe that our improved low-cost plaque assay can serve as an easy tool for researchers conducting HCoV research.

1   **Improved Plaque Assay for Human Coronaviruses 229E and OC43**

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Abstract

23
24 In light of the COVID-19 pandemic, studies that work to understand SARS-CoV-2 are urgently
25 needed. In turn, the less severe human coronaviruses such as HCoV-229E and OC43 are drawing
26 newfound attention. These less severe coronaviruses can be used as a model to facilitate our
27 understanding of the host immune response to coronavirus infection. SARS-CoV-2 must be
28 handled under biosafety level 3 (BSL-3) conditions. Therefore, HCoV-229E and OC43, which
29 can be handled at BSL-2 provide an alternative to SARS-CoV-2 for preclinical screening and
30 designing of antivirals. However, to date, there is no published effective and efficient method to
31 titrate HCoVs other than expensive indirect immunostaining. Here we present an improved
32 approach using an agarose-based conventional plaque assay to titrate HCoV 229E and OC43
33 with mink lung epithelial cells, Mv1Lu. Our results indicate that titration of HCoV 229E and
34 OC43 with Mv1Lu is consistent and reproducible. The titers produced are also comparable to
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37 faster growth rate. We believe that our improved low-cost plaque assay can serve as an easy tool
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39

Introduction

40

41 The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory

42 syndrome coronavirus 2 (SARS-CoV-2), has dramatically altered the way of life worldwide and

43 halted non-essential scientific research. As a result, the priority of the world is to develop

44 antiviral agents and vaccines against SARS-CoV-2 (Wu et al. 2020). Despite the catastrophic

45 effects brought on by SARS-CoV-2, other human coronaviruses (HCoV), such as the strains

46 229E and OC43, have been circulating for years and are one of the causative agents for the

47 common cold (Greenberg 2016). Due to the low lethality of non-severe coronavirus strains, they

48 tend to be neglected, and resources to investigate their pathogenesis and epidemiology are

49 relatively limited (Zeng et al. 2018; Zumla et al. 2016). However, the outbreaks of SARS in

50 2003, Middle East respiratory syndrome (MERS) in 2012, and COVID-19 in 2019-current, have

51 drawn an unprecedented amount of attention to coronavirus research, yet there is no effective

52 method to perform a low-cost plaque assay for HCoV 229E and OC43. Being able to titrate these

53 strains accurately would facilitate a plethora of coronavirus studies.

54

55 Due to the lack of effective antivirals and vaccines towards the highly contagious coronavirus

56 strains, such as SARS-CoV, MERS-CoV, and SARS-CoV-2, it is required to work with these

57 agents in a negative pressure equipped biosafety level 3 (BSL-3) laboratory or above. In addition

58 to work in these facilities, personnel have to be well-trained and wear proper personal protection

59 equipment (PPE), including Tyvek suits and respiratory protection such as powered air-purifying

60 respirators. However, a great number of institutes lack the budget or even the capability to

61 perform work in such a facility. In addition, PPE supplies have been in high demand during the

62 COVID-19 pandemic, limiting researchers' access to PPE. As a result, studies focusing on these

63 non-severe coronavirus strains have become a necessary alternative to prescreen potential

64 antiviral compounds in a BSL-2 setting. Utilizing these strains allows for a readily available low-

65 cost alternative that provides insight into the viral properties of the human coronaviruses.

66

67 While RT-qPCR has been widely adapted to determine the viral loads of CoVs due to its

68 sensitivity, particularly for SARS-CoV-2; it is also very costly for the required reagents for

69 researchers who lack the budget. Additionally, RT-qPCR is incapable of reflecting virucidal

70 activity of a given drug if it targets infectious virion production or egress instead of viral genome

71 replication. As a result, a conventional plaque assay can be a viable low-cost and complement

72 approach for RT-qPCR to test a potential antiviral compound and quantify infectious viral titers

73 as an indication of viral loads in terms of virological research.

74

75   Unlike non-severe human CoVs, SARS- and MERS-CoVs can easily form plaques using Vero

76   cells (Coleman & Frieman 2015; Harcourt et al. 2020; Vicenzi et al. 2004). Vero cells are a non-

77   cancerous cell line with a type I interferon production defect (Emeny & Morgan 1979) and are

78   the most commonly used cell line for the traditional plaque assays (Baer & Kehn-Hall 2014).

79   However, the traditional plaque assay appears to be inadequate to titrate HCoV 229E and OC43.

80   Without an accurate titration, it would be difficult to estimate drug efficacy and perform vaccine

81   challenge studies. However, there are alternative approaches to titrate HCoV 229E and OC43.

82   For example, an indirect plaque assay using an anti-CoV primary antibody and peroxidase to

83   visualize the virus-infected cell colonies has been developed (Lambert et al. 2008). Direct

84   observation of cytopathogenic effects (CPE) by the naked eye utilizing a compound light

85   microscope can even be performed to determine the TCID50 (Median Tissue Culture Infectious

86   Dose). However, the CPE-based titration is not sensitive enough to discern viral inhibition

87   resulting from potential antiviral compounds, and the indirect immunoperoxidase assay

88   consumes large amounts of antibodies, which makes this method expensive despite its high

89   sensitivity.

90

91   Approaches to improve the reliability of the traditional plaque assay include utilizing more

92   susceptible cell lines and replacing the overlay medium with cellulose materials such as low-

93   viscosity Avicel or methylcellulose (Funk et al. 2012; Matrosovich et al. 2006). For example,

94   Herzog et al. reported that while the LLC-MK2 cells, a rhesus monkey kidney cell line, is

95   suitable for propagation of HCoV-NL63, one of the non-severe strains isolated in 2007(Pyrc et

96   al. 2007), it is not suitable to titrate NL63 using conventional plaque assay due to no or little CPE

97   observed. A similar result was also observed with Vero cells, following NL63 infection. To

98   combat this, their study used Caco-2 cells as a monolayer and applied Avicel RC-581 as the

99   overlay medium to successfully titrate NL63 by traditional plaque assay (Herzog et al. 2008).

100   Despite their success, very limited information regarding the conventional plaque assays for

101   HCoV-229E and OC43 can be found. As a result, this study revisited the traditional plaque assay

102   method for HCoV 229E and OC43 and validated the proposed protocols. Human lung epithelial

103   cells, MRC-5 and ileocecal adenocarcinoma cells, HTC-8, were used to propagate 229E and

104   OC43, respectively (Gorse et al. 2009; Laude & Vautherot 1993; Warnes et al. 2015). Various

105   cell lines were then utilized to optimize an agarose-based conventional plaque assay. The goal of

106   this study was to create a plaque assay protocol that could stably and reproducibly titrate the non-

107   severe coronavirus strains HCoV 229E and OC43.

108

109   **Materials and Methods**

110   *Cells, Viruses, and Medium*

111   The cell lines used in this study were purchased from ATCC: MRC-5 (human lung epithelial
112   cell; ATCC: CCL-171), HCT-8 (human ileocecal adenocarcinoma cell; ATCC: CCL-244)
113   Mv1Lu (mink lung epithelial cell; ATCC: CCL-64), RD (human rhabdomyosarcoma cell;
114   ATCC: CCL-136), LLC-MK2 (rhesus monkey kidney cell; ATCC: CCL-7), Vero (African green
115   monkey kidney cell; ATCC: CCL-81). HCoV- 229E (Catalog No. FR-303) and OC43 (Catalog
116   No. FR-302) were obtained from International Reagent Resources (IRR) followed by
117   propagation in MRC-5 and HCT-8 cells for 3-5 days post infection, respectively.
118
119   Medium for MRC-5, Mv1Lu, RD, LLC-MK2, and Vero cells was DMEM (Quality Biological
120   Cat# 112-300) supplemented with 10% FBS, $1\times$ MEM nonessential amino acids (NEAA;
121   Corning, Cat# 25-025), 1 mM of sodium pyruvate (Corning, Cat# 25-000), 2 mM of L-glutamine
122   (Corning, Cat# 25-005), and $1\times$ penicillin and streptomycin (Corning, Cat#30-002) except
123   HCT-8 which was cultured with RPMI 1640 (Quality Biological, Cat# 112-025) containing 10%
124   FBS.
125
126   *Plaque assay*
127   1×10^6 cells per well were seeded in a 6-well plate a day before performing the plaque assay. The
128   culture media was removed and washed with PBS. Viral samples were diluted 10-fold in DMEM
129   without FBS and the appropriate dilution was added to the corresponding well and incubated for
130   1.5 h at 37° C and 5% CO_2. Overlay medium was prepared from a mixture of 1% of agarose
131   (Invitrogen, Cat#16500), unless indicated elsewhere, and $2\times$ EMEM (Quality Biological, Cat#
132   115-073) supplemented with 10 or 16% of FBS, $1\times$ NEAA, 1 mM of sodium pyruvate, 2 mM of
133   L-glutamine, and $2\times$ penicillin and streptomycin. Two mL of the overlay medium were added to
134   each well. The plates were incubated at 37° C or 33° C and 5% CO_2 for 5 days. At the end of
135   incubation, cells were fixed with 10% formalin for at least one hour and stained with 0.2%
136   crystal violet dissolved in 10% of absolute ethanol and 90% of PBS overnight. A diagrammatic
137   scheme is depicted in Figure 1.
138
139   **Results**
140   *1. Plaque assays of HCoV 229E and OC43 with MRC-5 cells.*
141   MRC-5 cells have successfully been used to titrate HCoV 229E and OC43 in previous studies
142   (Funk et al. 2012; Kim et al. 2019; Warnes et al. 2015). This study attempted to reproducibly test
143   whether MRC-5 cells may be used to titrate HCoV 229E and OC43 using the proposed agarose
144   plaque assay method. The ability of each strain to form plaques was analyzed. Additionally, it
145   was examined if the presence or absence of the inoculum in the wells had any effect on plaque
146   formation. It was found that when the inoculum remained in the wells throughout the 5-day

147 incubation period, the monolayer of the MRC-5 cells appeared to be intact and conducive to

148 plaque formation. Conversely, without the inoculum, MRC-5 cells hardly remained intact and

149 seemed unable to support the formation of viral plaques (Fig. 2). Various compositions of

150 overlay medium were tested by adjusting the concentrations of agarose and FBS. An optimal

151 concentration of 0.75% agarose and 8% FBS stood out among the various overlay mediums

152 tested since it formed the most discernable plaques on MRC-5 cells (Suppl. Fig. 1).

153

154 *2. Optimize agarose-based plaque assays for HCoV 229E and OC43*

155 Although countable plaques were formed in MRC-5 cells, we realized that MRC-5 cells are not

156 an ideal cellular material for plaquing HCoV 229E and OC43. MRC-5 cells are slow-growing

157 and are very sensitive to their surrounding growth conditions, which are not ideal for the

158 formation of a monolayer with a 5-day incubation. Therefore, other cell types were compared to

159 the MRC-5 cells for their susceptibility to HCoV 229E and OC43 by the observance of

160 cytopathic effects (CPE). Among the cell types tested, including LLC-MK2, which has been

161 previously described as permissive for SARS-CoV (Kaye 2006) and NL-63 (Schildgen et al.

162 2006), the mink epithelial cells, Mv1Lu, initially used in the detection of various respiratory

163 viruses (Huang & Turchek 2000), were selected due to their significant CPE at 4 days post

164 HCoV 229E and OC43 infection (Fig. 3). As a result, Mv1Lu cells were used to optimize the

165 plaque assay protocol.

166

167 For coronaviruses as well as many other respiratory viruses, 33° C appears to be the optimal

168 temperature for propagation and titration (Creager et al. 2018; Lamarre & Talbot 1989; Muller et

169 al. 2017). To confirm this, plaque assays were performed with Mv1Lu cells at both 33° C and

170 37° C, and the plaques formed were compared. Following the five day incubation period, it was

171 found that the plaque sizes of HCoV 229E and OC43 were smaller at 33° C than those at

172 37° C. Furthermore, the titer of OC43 was significantly reduced at 33° C while there was no

173 notable titer change for 229E at either temperature (Fig. 4A and 4B). These results indicate that

174 37° C appears to be an appropriate temperature for the modified plaque assay with Mv1Lu cells.

175

176 Next, various agarose concentrations were tested to determine which concentration formed the

177 most discernable plaques (Shurtleff et al. 2012). The results showed no obvious difference in

178 shape, size, or number of plaques generated with either HCoV 229E or OC43 at any of the final

179 agarose concentrations of 1%, 0.5%, or 0.3% (Fig. 4C). The final concentration of 0.5% agarose

180 was used in the next sequence of experiments due to its ease in handling and the clarity of

181 plaques formed. Therefore, the optimal conditions for the plaque assay protocol for 229E and

182 OC43 utilize Mv1Lu cells, incubated at 37° C with a final agarose concentration of 0.5%.

183

184 *3. Validation of Agarose-based Plaque Assays with Longitudinal Viral Passages*

185 To validate the proposed plaque assay protocol, two consecutive passages of HCoV 229E and

186 OC43 from MRC-5 and HCT-8 were cultured and collected, respectively. The corresponding

187 titers (Fig. 5A) and the plaque morphologies (Fig. 5B and 5C) were noted. The plaque assay

188 conditions used, including temperature and overlay medium composition were adequately able to

189 discern the differences in viral titers of HCoV 229E and OC43 between two passages. Both

190 strains were at least 10-fold greater after passaging. The data demonstrates that the current

191 modified plaque assay protocol with Mv1Lu cell can reproducibly differentiate a change in titer

192 of HCoVs.

193

194 *4. Comparison of Mv1Lu and RD cells for HCoV 229E and OC43 plaque assays*

195 Human rhabdomyosarcoma (RD) cells have also been reported as a conducive cell line for

196 HCoV- 229E and OC43 plaque formation (Schmidt et al. 1979), but over the years MRC-5 cells

197 have also become common cell lines for HCoVs plaque assays. To further characterize these cell

198 types' ability to form plaques, the optimized protocol utilizing both cell lines was performed

199 concurrently. Following the 5-day incubation, both cell lines exhibited a decent capability in

200 generating countable and discernable plaques (Fig. 6). Overall, both cell lines are adequate as

201 cellular material to titrate HCoV 229E and OC43 with slightly different properties in forming

202 clear plaques. That is, the monolayer of Mv1Lu cells was easily stained by the crystal violet

203 solution which yielded clearer and less hazy plaques than those on RD cells. Nevertheless, both

204 Mv1Lu and RD cells exhibited adequate and comparable capability in titrating 229E and OC43.

205

206 *5. Comparison of HCoV 229E and OC43 propagated from different cell lines*

207 Lastly, given that a virus can be propagated in cell lines that may not be permissive for plaque

208 formation, three cell lines, MRC-5 (human lung epithelial cell), Mv1Lu (mink lung epithelial

209 cell), and LLC-MK2 (rhesus monkey kidney epithelial cell) cells, were screened for their ability

210 to propagate HCoV 229E and OC43. Viral titers yielded from the 3 distinct cell lines were

211 determined utilizing the proposed modified plaque assay method. Results showed that the viral

212 titers of HCoV 229E and OC43 generated from MRC-5 were comparable to those from Mv1Lu

213 cells. Whereas the titer obtained for OC43 propagated in LLC-MK2 cells was down by nearly 2

214 logs and no virus could be detected for 229E propagated in LLC-MK2 cells (Fig. 7). This

215 experiment was then repeated using LLC-MK2 as the monolayer for the modified plaque assay

216 at 37° C and 33° C. Our results indicate that LLC-MK2 cells showed a limited capability to

217 support plaque formation of HCoV OC43 and did not support plaque formation of HCoV 229E

218 at either temperature (Suppl. Fig. 2). The plaque assays results correspond with the inability of

219 HCoV 229E to replicate in LLC-MK2.

220

221 **Discussion**

222 In this study, we demonstrated that HCoV 229E and OC43 can be reproducibly titrated by a

223 modified conventional plaque assay protocol using Mv1Lu cells. It was also shown that the use

224 of Mv1Lu cells produces plaques that are comparable and even in cases superior to those of RD

225 cells. Mv1Lu cells are derived from American mink lung epithelial cells and they have been

226 adapted to support propagation of various viruses, specifically influenza virus and respiratory

227 syncytial virus (Schultz-Cherry et al. 1998; Tsai et al. 2019; Yeolekar et al. 2002). From our

228 observations, Mv1Lu cells seem to grow faster (subculture frequency on average of 3 days) and

229 are more tolerant of cell-cell contact stress. It was also observed that Mv1Lu cells in a culture

230 flask remain morphological intact for at least 7-9 days without the addition of new culture

231 medium or subculturing. Whereas MRC-5 cells grow relatively slow (subculture frequency on

232 average of 5 days; Suppl. Fig. 3) and require more meticulous culture conditions to prevent

233 monolayer breakdown during incubation. RD cells are another adequate cell line to support

234 plaque formation of HCoV 229E and OC43. However, the morphology of the plaques formed in

235 RD cells is not as defined as those formed in Mv1Lu cells for OC43 (Fig. 6B). Vero cells were

236 also tested for their ability to form plaques upon infection with HCoV at 33°C with a similar

237 but distinct protocol (see supplemental protocol). Unfortunately, Vero cells were not supportive

238 of plaque formation for either strain (data not shown). An altered protocol might enable plaque

239 formation in Vero cells since extensive optimization of this protocol was not performed.

240

241 Agarose is a widely used viscous material found in many overlay mediums. It is efficient at

242 restraining diluted viral particles which aid in the formation of distinct countable plaques. Avicel

243 and methylcellulose are other widely used materials for overlay medium. Avicel is a nonfibrous,

244 microcrystalline cellulose derivative commonly used by the food industry, one example of its use

245 is in ice cream production (Kennedy & Law 1999). The use of Avicel, as a low viscosity overlay

246 medium, provides optimal conditions for indirect immunoperoxidase assays which is ideal for

247 those viruses that don't readily form plaques like influenza (Lin et al. 2017; Matrosovich et al.

248 2006). Previously, Avicel has been reported to be an outstanding overlay medium for titrating

249 another HCoV strain, NL63, compared to agarose and methylcellulose (Herzog et al. 2008).

250 However, Avicel powder is not easily acquired and the preparation of a homogenous solution is

251 more complicated and time-consuming than agarose. In this study plaques of NL63 could not be

252 visualized with Mv1Lu or RD cells using an agarose-based protocol (data not shown).

253

254 It was observed that MRC-5 is not the ideal cell type for conducting our modified plaque assay

255 for HCoVs due to the following reasons: 1) inconsistent plaque formation of HCoVs; 2) The cell
256 line possesses a narrow window of observation to obtain clear and readable plaques; and 3)
257 MRC-5 cells have a higher demand for culture conditions. In a 10-fold dilution of HCoV 229E
258 and OC43 samples, the countable plaques obtained in a 6-well plate are present in only one
259 single dilution. This means a 12-well plate format is not recommended for plaque assays
260 performed with MRC-5 cells because this format yields an insufficient resolution for counting
261 plaques. Discernable plaques were not formed with the HCoV 229E in MRC-5 cells until 4 to 5
262 days post incubation, which differs from the previously described 48 h protocol (Funk et al.
263 2012). In our experience, a 4-day incubation with Mv1Lu or RD cells is also a viable option to
264 yield countable plaques. With MRC-5 cells, even an incubation as short as 4 days requires a
265 higher concentration of nutrients (8% of FBS) compared to the other cell lines used in this study
266 (2.5% to 5% of FBS). The aforementioned factors would substantially increase the variations and
267 costs due to the increased labor, supplies, and reagents needed to perform large-scale anti-HCoV
268 drug testing. However, if it is necessary to perform plaque assays on MRC-5 cells, using an
269 alternative overlay such as 0.8% of methylcellulose may allow for a higher resolution.
270
271 It is important to note that cell lines that support plaque formation are not necessarily the ideal
272 candidate for viral stock propagation. For example, it has been reported that HCoV OC43 can be
273 propagated in HCT-8 and Caco-2 (both are human epithelial colorectal adenocarcinoma cell
274 lines) cells (2020), but neither of those cell types serves as a suitable monolayer for modified
275 agarose-based plaque assay proposed in this study. Likewise, LLC-MK2 cells can generate more
276 HCoV NL63 virus but are incompetent for plaque production of HCoV 229E (Fig. Suppl. Fig. 3)
277 despite both being alpha coronaviruses (Liu et al. 2020). Nevertheless, Mv1Lu cells and the
278 current improved agarose-based conventional plaque assay protocol are adequate for performing
279 a low-cost consistent titration of HCoV OC43 and 229E. The development of this protocol is a
280 significant step forward in the ability to titer HCoV strains and is a necessary step along the path
281 of identifying effective antiviral agents against HCoV.
282
283 **Supplemental protocol**
284 *Plaque assay (Vero only)*
285 5×10^6 Vero E6 cells were seeded in a 6-well plate for 24 h incubation at 33°C. Prior to
286 inoculation, the medium was removed and washed twice with PBS. Diluted viral samples were
287 added to the cells for 1 h with rocking every 10 mins. Upon removal of the inoculum cells were
288 washed with PBS twice and 3 mLs of overlay media, composed with MEM, 2% FBS, and 0.3%
289 agarose, was added to each well. Plates were incubated at 33°C for 3 days followed by fixation
290 with 4% formaldehyde containing methanol at RT for 2 hours. The plates were subsequently

291   stained with a crystal violet solution.

292

293   **Acknowledgments**

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295   OC43 (ATCC® VR-1558™), FR-302 were obtained through the International Reagent

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297   Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA.

298

299   **References**

300

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391

392 **Figure legends**

393 **Fig. 1.** Diagrammatic scheme for illustration of improved plaque assay procedures and optimal

394 conditions.

395

396 **Fig. 2.** Plaque assays with MRC-5 cells. Plaque assay for (A) 229E and (B) OC43 on MRC-5

397 monolayer with or without inoculum left in wells. The inoculum was incubated for 1.5hr at 37C.

398 Overlay medium was composed of a final conc. of agarose ~0.75% and ~8% of FBS.

399

400 **Fig. 3.** Cell morphology of susceptible cell lines. Cell morphology of susceptible cell lines,
401 MRC-5, Mv1Lu, and LLC-MK2 with or without infections of (A) 229E and (B) OC43 at 4 dpi.
402 Magnification 200x; Insert 100x.

403

404 **Fig. 4.** A tune-up for plaque assay conditions of hCoVs. Plaques observed at 5 dpi of (A) 229E
405 and (B) OC43 on Mv1Lu monolayer at 33°C and 37°C with overlay media of 0.5% agarose and
406 5% FBS. (C) A representative sample of plaques formed by 229E and OC43 using various
407 overlay mediums where indicated final agarose concentrations were mixed with 2X EMEM
408 media supplemented with 10% FBS and other ingredients described in Material and Methods.

409

410 **Fig. 5.** Functional evaluation with improved plaque assay. Titration of consecutive passages of
411 (A&B) 229E and (A&C) OC43 by plaque assay at 37°C with overlay media of 0.5% agarose and
412 5% FBS.

413

414 **Fig. 6.** Comparison of susceptible cell lines with the improved plaque assay. Plaques formed at 5
415 dpi by (A) 229E and (B) OC43 using a Mv1Lu and RD cell monolayer using an EMEM-based
416 overlay media of 0.5% of agarose and 5% of FBS.

417

418 **Fig. 7.** Titration of hCOV propagation from different cells. Differential titers of (A) 229E and
419 (B) OC43 propagated from three distinct cell lines. Virus-containing supernatants were collected
420 on 4 dpi and titrated by using the 5 day plaque assays protocol. Values are presented as mean $\pm$
421 SEM from at least two independent experiments.

422

423 **Suppl. Fig. 1.** Various conditions for plaque assays with MRC-5 cells. Plaques formed at 5 dpi
424 for 229E on MRC-5 monolayer with inoculum left and the EMEM-based overlay mediums with
425 following indicated final concentrations: (A) 0.3% of agarose and 5% of FBS; (B) 0.5% of
426 agarose and 8% of FBS; (C) 0.75% of agarose and 8% of FBS; (D) 0.75% of agarose and 5% of
427 FBS.

428

429 **Suppl. Fig. 2.** Plaque assays for 229E and OC43 with LLC-MK2 cellular monolayer. The plaque
430 assays were incubated at (A) 37C and (B) 33C for five days with a final concentration of 0.3% of
431 agarose and 2.5% of FBS.

432

433 **Suppl. Fig. 3.** Comparison of growth condition of MvlLu and RD cells. (A) Growth curve and
434 (B) cell morphology at a low and high density of Mv1Lu and RD cells. Both cells were seeded

435   with $2x10^4$ cells/well and count cell numbers every two days. The data were presented as mean

436     $\pm$ SEM and the cell number of y-axis was plotted in log2 scale. Insert images: 200x;

437   background image: 100x.

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Figure 1

Diagrammatic scheme for illustration of improved plaque assay procedures and optimal conditions.
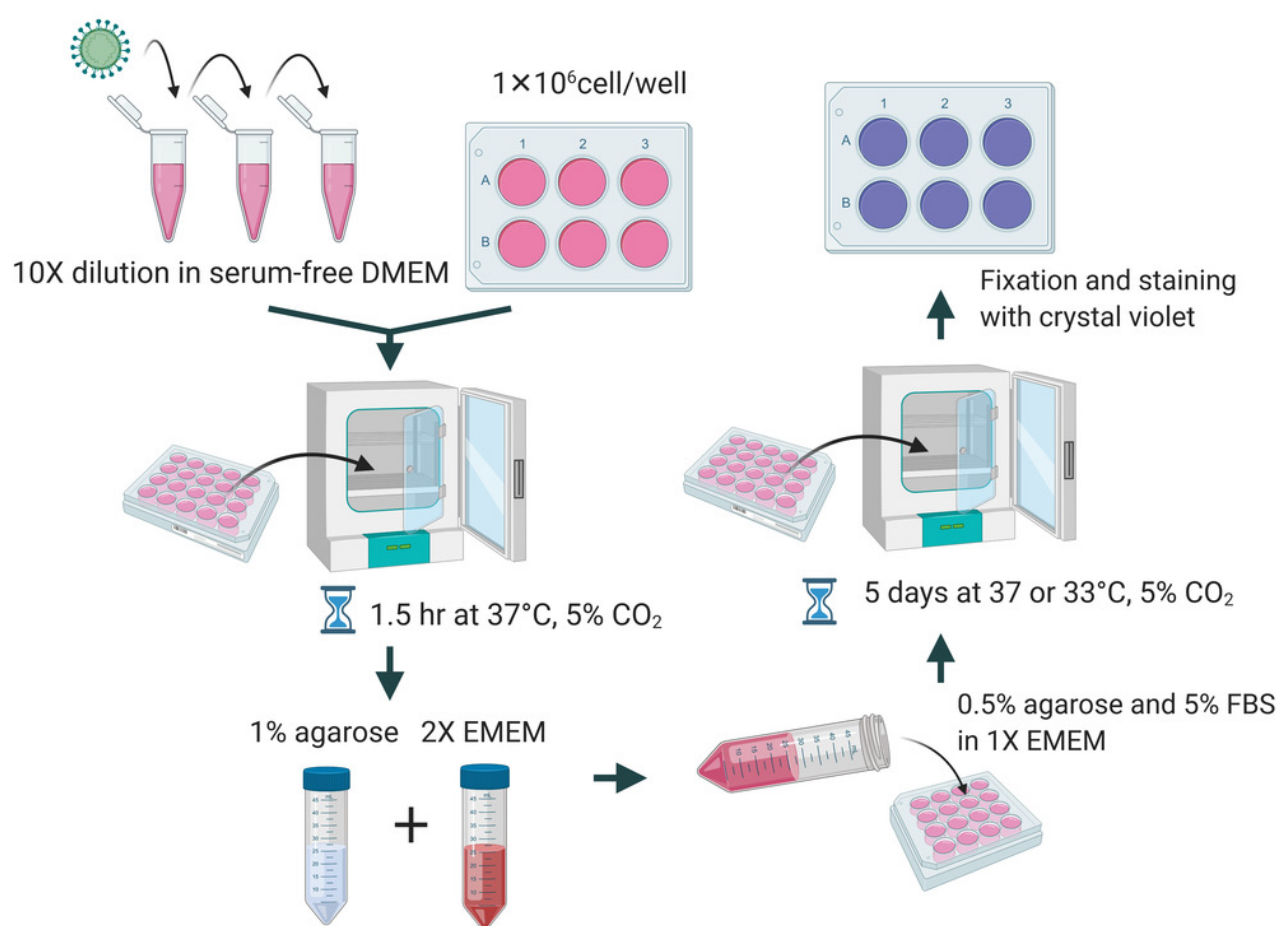
Figure 2

Plaque assays with MRC-5 cells.

Plaque assay for (A) 229E and (B) OC43 on MRC-5 monolayer with or without inoculum left in wells. The inoculum was incubated for 1.5hr at 37C. Overlay medium was composed of a final conc. of agarose ~0.75% and ~8% of FBS.
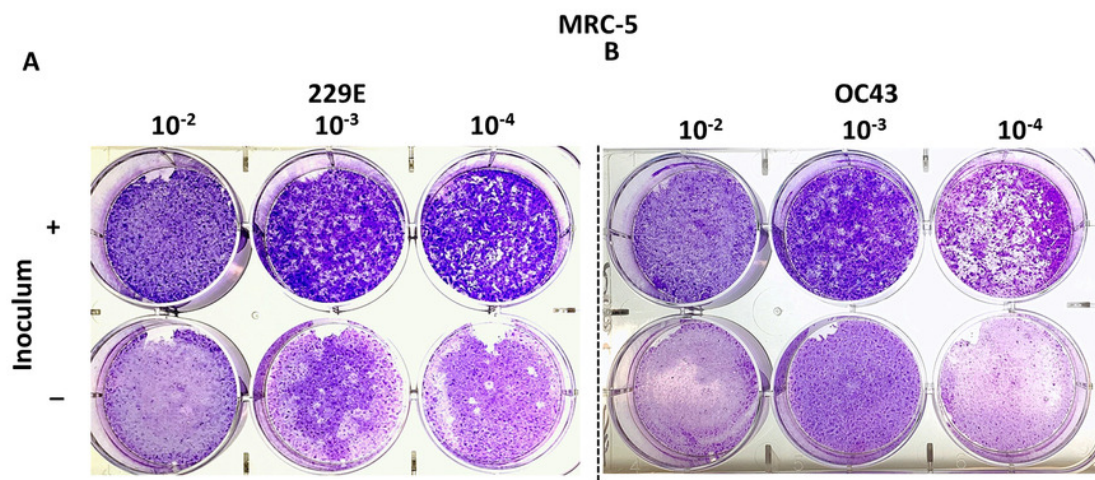
Figure 3

Cell morphology of susceptible cell lines.

Cell morphology of susceptible cell lines, MRC-5, Mv1Lu, and LLC-MK2 with or without infections of (A) 229E and (B) OC43 at 4 dpi. Magnification 200x; Insert 100x.
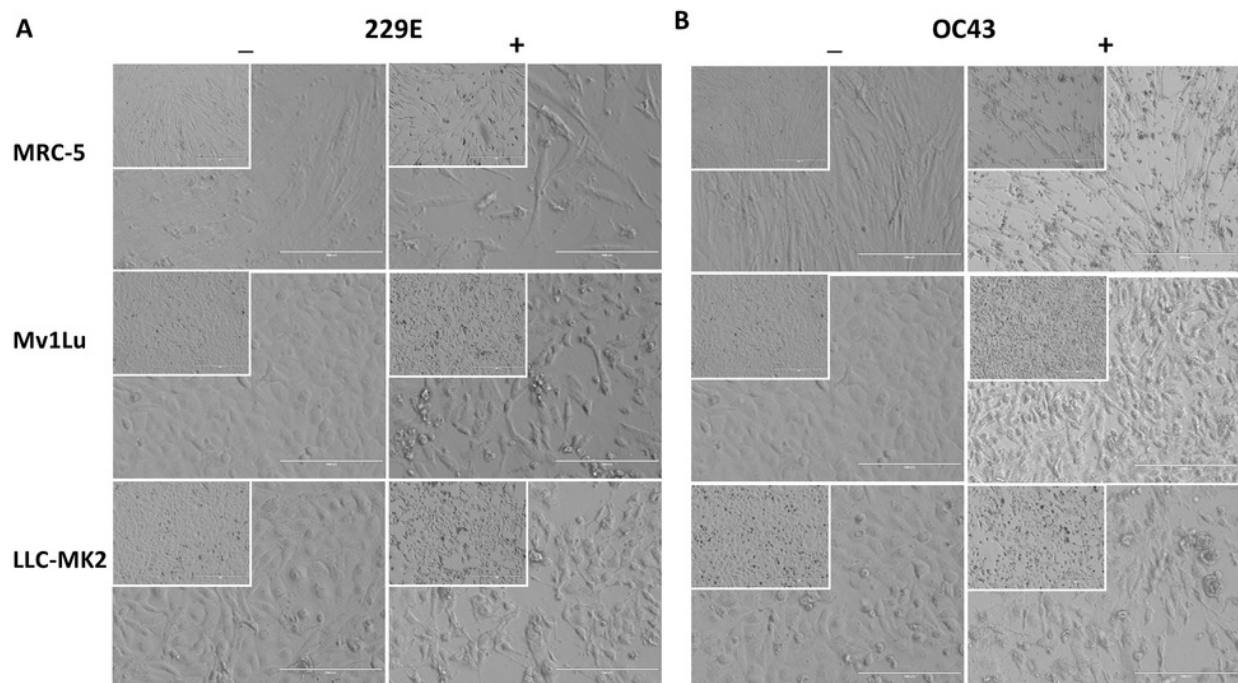
Figure 4

A tune-up for plaque assay conditions of hCoVs.

Plaques observed at 5 dpi of (A) 229E and (B) OC43 on Mv1Lu monolayer at 33°C and 37°C with overlay media of 0.5% agarose and 5% FBS. (C) A representative sample of plaques formed by 229E and OC43 using various overlay mediums where indicated final agarose concentrations were mixed with 2X EMEM media supplemented with 10% FBS and other ingredients described in Material and Methods.
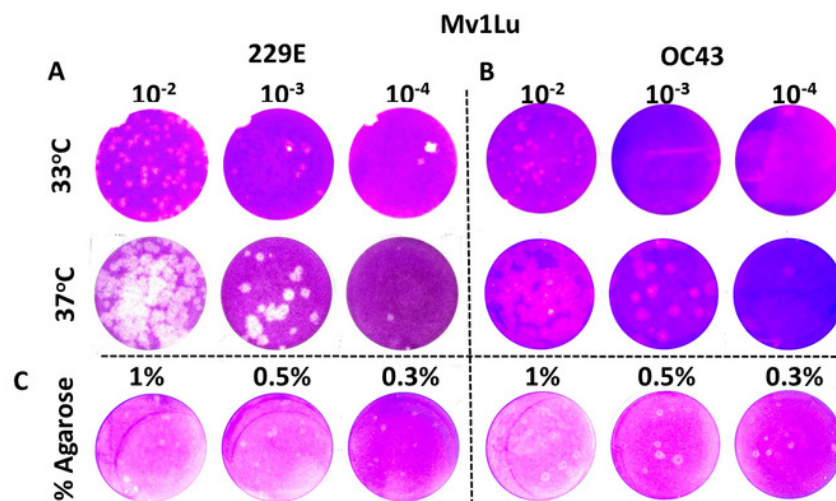
Figure 5

Functional evaluation with improved plaque assay.

Titration of consecutive passages of (A&B) 229E and (A&C) OC43 by plaque assay at 37°C with overlay media of 0.5% agarose and 5% FBS.
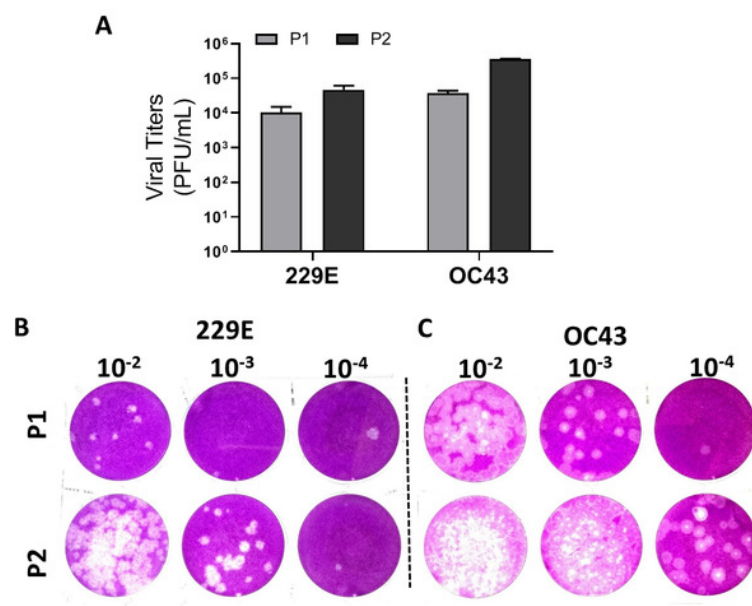
Figure 6

Comparison of susceptible cell lines with the improved plaque assay.

Plaques formed at 5 dpi by (A) 229E and (B) OC43 using a Mv1Lu and RD cell monolayer using an EMEM-based overlay media of 0.5% of agarose and 5% of FBS.
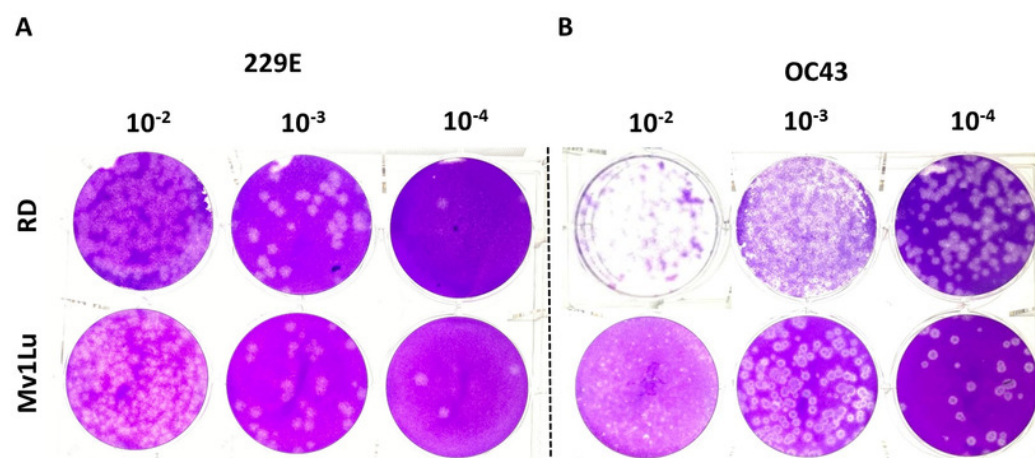
Figure 7

Titration of hCOV propagation from different cells.

Differential titers of (A) 229E and (B) OC43 propagated from three distinct cell lines. Virus-containing supernatants were collected on 4 dpi and titrated by using the 5 day plaque assays protocol. Values are presented as mean ± SEM from at least two independent experiments.