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1 **Ready for Production**

2

3 **H1 Genetic manipulation of giant viruses and their host *Acanthamoeba castellanii***

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10

11 **H1 Editorial Summary:** This protocol enables genetic manipulation of nuclear and cytoplasmic giant
12 viruses, and their host *Acanthamoeba castellanii*, using either CRISPR/Cas9 or homologous
13 recombination.

14

15 **H1 Key references**

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17 4237-4250 e4219 (2021). <https://doi.org:10.1016/j.cell.2021.06.032>

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19 <https://doi.org:10.1038/s41467-023-36145-4>

20 Alempic, J-M. et al. No fitness impact of the knockout of the two main components of mimivirus genomic
21 fiber and fibril layer. *bioRxiv*, 2023.04.28.538727 (2023). <https://doi.org/10.1101/2023.04.28.538727>

22

23 **H1 Key points**

- 24
- 25 • This protocol enables genetic manipulation of nuclear and cytoplasmic giant viruses, and their
26 host *Acanthamoeba castellanii*, using either CRISPR/Cas9 or homologous recombination.
 - 27 • The methodology allows the generation of stable gene modifications, which wasn't possible
using RNA silencing, the only previously available reverse genetic tool.

28 **H1 ABSTRACT**

29 Giant viruses (GVs) provide an unprecedented source of genetic innovation in the viral world and are thus,
30 besides their importance in basic and environmental virology, in the spotlight for bioengineering advances.
31 Their host, *Acanthamoeba castellanii* is an accidental human pathogen that acts as a natural host and
32 environmental reservoir of other human pathogens. Tools for genetic manipulation of viruses and host
33 were lacking. Here, we provide a detailed method for genetic manipulation of *A. castellanii* and the GV it
34 plays host to by utilizing either CRISPR/Cas9 and/or homologous recombination. We detail the steps of

35 vector preparation (4 days), transfection of amoeba cells (1 h), infection (1 h), selection (5 days for viruses,
36 2 weeks for amoebas), and cloning of recombinant viruses (4 days) or amoebas (2 weeks). This procedure
37 takes about 3 weeks or a month for the generation of recombinant viruses or amoebas, respectively. This
38 methodology allows the generation of stable gene modifications, which wasn't possible using RNA
39 silencing, the only previously available reverse genetic tool. We also include detailed sample-preparation
40 steps for protein localization by immunofluorescence (4 hs), western blotting (4 hs), quantification of viral
41 particles by optical density (15 min), calculation of viral lethal dose 50 (7 days) and quantification of DNA
42 replication by quantitative PCR (4 hs) to allow efficient broad phenotyping of recombinant organisms. This
43 methodology allows the function of thousands of ORFan genes present in GVs, as well as the complex
44 pathogen-host, pathogen-pathogen, or pathogen-symbiont interactions in *A. castellanii* to be studied *in*
45 *vivo*.

46

47 **H1 INTRODUCTION**

48 *Acanthamoeba spp.* is an opportunistic human pathogen with increasing importance for ocular health¹.
49 Moreover, this organism has been largely recognized as a host and environmental reservoir for pathogenic
50 organisms like Legionella, Mycobacterium, or Cryptococcus². More recently, *Acanthamoeba castellanii* has
51 also been utilized as an experimental host for the discovery of several giant viruses (GVs), which contain
52 genomes of up to 2.8 megabases and viral particles exceeding 1 μm in size³⁻⁵. Different examples of GVs
53 include Pandoravirus salinus (linear 2.8 Mb genome, 1 μ particle)⁴, Mollivirus sibericum (linear 0.65 Mb
54 genome, 500nm particle)⁶, Pithovirus sibericum (circular 0.6 Mb genome, 1.5 μM particles)⁵ and Mimivirus
55 (linear 1.2 Mb genome, 400nm particle)³. Importantly, GVs not only play key roles in ecology but also 70%
56 of the thousands of genes encoded by these organisms are ORFan genes (unrelated to any other protein
57 known so far)⁷. Thus, these viruses represent an unexplored resource of innovations for bioengineering
58 advances⁸. Yet, genetic manipulation of both GVs and their host *A. castellanii*, has been largely neglected
59 due to the difficulty in implementing it on these organisms. Specifically, *A. castellanii* is a highly polyploid
60 organism (25n approx.)⁹ with amitotic nuclear division leading to aneuploidy and random segregation of
61 genetic material^{10,11}. Moreover, selection markers for drug resistance are scarce (currently limited to two,
62 geneticin¹² and nourseothricin¹³). Cytoplasmic GVs protect their DNA by establishing viral factories¹⁴ which
63 segregate their DNA from protein effectors like Cas9 (ref. 15). Thus, previous gene functional assessment
64 was mostly accomplished by RNA silencing^{16,17}. Here we describe a protocol for rapid genetic engineering
65 of both fully cytoplasmic and nuclear GVs as well as their host *A. castellanii*. CRISPR/Cas9 can be utilized
66 for the modification of nuclear GVs and *A. castellanii*, while both cytoplasmic and nuclear GVs can also be
67 genetically engineered by recombination in combination with selection markers. Investigators should be
68 able to obtain recombinant viruses in 3 weeks and recombinant amoebas in a month.

69

70 **H2 Overview of the genetic manipulation techniques.**

71 A schematic overview of our protocols is provided in [Figure 1](#). Our methods utilise both homologous
72 recombination (HR) and non-homologous-end joining (NHEJ) for modifications of the genome of amoeba
73 and viral DNA^{13,15,18}. CRISPR-Cas (Clustered Regularly Interspersed Short Palindromic Repeats) has proved
74 invaluable for the genetic manipulation of many organisms, based on the tunable selectivity of a dsDNA
75 endonuclease (like Cas9) for a 20 base pair sequence-specific region of the genome¹⁹. Such selectivity is

76 accomplished by annealing of the DNA with a guide RNA (gRNA) which binds to the endonuclease and
77 directs it to its targeting site¹⁹. Double strand breaks generated by Cas9 can consequently be repaired by
78 two major mechanisms: HR and NHEJ. Particularly relevant for this protocol, we have demonstrated that
79 CRISPR/Cas9 can be efficiently used for manipulation of the genome of *A. castellanii* and nuclear GV
80 mollivirus and pandoravirus¹⁵. We have utilized this technology to generate knockouts in *A. castellanii*
81 and nuclear GVs by inducing NHEJ repair¹⁵. In addition, HR can also be exploited in the absence of double-
82 strand breaks to prompt modifications on the genomic DNA. Thus, when combined with selection markers
83 for the enrichment of recombinant organisms, it can also be utilized for genetic engineering independently
84 of CRISPR-based modifications. We have demonstrated that HR can be efficiently used for the modification
85 of cytoplasmic GVs like marseillevirus and mimivirus¹⁸, but also for nuclear GVs like mollivirus and
86 pandoravirus¹⁵. HR allows not only the generation of gene knockouts but also the endogenous tagging of
87 genes (for protein localization) or the introduction of exogenous DNA (*i.e.* for cis-complementation)¹⁵.

88

89 **H2 Comparison with previous reverse genetic techniques.**

90 Reverse genetics of GVs and *A. castellanii* were limited to RNA silencing before we developed genetic tools
91 for these organisms. While silencing has proven efficient for the functional analysis of *A. castellanii* genes,
92 particularly for genes involved in encystment^{16,20}, gene knockdowns are usually only partial and transient²¹.
93 Thus, silencing is mostly limited to a certain window of time and the study of vulnerable genes²² (*i.e.* genes
94 where low expression inhibition will result in a decrease in organismal fitness). Moreover, highly stable
95 proteins might also be difficult to downregulate, hampering their study by RNA silencing (*i.e.* highly stable
96 proteins might take days for efficient protein downregulation²³). On the other hand, gene knockout of
97 essential genes is lethal and thus provides only partial information about the functionality of a gene. In
98 such cases, RNA silencing might result in a better way to address the functionality of a gene. Since
99 conditional knockout in *A. castellanii* has not been developed so far, RNA silencing is the only available
100 technique for the study of essential genes.

101 Gene function has also been previously studied by RNA silencing in mimivirus (a cytoplasmic GV)^{17,24,25}.
102 While the same pros and cons that were discussed for amoeba reverse genetics apply to GVs, only 3
103 publications have been shown to be successful in applying this technology for the silencing of GVs genes
104 (all in mimivirus)^{17,24,25}. Reports associated to the use of RNA silencing of mimiviral genes were generated
105 by a single research team and no other group has reported successful reverse genetics using this tool.
106 Moreover, the study of the essential genes of GVs can be studied by generating gene knock-out viruses in
107 trans-complementing amoeba cells¹³, eliminating the usual advantage of gene knockdown over gene
108 knockout to study essential genes. In addition, a previous study has described the replacement of the
109 mimiviral gene R349 by green fluorescent protein using homologous recombination²⁶. Unfortunately, even
110 when infected cells were sorted by the presence of GFP, the strategy did not allow the isolation of stable
111 recombinant viruses²⁶ and has not been applied since its publication. We have overcome such limitations
112 by introducing a selection cassette to allow enrichment of the recombinant viruses^{13,15,18}.

113 Overall, while we believe that the field will be inclined to use the tools described in this protocol, traditional
114 silencing approaches and emerging genetic tools will likely also be necessary to unveil different aspects of
115 viral and host biology.

116

117 **H2 Potential applications and limitations of the method.**

118 The versatility and efficiency of CRISPR/Cas9 are exploited in this protocol for the genetic manipulation of
119 *A. castellanii*. This organism possesses about 25 copies of each chromosome⁹, thus 25 alleles of every gene
120 in their genome need to be modified to achieve successful gene knockout. Regardless, we have recently
121 shown that it is possible to target multicopy genes in a single experiment, using CRISPR/Cas9 with 100%
122 efficiency¹⁵. Specifically, we targeted the three genes of the cellulose synthase using 2 guide RNAs (gRNAs)
123 simultaneously and demonstrated modification in the 125 targeting sites (1 gRNA targeting the 3 genes
124 (3x25=75 target locus) and 1 gRNA targeting 2 of the genes (2x25=50 target locus)) without traces of the
125 wild-type alleles¹⁵. Such modifications were performed by NHEJ, generating random indels (insertion-
126 deletions). Moreover, we have shown that upon Cas9-mediated cleavage of *A. castellanii* genome, small
127 indels are preferentially generated upon repair of the targeting site¹⁵, thus, making it necessary to perform
128 extensive genotyping to demonstrate non-sense modification and complete gene knockout. Importantly,
129 while this limitation of the technology needs to be addressed in every experiment by cloning of
130 recombinant amoebas and sequencing to identify amoebas with non-sense mutations, the use of
131 polycistronic gRNA expression allows users to partially bypass such issues. Specifically, the use of multiple
132 gRNAs targeting a single gene significantly decreases the chances of getting functional recombinant genes
133 by increasing the sites to be repair by NHEJ and thus, the generation of potential non-sense mutations.
134 Thus, we expect that the use of such tools for the manipulation of *A. castellanii* will allow gene functionality
135 to be studied in this human-pathogenic organism as well as the complex sets of interactions among
136 microorganisms resistant to amoeba digestion which have made *A. castellanii* their environmental host.

137 Nuclear and cytoplasmic GVs modification can be easily achieved by recombination techniques^{13,15,18}. We
138 have currently set up selection cassettes that allow efficient selection of recombinant viruses in
139 pandoravirus/mollivirus¹⁵ and mimivirus/megavirus¹⁸. Importantly, promoters from pandoravirus have
140 been used for the expression of selection cassettes in different pandoravirus and mollivirus¹⁵, while
141 megavirus promoters have been used to express selection cassettes in mimivirus and megavirus¹⁸. Thus,
142 our arsenal of vectors shows a broad usage for different viruses. Regulatory elements can also easily be
143 exchanged to modify other GVs. To achieve efficient drug resistance by virally encoded drug selection
144 cassettes, we have selected promoters with early and constant expression during the infectious cycle of
145 the virus. Such traits in viral promoters should be prioritized by any investigator wishing to adapt our
146 vectors for the modification of different viruses. Recombination allows precise modifications of the
147 genome of all viruses and has shown high rates of success in our experience. The main limitation of the
148 system lies in the limited amount of selection cassettes currently available (limited to two, geneticin¹² and
149 nourseothricin¹³). Thus, we are currently limited to two modifications per genome (only one if trans-
150 complementation is needed). Finally, the modification of essential genes remains challenging. It is worth
151 mentioning that marker-free recombinant viruses cannot be generated for cytoplasmic viruses since
152 selection cassettes are indispensable to achieve selection of recombinant viruses. On the other hand,
153 nuclear viruses can be generated marker-free by combining CRISPR/Cas9 and homologous recombination
154 to repair the cleaved DNA. Another limitation of the technique involves the study of essential genes due
155 to the current lack of conditional systems for gene inactivation. In order to address this issue, we have
156 developed a trans-complementation strategy to allow the expression of the gene to be knocked-out by
157 the amoeba¹³, bypassing the essentiality of the genes. The basis of trans-complementation is related to
158 the fact that independently of where transcription occurs, viral and amoeba mRNAs are transported into
159 the cytoplasm for translation. Thus, localization of the gene coding sequence is irrelevant for protein

160 production and functionality. This allows ectopic expression of viral genes from plasmids localized at the
161 nucleus of the amoeba to produce fully functional proteins. We have experienced that early expressed
162 genes are easier to trans-complement, while genes expressed late in the infectious cycle of the virus tend
163 not to be efficiently complemented in trans. These differences are likely related to protein expression
164 levels and/or timing. Thus, the success of this strategy is gene dependent and should be tested empirically.
165 Importantly, we suggest trans-complementation only as a tool to generate recombinant viruses with
166 modifications that otherwise would impair viral replication. To confirm that the phenotype observed upon
167 gene knockout is specific to the gene modification and not an off-target effect would be accomplished by
168 cis-complementation, by inserting the gene back at the right locus with the endogenous promoter.

169 Finally, CRISPR/Cas9 can also be used for the modification of nuclear GVs¹⁵. Cytoplasmic giant viruses
170 efficiently protect their DNA, rendering the use of Cas9 impossible¹⁵. Cas9 can be used to induce double-
171 strand breaks in the genome of nuclear GVs and allow NHEJ to generate recombinant viruses¹⁵. This
172 technique allows the generation of marker-free viruses and thus, an unlimited number of modifications.
173 NHEJ is however rather inefficient in pandoravirus and mollivirus and usually generates large deletions.
174 Accordingly, mutant viruses should be screened to isolate viruses with short deletions and avoid the
175 knockout of neighboring genes. Importantly, HR and CRISPR/Cas9 can be combined to generate marker-
176 free viruses with precise modifications, bypassing most of the limitations of the system and might be
177 preferred to NHEJ repair depending on the aims of the researcher. Regardless of the tools used for gene
178 modification, gene knockout of essential genes remains still challenging and the success of trans-
179 complementation should be empirically tested. Future efforts should be directed to generate vectors and
180 protocols to achieve conditional depletion of genes (like Cre recombinase²⁷, auxin-inducible degron²⁸ or
181 mRNA degradation²⁹) in order to attain the full potential of genetic manipulation in GVs.

182

183 **H2 Experimental design.**

184 **H3 Endogenous tagging of viral genes with epitope tags (HA or FLAG)**

185 We have currently designed vectors for endogenous tagging (HA and FLAG epitope tags) at the C-terminus
186 of proteins. These vectors can be adapted for N-terminal tagging or internal tagging if necessary (vector
187 maps and sequences are available at Addgene. IDs are provided in Table 1). A schematic representation of
188 the design is shown in Figure 2a. After the identification of a gene of interest, homology arms (250-500
189 bp) should be selected in the area where the tag will be inserted. If the tag is to be introduced at the C-
190 terminal of the protein, the stop codon should be excluded from the 5'-homology arm. Homology arms
191 can be amplified by PCR from genomic DNA (gDNA) using primers bearing homology to the tagging
192 acceptor vector (Table 1). These flanking regions allow the cloning of the homology arms by In-Fusion®
193 seamless cloning (TaKaRa) into the desired vector (Primer templates are shown in Table 2). Other cloning
194 methods can also be utilized to insert the homology arms in the vector.

195 Several critical steps should be considered. First, confirm that the tag is in frame with the open reading
196 frame of the gene of interest (goi) and that the endogenous stop codon was removed. We currently utilize
197 ApE plasmid editing software with this aim (<https://jorgensen.biology.utah.edu/wayned/ape/>). Second,
198 ensure that the homology arms do not contain the restriction sites used for the linearization of the vector.
199 If that is the case, a second restriction site (absent in the homology arms and the acceptor vector) can be
200 introduced in the primers. Alternatively, PCR amplification can be used as a substitute to plasmid digestion.

201 Finally, in order to efficiently assess integration and clonality of recombinant lines, a negative control must
202 be produced for every independent set of transfections. This negative control is done by incubating
203 amoebas with PolyFect Transfection Reagent but no DNA. Infection and drug selection are performed in
204 parallel with other samples. Differences in viral growth as well as DNA amplification by PCR will be critical
205 to assess if the experiment is successful or not.

206 **H3** *Gene knockout by HR*

207 Vectors for gene knockout using HR in mimivirus/megavirus and pandoravirus/mollivirus are currently
208 available (Table 1). Homology arms of about 250-500 bp should be selected to target a gene of interest as
209 shown in Figure 2b. We recommend designing two homology arms, wherein 5' arm covers the beginning
210 of the gene whereas the 3' arm covers the end of the gene, allowing the deletion of a section of the coding
211 region. Homology arms can be amplified by PCR from gDNA using primers bearing homology to the tagging
212 acceptor vector (Table 1). These flanking regions allow the cloning of the homology arms by In-Fusion®
213 seamless cloning (TaKaRa) into the desired vector (Primer templates are shown in Table 2). Any other
214 cloning method can also be used.

215 Several critical steps should be considered. First, ensure that the homology arms do not contain the
216 restriction sites used for the linearization of the vector. If that is the case, a second restriction site (absent
217 in the homology arms and the acceptor vector) can be introduced in the primers. Alternatively, PCR
218 amplification can be used as a substitute to plasmid digestion. Finally, negative control as described in
219 *Endogenous tagging* section must be included.

220 **H3** *Gene knock out by CRISPR/Cas9*

221 The basic principles of CRISPR/Cas9 and the rules to select appropriate gRNA sequences can be found in³⁰.
222 gRNA target sequences must include a PAM (protospacer adjacent motif) sequence and be significantly
223 different from other sequences in the genome of *A. castellanii* (if gene knockout will be performed in this
224 organism) or both *A. castellanii* and nuclear GV (if gene knockout will be performed in a nuclear GV). We
225 currently utilize the Eukaryotic Pathogen CRISPR gRNA Design Tool
226 (EuPaGDT)(<http://grna.ctegd.uga.edu/>), which allows users to rank gRNA target sequences (by analyzing
227 GC content and flanking microhomology pair score) and analyzes potential off-target editing in the genome
228 of *A. castellanii*. Potential off-target sites are manually identified by BLASTn using the reference genome
229 of the GV to be genetically modified. We remind any researcher interested in carrying out gene editing by
230 CRISPR/Cas9 that potential off-target sites have a lower risk of cleavage when mismatches with the 3'
231 (seeding region) of the gRNA or lack of a PAM are observed. Once one or multiple gRNAs are selected,
232 these can be inserted into vector vHB8 by mutagenesis (Figure 3). Primers should be designed containing
233 the new gRNAs and bear homology sequences to clone the gRNAs by In-Fusion® seamless cloning (TaKaRa)
234 into vHB8 (Primer templates are shown in Table 2).

235 **Importantly**, a second plasmid encoding for Cas9 and gRNAs which do not target the desired genome
236 should be generated and transfected simultaneously with any set of transfections. Selection and
237 genotyping must be done in parallel with other samples in order to assess if the experiment is successful
238 or not. If amoebas are being genetically modified, this strain will be the baseline control for any phenotypic
239 characterization.

240 **H3** *Complementation in cis or in trans*

241 Each time a gene is knocked out and mutant organisms are generated, a confirmation that the phenotype
242 observed is due to the expected modification and not to any off-target modifications is needed. A
243 traditional way to assess this is by complementation of the gene function through reintroducing the coding
244 sequence of the gene knocked out. If the phenotype is restored, it can be concluded that the phenotype
245 associated with the genome modification is due to the knockout of the gene being studied. Importantly,
246 complementation in cis, at the endogenous locus and utilizing the endogenous promoter, is recommended
247 and can be achieved by re-introducing the coding sequence of the gene by homologous recombination.
248 Genome sequencing might also be explored if gene complementation is not possible. Trans-
249 complementation allows the generation of recombinant viruses lacking essential genes. In order to trans-
250 complement the lack of these genes, we have generated a battery of vectors that allows the expression of
251 genes under the regulation of *Acanthamoeba castellanii* weak, medium, or strong promoters (vectors
252 modified from³¹); specific expression levels are usually important for efficient complementation of gene
253 function). Moreover, vectors encoding geneticin¹² or nourseothricin¹³ are available (see Table 1). The
254 coding sequence of the gene of interest should be cloned into one (or multiple) of these vectors, which
255 are consequently transfected into amoebas for expression of the recombinant protein.

256 Gene knockout of essential viral genes can be obtained if recombination/CRISPR is performed in an *A.*
257 *castellanii* host expressing a trans-complementing copy of the gene (mutagenesis or codon optimization
258 are required in order to avoid cleavage by CRISPR/Cas9). Many companies provide services for gene
259 synthesis (which includes codon optimization upon providing a codon usage table for the organism of
260 interest) including Integrated DNA technologies (<https://eu.idtdna.com/pages>), Genewiz
261 (<https://www.genewiz.com/en-GB/>) or GeneScript (<https://www.genscript.com/>). In this case, all steps of
262 selection and propagation of the recombinant viruses might be performed in trans-complementing
263 amoebas.

264 Several critical steps should be considered. First, codon optimization for expression in *A. castellanii* is
265 highly recommended to achieve optimal expression levels. Second, if trans-complementation is designed
266 to complement gene knockout by CRISPR/Cas9, silent mutations at the gRNA targeting site are necessary
267 to avoid cleavage of the vector by Cas9. Finally, in order to assess efficiency of drug selection, a negative
268 control must be produced for every independent set of transfections. This negative control is done by
269 incubating amoebas with PolyFect Transfection Reagent but no DNA. After one or two days of drug
270 selection, no amoebas in the trophozoite stage should be detected. No amoebal growth should appear
271 until the end of the experiment.

272 **H3** Selection of mutant viruses by drug cassettes

273 Selection of viral mutants using selection cassettes is currently performed by the utilization of drugs
274 targeting the translation of their host *A. castellanii*. Thus, amoebas infected by WT viruses and non-
275 infected amoebas will succumb to the inhibition of protein synthesis. Only amoebas infected by a virus
276 conferring resistance to such drugs will be able to complete the round of infection and release a pool of
277 viruses enriched in recombinant ones. Such a strategy allows a single round of infection per culture,
278 because freshly released viruses will not be able to infect amoebas in their neighborhood for a second
279 round, as the selection killed them. Thus, viral growth in the presence of the drug is slow. In order to speed
280 up the process, we have designed a protocol that alternates rounds of infection under selection pressure
281 and in the absence of the drug, allowing faster amplification of the virus.

282 Importantly, multiplicity of infection (MOI) must be kept 0.1 to 1 when the selection of recombinant
283 viruses is attempted. If a recombinant virus and a wild-type virus infect the same cell, production of the
284 selection cassette by the recombinant virus will trans-complement the lack of it in the wild-type virus,
285 defeating the purpose of the drug selection.

286 **H3** *Selection of amoebas by drug selection cassettes.*

287 *A. castellanii* segregates its genetic material randomly^{10,11}. Concordantly, the level of expression of genes
288 from vectors in *A. castellanii* shows intrinsic variation which cannot be corrected by cloning the amoebas.
289 Therefore, to select transfected amoebas, we utilize a protocol increasing the concentration of the
290 selective drug at different passages of the transfected amoebas³². This allows an increase in the production
291 of expressed genes, likely by increasing the copy number of the plasmids.

292

293 **H2** **Expertise needed to implement the protocol.**

294 The protocols here described are simple and do not require particular expertise in molecular biology to be
295 carried out. *A. castellanii* is however a human pathogen and thus requires L2 biosafety measures.

296

297 **H1** **MATERIALS**

298 **H2** **BIOLOGICAL MATERIALS**

- 299 • *Acanthamoeba castellanii* strain NEFF (ATCC 30010™) (<https://www.atcc.org/products/30010>)
- 300 • *Escherichia coli* Stellar™ competent cells (TaKaRa, cat. no. 636763)
- 301 • Mimivirus reunion (MW004169.1)¹⁸
- 302 • Pandoravirus neocaledonia (MG011690.1)/ Mollivirus kamchatka (MN812837.1)¹⁵

303

304 **H2** **REAGENTS**

305 **H3** *Growth media and buffers*

- 306 • Proteose peptone (Merck, cat. no. 82450)
- 307 • Yeast extract (ThermoFisher Scientific, cat. no. BP1422-500)
- 308 • MgSO₄ - 7 H₂O (Merck, cat. no. 63138)
- 309 • CaCl₂ anhydrous (Merck, cat. no. 1.02378)
- 310 • Fe(NH₄)₂(SO₄)₂ (Merck, cat. no. 215406)
- 311 • Na₂HPO₄ (Merck, cat. no. S9763)
- 312 • KH₂PO₄ (Merck, cat. no. P5379)
- 313 • Glucose (Merck, cat. no. G8270)
- 314 • NaCl (Euromedex, cat. no. 1112)
- 315 • KCl (Merck, cat. no. P3911)
- 316 • NaOH (Merck, cat. no. S8045)
- 317 • LB (Luria/Miller) (Carl-Roth, cat. no. X968.4)
- 318 • Agar (ThermoFisher Scientific, cat. no. BP1423-500)
- 319 • TAE 10X – Tris Acetate EDTA Buffer 10X (Merck, cat. no. T9650-1L)

320 **H3** *Antibiotics*

- 321 • Geneticin™ (G418 sulfate, 50 mg / ml) (ThermoFisher Scientific cat. no. 10131035)
- 322 • Nourseothricin (Jena Biosciences, cat. no. AB-102L)
- 323 • Ampicillin (Euromedex, cat. no. EU0400)
- 324 • Kanamycin monosulfate (Euromedex, cat. no. UK0010-D)

325 **H3** *PCR and cloning*

- 326 • SsoFast™ EvaGreen® Supermix for qPCR (Biorad, cat. no. 1725202)
- 327 • PureLink® Genomic DNA Kit from Invitrogen (ThermoFisher Scientific, cat. no. K182001)
- 328 • Wizard® Genomic DNA Purification Kit (Promega, cat. no. A1120)
- 329 • Monarch plasmid miniprep kit (New England Biolabs, cat. no. T1010S)
- 330 • Monarch PCR & DNA Cleanup Kit (New England Biolabs, cat. no. T1030S)
- 331 • QIAEX II Gel Extraction Kit (Qiagen, cat. no. 20021)
- 332 • Phusion™ High-Fidelity DNA Polymerase (2 U/μL) (ThermoFisher Scientific, cat. no. F530L)
- 333 • Terra™ PCR Direct Red Dye Premix (TaKaRa cat. no. 639286)
- 334 • GoTaq® DNA polymerase (Promega, cat. no. M3008)
- 335 • In-Fusion® Snap Assembly Master Mix (TaKaRa cat. no. 638943)
- 336 • Agarose (Merck, cat. no. A9539-500G)
- 337 • Nuclease free water (Dutscher, cat. no. 972090)
- 338 • Ethidium bromide (Merck, cat. no. E1510-10ML) **!CAUTION** *Ethidium Bromide is a known mutagen. Wear a lab coat, eye protection and gloves when working with this chemical. You may prefer to use safe products as SYBR Safe dye instead of ethidium bromide according to the manufacturer's instructions.*
- 339
- 340
- 341
- 342 • SYBR™ Safe DNA Gel Stain (ThermoFisher Scientific, cat. no. S33102)
- 343 • 1kb DNA Ladder Plus (Euromedex, cat. no. 03B-1411)
- 344 • 6X Loading Dye Solution (Euromedex, cat. no. 10-0111)
- 345 • All restriction enzymes are ordered from New England Biolabs (<https://international.neb.com/>)
- 346 • PCR Primers are ordered from Eurofins Genomics (<https://eurofinsgenomics.eu/>)

347 **H3** *Transfection*

- 348 • PolyFect Transfection Reagent (Qiagen, cat. no. 301105)

349 **H3** *Immunofluorescence*

- 350 • HA Tag Recombinant Rabbit Monoclonal Antibody (Thermo Fisher Scientific Cat# MA5-27915, RRID:AB_2744968)
- 351
- 352 • Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594 (Thermo Fisher Scientific Cat# A-21207, RRID:AB_141637)
- 353
- 354 • Triton X-100 (EUROMEDEX, 2001-C)
- 355 • 37% Formaldehyde (Sigma-Aldrich, 252549-100ml)
- 356 • Hoechst 33258 Pentahydrate (ThermoFisher Scientific, H1398)
- 357 • BSA (Euromedex, cat. no. 04-100-812-C)

358 **H3** *SDS-PAGE and Western-blot*

- 359 • Acrylamide:bisacrylamide 40% (37,5:1) (Euromedex, cat. no. EU0062-C)
- 360 • Tween 20 (Euromedex, cat. no. 2001-C)

- 361 • Tris-Glycine-SDS buffer 10x (Euromedex, cat. no. EU0510-B)
- 362 • SDS (Merck, cat. no. 436143)
- 363 • TEMED (N,N,N',N'-Tetramethylethylenediamine) (Merck, cat. no. T9281-25ml)
- 364 • APS (Ammonium persulfate) (Merck, cat. no. A7460-100G)
- 365 • 2-Mercaptoethanol (Merck, cat. no. 63689-100ML-F)
- 366 • Coomassie brilliant blue G250 (ThermoFisher Scientific, cat. no. 20279)
- 367 • Acetic acid glacial (Carlo Erba, cat. no. 401424)
- 368 • Ethanol 96° (Carlo Erba, cat. no. 528151)
- 369 • Ethanol absolute (Carlo Erba, cat. no. 528151)
- 370 • BSA (Euromedex, cat. no. 04-100-812-C)
- 371 • cOmplete™, EDTA-free Protease Inhibitor Cocktail (Merck, cat. no. 5056489001)
- 372 • Prestained Protein Ladder 10-180 kDa (Euromedex, cat. no. 06P-0111)
- 373 • Unstained Prot MW Marker 14.4-116 kDa (Euromedex, cat. no. 06U-0511)
- 374 • Western Blotting Filter Papers (ThermoFisher Scientific, cat. no. 88600)
- 375 • nitro blue tetrazolium / 5-bromo-4-chloro-3-indolyl-1-phosphate (NBT / BCIP) (Sigma-Aldrich, B1911-100ml)
- 376
- 377 • Invitrogen™ Power Blotter 1-Step™ Transfer Buffer (5X) (ThermoFisher Scientific, cat. no. PB7300)
- 378

378

379 H2 REAGENT SETUP

- 380 • **PYG** In a 2 l glass bottle, add 40 g of proteose peptone and 2 g of yeast extract. Complete with water to ~1.8 l and autoclave at 121°C for 20 min. Prepare 1 l stock solutions of MgSO₄ (400mM), CaCl₂ (50 mM), Fe(NH₄)₂(SO₄)₂ (5 mM), Na₂HPO₄ (250 mM), KH₂PO₄ (250 mM) and glucose (2 M), sterilized with filtration units Stericups. In the autoclaved solution, add 20 ml of MgSO₄, Fe(NH₄)₂(SO₄)₂, Na₂HPO₄ and KH₂PO₄, 16 ml of CaCl₂, and 50 ml of glucose. Complete to 2 l with distilled water (use the graduations of the bottle). Mix well and sterilize on 2 filtration units of 1 l. Work in a class II BioSafety cabinet to ensure sterility of the medium. Medium and stock solutions can be stored at room temperature (22°C approx.) for several months, as long as they are not contaminated.
- 381
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- 388 • **LB-agar** Prepare LB as described by the supplier. In a 500 ml glass bottle, add 4.8 g of agar, and 400 ml of LB. Don't mix. Autoclave at 121°C for 20 min. Mix and use to prepare petri dishes for bacterial culture. Medium can be stored at room temperature for several months, as long as it is not contaminated.
- 389
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- 392 • **Tris Acetate EDTA (TAE) 0.5X electrophoresis solution** Dilute TAE buffer 10X in distilled water to 0.5X working solution for casting agarose gels and for use as a buffer for gel electrophoresis. Buffer can be stored at room temperature for several months.
- 393
- 394
- 395 • **Tris-Glycine-SDS buffer 1X** Dilute TG-SDS 10X in distilled water to 1X working solution for use as a buffer for SDS-PAGE acrylamide gel electrophoresis. Buffer can be stored at room temperature for several months.
- 396
- 397
- 398 • **PBS** For 1 l PBS, in a beaker, mix 900 ml distilled water with 8 g NaCl (136.8 mM final), 0.4 g KCl (5.36 mM final), 1.44 g Na₂HPO₄ (10.1 mM final), 0.24 g KH₂PO₄ (1.76 mM final). Adjust the pH to 7.4 with NaOH 1M. Adjust the volume to 1 l with distilled water. Filter sterilize or autoclave at 121°C for 20 min. Buffer can be stored at room temperature for several months.
- 399
- 400
- 401

- 402 • **PBS Triton X-100 0.2% (PBST)** For 1 l PBST, in a beaker, mix 900 ml distilled water with 8 g NaCl (136.8
 403 mM final), 0.4 g KCl (5.36 mM final), 1.44 g Na₂HPO₄ (10.1 mM final), 0.24 g KH₂PO₄ (1.76 mM final)
 404 and 2mL of Triton X-100 (final 0.2%). Adjust the pH to 7.6 with NaOH 1M. Adjust the volume to 1 l
 405 with distilled water. Buffer can be stored at room temperature for several months.

406

407 **H2 EQUIPMENT**

- 408 • Motic™ AE31 Main Body, Binocular Microscope (ThermoFisher Scientific, cat. no. 15485342)
- 409 • Epifluorescence microscope AXIO Observer Z1 Zeiss
 410 <https://www.zeiss.com/corporate/int/home.html>
- 411 • Class II Biosafety cabinet for *Acanthamoeba* and virus manipulations. ADS Laminaire, Optimale 12
- 412 • Incubator for amoebas and viruses. Froilabo, Bioconcept <https://www.froilabo.com/>
- 413 • Incubator for bacteria plates. Froilabo, Bioconcept <https://www.froilabo.com/>
- 414 • Shaking incubator for bacteria. InFors Multitron Eco <https://www.infors-ht.com/fr/>
- 415 • Transilluminator for agarose gels and western-blotting imaging. Vilber Fusion FX Spectra
 416 <https://www.vilber.com/fusion-fx-spectra/>
- 417 • Electrophoresis system for agarose gels. Mupid-One (Dutscher, cat. no. 088900)
- 418 • Electrophoresis system for acrylamide gels from Biorad (Power supplier : PowerPac Basic,
 419 Electrophoresis chambers and accessories : Mini-PROTEAN Tetra Handcast Systems)
 420 <https://www.bio-rad.com/fr-fr/category/electrophoresis-chambers?ID=N3F2N0E8Z>
- 421 • Western-Blot transfer system (ThermoFisher Scientific, Power Blotter Station cat. no. 15896152)
- 422 • Thermocycler (Analytik Jena, Biometra Trio) [https://www.analytik-jena.com/products/life-](https://www.analytik-jena.com/products/life-science/pcr-qpcr-thermal-cycler/thermal-cycler-pcr/biometra-trio-series/)
 423 [science/pcr-qpcr-thermal-cycler/thermal-cycler-pcr/biometra-trio-series/](https://www.analytik-jena.com/products/life-science/pcr-qpcr-thermal-cycler/thermal-cycler-pcr/biometra-trio-series/)
- 424 • Water bath (ThermoFisher Scientific, Precision GP 05, cat. no. 15385857)
- 425 • NanoDrop spectrophotometer (ThermoFisher Scientific)
- 426 • Centrifuge 5425R (Eppendorf)
- 427 • Centrifuge 4-16kS (Sigma-Aldrich)
- 428 • High-definition Neubauer counting chamber (hemocytometer) (Dutscher, cat. no. 900504)
- 429 • Cell scrapers 24 cm (Sarstedt, cat. no. 83.3951)
- 430 • Cell culture flask Cellstar 25 cm² filter cap Greiner Bio-One (Dutscher, cat. no. 690175)
- 431 • Plates 6 wells cell culture Cellstar standard Greiner Bio-One (Dutscher, cat. no. 657160)
- 432 • Plates 96 wells flat bottom for cell culture Cellstar standard Greiner Bio-One (Dutscher, cat. no.
 433 020035)
- 434 • Greiner Bio-One 96-well sterile cell culture plate with flat bottom, black with transparent bottom , for
 435 fluorescence imaging (Dutscher, cat. no. 655090)
- 436 • Greiner Bio-One EASYseal™ adhesive foil for seal microtiter plate (Dutscher, cat. no. 676001)
- 437 • Parafilm tape (Dutscher, cat. no. 090260)
- 438 • Precision wipes Kimtech Science (Dutscher, cat. no, 002907B)
- 439 • Petri dishes 90mm (Sarstedt, cat. no. 82.1472)
- 440 • Filtration unit Stericup 1L (Dutscher, cat. no. 051248B)
- 441 • Tubes 15 ml (Sarstedt, cat. no. 62.554.502)
- 442 • Microtubes 1.5 ml (Sarstedt, cat. no. 72.690.001)

- 443 • Microtube Multiply® Pro 0,2 ml with cap (Sarstedt, cat. no. 72.737.002)
- 444 • Microtube Multiply® µStrip 0,2 ml (Sarstedt, cat. no. 72.985.002)
- 445 • Caps for PCR tubes strips (Sarstedt, cat. no. 65.989.002)
- 446 • Filtration unit Stericup PES 0,2µm 1L (Dutscher, cat. no. 051248B)

447

448 **H2 SOFTWARE**

- 449 • Primer design and tools for In-Fusion® cloning <https://www.takarabio.com/learning-centers/cloning/primer-design-and-other-tools>
- 450
- 451 • New England Biolabs Tm calculator <https://tmcalculator.neb.com/#!/main>
- 452 • New England Biolabs double digestion <https://nebcloner.neb.com/#!/redigest>
- 453 • gRNAs design <http://grna.ctegd.uga.edu/>
- 454 • Blastn sequence alignment tool https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=BlastSearch
- 455
- 456
- 457 • Expasy translate tool <https://web.expasy.org/translate/>
- 458 • Clustal omega multiple sequence alignment tool <https://www.ebi.ac.uk/Tools/msa/clustalo/>
- 459 • Reverse complement tool https://www.bioinformatics.org/sms/rev_comp.html
- 460 • ApE plasmid editing software³³ <https://jorgensen.biology.utah.edu/wayned/ape/>

461

462 **H1 PROCEDURE**

463 **H2 Mutagenesis of nuclear viruses and *A. castellanii* using CRISPR/Cas9**

464

465 **H3 Vector preparation for Cas9 and gRNAs expression in *A. castellanii* ● TIMING 5 days (5-6 hs hands on time)**

467 The following protocol describes how to clone 2 gRNAs in vHB8 starting from primers containing the gRNAs
468 and vHB16 as a template (See Figure 3).

- 469 1. Prepare a PCR mix for gRNAs amplification according to [table below](#) and using the primer
470 templates shown in [Table 2](#).

	Amount	Final Concentration
Nuclease-free water	Make up to 50 µl	
5X Phusion HF or GC Buffer	10 µl	1x
10 mM dNTPs	1 µl	200 µM
10 µM Forward Primer 1	2.5 µl	0.5 µM
10 µM Reverse Primer 2	2.5 µl	0.5 µM
Template vHB16	20 ng	0.4 ng/µl
DMSO (optional)	(1.5 µl)	3 %
Phusion DNA Polymerase	0.5 µl	1 unit/50 µl PCR

471 ▲ **CRITICAL STEP** DMSO is used to inhibit the formation of secondary structures on the DNA
472 templates and should be considered for GC rich templates or templates with predicted secondary
473 structures.

474

475 2. Perform PCR following the cycling conditions below.

Cycle number	Denature	Anneal	Extend	Final
1	98°C, 30 sec			
2-30	98°C, 10 sec	55°C, 30 sec	72°C, 15 sec	
31			72°C, 5 min	
32				10°C, Hold

476 3. Analyze the product by migrating 1 µl of the PCR reaction on a 2 % agarose gel using standard gel
477 electrophoresis conditions (135V, 20 min). Purify the rest of the PCR reaction with Monarch® PCR
478 & DNA Cleanup Kit (see Supplementary Methods). Elute with nuclease-free water.

479 ■ **PAUSE POINT** Purified DNA can be stored at -20°C.

480 ?**TROUBLESHOOTING**

481
482 4. Digest the PCR product purified from Step 3 with DpnI in order to eliminate remaining parental
483 plasmid and 1 µg of vHB8 with NotI in parallel at 37°C for 3 hours following the reactions:

	Amount	Final Concentration
PCR product or vHB8	All purified PCR or 1 µg of vHB8	
NEB CutSmart buffer	2 µl	1x
DpnI or NotI (HF)	20 U	1 U/µl
Nuclease-free water	To 20 µl	

484 ■ **PAUSE POINT** Digestion can be performed overnight.

485 5. Analyze digestion efficiency of vHB8 by NotI by migrating 1 µl of the mixture using standard gel
486 electrophoresis (135V, 20 min). Purify the rest of digested plasmid with Monarch® PCR & DNA
487 Cleanup Kit according to the manufacturer's instructions (Supplementary Methods)

488 ?**TROUBLESHOOTING**

489 6. Assemble an In-Fusion® seamless cloning reaction as follows:

	Amount	Final Concentration
Digested and purified PCR fragment from Step 4	200 ng	20 ng/µl
Linearized vector from Step 5	50 ng	5 ng/µl
5X In-Fusion® HD Enzyme Premix	2 µl	1x
Nuclease-free water	Up to 10 µl	

490 7. Incubate the reaction for 15 min at 50 °C, then place it on ice.

491 8. Transform Stellar™ Competent Cells (according to manufacturer's specifications) using 5 µl of the
492 In-Fusion® reaction and at least 25 µl of chemically competent bacteria.

493 ▲ **CRITICAL STEP** The incubation allowing the recovery of bacteria after transformation is not
494 necessary when using plasmids containing ampicillin resistance (bacteriostatic).

495 9. Plate cells on LB agar dishes containing Ampicillin 100 µg.ml⁻¹

496 10. Incubate plates overnight at 37 °C.

497 11. Perform a colony screening PCR to identify positive clones. The PCR mixture is assembled as
498 follows (one reaction per colony tested):

	Amount	Final Concentration
5X Green GoTaq® Buffer	3 µl	1x
10 mM dNTPs	0.3 µl	200 µM

10 μ M Forward Primer from Step 1	0.75 μ l	0.5 μ M
10 μ M Reverse Primer (11, see Table 1)	0.75 μ l	0.5 μ M
GoTaq [®] DNA Polymerase	0.075 μ l	0.375 units
Nuclease-free water	Up to 15 μ l	
Template	See Step 12	

499 **▲ CRITICAL STEP** Primer 11 was designed next to the insertion site on vHB8 plasmid. Forward
500 primer used in step 1 can be used in combination.

501 12. To perform the PCR reactions, number each colony to be analyzed by PCR using a marker pen on
502 the petri dish, to later recognize the colonies bearing the desired vector. Using a pipette tip, collect
503 approximately half of each colony and vigorously shake it in the PCR reaction solution.

504 13. Perform PCR following the cycling conditions below, and analyze the products by running a
505 standard gel electrophoresis (135V, 20 min).
506

Cycle number	Denature	Anneal	Extend	Final
1	95°C, 2 min			
2-30	95°C, 45 sec	42–65°C, 45 sec	72°C, 1 min/kb	
31			72°C, 5 min	
32				10°C, Hold

507 **▲ CRITICAL STEP** Calculate annealing temperature using the NEB Tm Calculator
508 (<https://tmcalsulator.neb.com/#!/main>), selecting Taq DNA Polymerase from the given options

509 14. Pick individual colonies bearing the desired plasmid from the plates in Step 10 and incubate them
510 in 5 ml of LB (Luria-Bertani) medium in presence of 100 μ g.ml⁻¹ ampicillin at 37 °C, 200–250 RPM
511 agitation for 12-16 hours (one colony per tube).

512 15. Centrifuge the tubes at 3000 g for 10 min at 4°C, remove the supernatant and purify the plasmid
513 in the pellet using Monarch[®] Plasmid DNA Miniprep Kit Protocol, following manufacturer’s
514 instructions.

515 16. Send an aliquot of the plasmid for sequencing and confirm the absence of undesirable mutations.
516

516

517 **H3 Transfection of *A. castellanii* ● TIMING 2 days (2-3 hs hands on time)**

518

519 **▲ CRITICAL** This protocol can be used to transfect any kind of DNA into *Acanthamoeba castellanii*, i.e.
520 linear DNA serving as a template for recombination or circular DNA (plasmid) designed for the stable
521 expression of a transgene like *cas9*, gRNAs, or any other fusion gene. The vector should also contain a
522 selection marker such as *neo* (Neomycin phosphotransferase) conferring resistance to geneticin, or *NAT*
523 (Nourseothricin N-Acetyl Transferase) conferring resistance to nourseothricin (Table 1).
524

524

525 17. In a 25 cm² flask, inoculate 5 x 10⁵ fresh *A. castellanii* cells in 5 ml PPYG medium (2 x 10⁴ cells /
526 cm²). Incubate at 32 °C for 24 hs.

527 18. Recover the amoebas into a 15 ml tube after scraping the flask with a cell scraper.

528 19. Count the cells and determine their concentration with a hemocytometer.

- 529 20. In a 6-well dish, add 1.5×10^5 cells in one well per assay containing 2 ml PYG. Incubate for a few
530 minutes to allow the cells to adhere at the bottom (5 minutes is usually enough for healthy cells).
531 ▲ **CRITICAL STEP** Include an additional well for the negative control which will be transfected with
532 no DNA. Such amoebas will be treated using the same selection procedure as other samples. No
533 trophozoites should be observed after 1 or 2 days of drug selection (refer to ³⁴ for brightfield
534 images of different stages of *Acanthamoeba*). At the end of the experiment, no cells should be
535 observed in the negative control sample.
536 ▲ **CRITICAL STEP** If CRISPR/Cas9 vectors are transfected, include a vector expressing Cas9 and
537 gRNAs which do not target the organism of interest as negative control for future genotyping or
538 phenotypic characterization.
539 ▲ **CRITICAL STEP** Before using the culture, with a bench microscope, check that the cells in the
540 flask are in a good shape, i.e. adherent, in trophozoite stage, and in monolayer. This drastically
541 enhances the transfection efficiency.
- 542 21. Simultaneously, in a 1.5 ml microtube, mix $\sim 4 \mu\text{g}$ of DNA diluted in PBS to a final volume of 100 μl ,
543 then add 10 μl PolyFect (Qiagen), vortex for 20 sec, and incubate for 5-10 min at room temperature
544 to allow the formation of transfection complexes.
545 ▲ **CRITICAL STEP** The use of PBS is critical to ensure maximum transfection efficiency. Homogenize
546 the PolyFect solution by tapping before pipetting.
547
- 548 22. During the incubation of the transfection mix, remove the supernatant from the cells from Step
549 20, carefully wash them by adding 1 ml of PBS, and gently rock the plate.
550 ▲ **CRITICAL STEP** Add the liquid on the walls of the wells, not directly onto the cells, to avoid their
551 detachment from the plate. The use of PBS is critical to ensure maximum transfection efficiency.
- 552 23. Repeat Step 22 and leave the PBS in the wells.
- 553 24. Delicately add the transfection mix from Step 21 (110 μl) drop by drop onto the cells. Homogenize
554 by gently rocking the plate.
- 555 25. Incubate for 1 h at 32 °C.
- 556 26. Gently aspirate the PBS using a pipette tip without disturbing attached cells, and delicately add 2
557 ml PYG.
558 ▲ **CRITICAL STEP** Add the liquid on the walls of the wells, not directly onto the cells, to avoid their
559 detachment from the plate.
- 560 27. For stable transfection in *A. castellanii*, to express Cas9 and gRNAs for example, continue to the
561 next section.
562 For genomic manipulations of viruses by homologous recombination, infect the cells directly with
563 the desired virus (Steps 37-75).
564
- 565 **H3 Selection of amoebas stably expressing a transgene such as cas9 and gRNAs** ● **TIMING 7-10 days (2-3**
566 **hs hands on time)**
567
- 568 28. Seal the plate with parafilm to avoid evaporation and incubate overnight at 32 °C. Overnight
569 growth allows the recovery of the cells and the expression of the selection marker.

570 ▲ **CRITICAL STEP** Check the appearance of the cells with a microscope: they should be mainly at
571 the trophozoite stage.

572 29. Remove the supernatant by pipetting/aspiration and replace it delicately with 2 ml PYG containing
573 30 $\mu\text{g}\cdot\text{ml}^{-1}$ geneticin or 30 $\mu\text{g}\cdot\text{ml}^{-1}$ nourseothricin (according to the vector selection cassette (see
574 Table 1)).

575 30. Incubate for 2 days at 32 °C.

576 31. Check the appearance of the cells using an inverted microscope magnification x 400.

577 ▲ **CRITICAL STEP:** As the transfection efficiency is quite low, most cells should appear dead, i.e.
578 round and detached from the bottom of the dish, and a few should remain as healthy trophozoites.
579 These ones are most probably containing the vector and stably expressing the selection marker.

580 ?TROUBLESHOOTING

581 32. Remove dead cells by replacing the culture media every 2-3 days with fresh PYG containing 30
582 $\mu\text{g}\cdot\text{ml}^{-1}$ of the selection drug until a confluent monolayer of trophozoites is observed. The timing
583 needed to obtain a confluent monolayer is highly variable according to transfection efficiency and
584 the effect of the transgene on the growth of the amoebas. A minimum of one week is needed.

585 33. Recover the cells from the wells either by scraping or by carefully pipetting up and down.

586 34. Prepare two 25 cm^2 flasks per culture, one containing 5 ml PYG and 30 $\mu\text{g}\cdot\text{ml}^{-1}$
587 geneticin/nourseothricin, and one with 5 ml PYG and 100 $\mu\text{g}\cdot\text{ml}^{-1}$ geneticin/nourseothricin.

588 35. Distribute 1 ml of cells in each flask. Visualization of the amoebas by fluorescence microscopy
589 should be used to confirm expression of GFP-Cas9. Clear nuclear localization should be observed.
590 Change the medium every 2-3 days until the cultures can be used for further experiments and
591 passage into a new dish whenever cultures reach confluency. Amoebas can be maintained
592 indefinitely in this condition.

593 ▲ **CRITICAL STEP:** The medium containing 100 $\mu\text{g}\cdot\text{ml}^{-1}$ antibiotic allows increased expression of
594 plasmid-contained genes likely by increasing copy number of the plasmid. The medium containing
595 30 $\mu\text{g}\cdot\text{ml}^{-1}$ of antibiotic will serve as a backup in case the amoebas do not grow properly in the first
596 one.

597 36. At this point, there are two options according to the aim of the experiment: Option A, Mutagenesis
598 of nuclear viruses with CRISPR/Cas9 or Option B, Mutagenesis of amoebas using CRISPR/Cas9.

599

600 A. Mutagenesis of nuclear viruses with CRISPR/Cas9 ● **TIMING 7-10 days (3-4 hs hands on time)**

601

602 i. In a 25 cm^2 flask, inoculate 5×10^5 fresh *A. castellanii* cells expressing Cas9 and gRNAs
603 produced during Step 35 in 5 ml PYG medium containing geneticin 100 $\mu\text{g}\cdot\text{ml}^{-1}$ to keep
604 selecting for Cas9 expression (2×10^4 cells / cm^2). Incubate at 32 °C for 24 hs.

605 ii. Infect the culture with the virus you wish to mutagenize with an approximate MOI of 5-10
606 for pandoravirus and mollivirus. Gently rock the plate to homogenize.

607 iii. Incubate 1 h at 32 °C.

608 iv. Remove the supernatant by pipetting/aspiration to remove the viruses that were not
609 internalized and replace with 5 ml PYG containing geneticin 100 $\mu\text{g}\cdot\text{ml}^{-1}$.

- 610 v. Incubate at 32 °C until you start to observe cell lysis and production of new viruses
611 (approximately 2 to 5 days). Evaluate the production of viruses by observing the flask with
612 an inverted microscope magnification x 400.
- 613 ▲ **CRITICAL STEP** Part of the suspension corresponds to mutant viruses.
- 614 ▲ **CRITICAL STEP** Infected cells appear lysed or round, and the viruses appear as small dots
615 between the cells. This step is important to evaluate roughly how much suspension is needed for
616 next round of infection.
- 617 vi. Recover the viruses by pipetting up and down to resuspend viral particles and transfer into
618 a 15 ml tube, transfer 500 µl into two separate 1.5 ml microtubes, and proceed to cloning
619 and genotyping to detect mutant viruses (see Steps 76-91 and 97-102). Keep the
620 remaining suspension at 4 °C. PAUSE POINT: Samples are stable for at least 1 year if there
621 is no bacterial contamination.
- 622 vii. Prepare a culture of *A. castellanii* expressing Cas9 and gRNAs by repeating Step 36Ai and
623 infect with the viral suspension obtained in Step 36Avi (use 500 µl from one of the
624 microtubes), as described in Step 36Aii. Continue to Step 36Aiii-vi.
- 625 viii. Repeat step 36Avii two more times.
- 626 ▲ **CRITICAL STEP** Due to the low efficiency of NHEJ in nuclear giant viruses and its tendency to
627 generate large deletions, the number of passages needed to increase the proportion of mutants
628 with discrete deletions needs to be analyzed empirically for each gene (usually between 1 and 3
629 passages). Over 5 consecutive passages might increase natural mutations in the gRNA targeting
630 site and thus, should also be avoided.
- 631 ▲ **CRITICAL STEP** Cloning (Steps 76 - 91) and genotyping of clones (Steps 97 - 102) should be
632 performed at each passage to maximize the success rate of the experiment. Viral lines should be
633 collected and passaged every day.

634

635 **B. Mutagenesis of amoebas using CRISPR/Cas9 ● TIMING 3 weeks (10-15 hs hands on time)**

- 636 i. Prepare a vector designed for CRISPR/Cas9 mutagenesis targeting the gene of interest as
637 described in Steps 1-16
- 638 ii. Transfect *A. castellanii* with this plasmid as described in Steps 17-27.
- 639 iii. Select and amplify cells expressing Cas9 and gRNAs as described in Steps 2 8-36.
- 640 iv. When a confluent population is obtained, proceed to cloning of amoebas (see Steps 92-
641 96). ▲ **CRITICAL STEP** Modification of the genome of *A. castellanii* occurs during the
642 selection and amplification process.
- 643 v. Identify mutant strains by genotyping (see Steps 97-102).
- 644 ▲ **CRITICAL STEP** Ensure that the mutations obtained create an efficient knock-out. *i.e.* the indels
645 generate frameshifts or the absence of protein production is confirmed by detection with
646 antibodies (Western blot).

647

648 **H2 Mutagenesis of nuclear and cytoplasmic viruses using recombination**

649

650 **H3 Vector preparation for homologous recombination experiments ● TIMING 10 days (4-6 h hands-on** 651 **time)**

652 37. Prepare a PCR mix for amplification of each homology arm according to the following [table](#) and
 653 using the primer templates shown in [Table 2](#). The genomic DNA of the selected virus must be used
 654 as a template for PCR amplification. gDNA is obtained using PureLink® Genomic DNA Kit from
 655 Invitrogen by extracting it from purified viral particles or infected cells, following manufacturer's
 656 instructions.

	Amount	Final Concentration
Nuclease-free water	Up to 50 µl	
5X Phusion HF or GC Buffer	10 µl	1x
10 mM dNTPs	1 µl	200 µM
10 µM Forward Primer 1, 3, 5 or 7	2.5 µl	0.5 µM
10 µM Reverse Primer 2, 4, 6 or 8	2.5 µl	0.5 µM
Template gDNA	100 ng	2 ng/µl
DMSO (optional)	(1.5 µl)	3%
Phusion DNA Polymerase	0.6 µl	1 unit/50 µl PCR

657 ▲ **CRITICAL STEP** DMSO is used to inhibit the formation of secondary structures on the DNA
 658 templates and should be considered for GC rich templates or templates with predicted secondary
 659 structures.

660 38. Perform PCR following the cycling conditions below, and analyze the products by running
 661 1 µl of the PCR reaction using standard agarose gel electrophoresis (135V, 20 min).
 662 .

Cycle number	Denature	Anneal	Extend	Final
1	98°C, 30 sec			
2-30	98°C, 10 sec	45-72°C, 30 sec	72°C, 15 sec	
31			72°C, 5 min	
32				10°C, Hold

663 ▲ **CRITICAL STEP** Calculate the annealing temperature using the NEB Tm Calculator
 664 (<https://tmcalsculator.neb.com/#!/main>) without considering the 15 bp corresponding to the receptor
 665 vector and used for In-Fusion® recombination

666 ?TROUBLESHOOTING

667 39. Purify the rest of the PCR reaction with Monarch® PCR & DNA Cleanup Kit according to the
 668 manufacturer's recommendations (Supplementary Methods). ■ **PAUSE POINT** Purified DNA can
 669 be stored at -20°C .

670 40. In parallel, linearize at least 1 µg of the desired receptor vector ([Table 1](#)) by digesting it with
 671 HindIII or EcoRI for the cloning of 5' homology arm at 37°C for 3 hours following the reaction:

	Amount	Final Concentration
Vector	1 µg	50 ng/µl
NEB 3.1 or NEB CutSmart buffer	2 µl	1x
HindIII or EcoRI	20 U	1 U/µl
Nuclease-free water	To 20 µl	

672 ■ **PAUSE POINT** Digestion can be performed overnight.

- 673 41. Analyze digestion efficiency by running 1 μ l of the digested plasmid using standard agarose
 674 gel electrophoresis (135V, 20 min). Purify the rest of the digested plasmid with Monarch[®]
 675 PCR & DNA Cleanup Kit according to the manufacturer's instructions (Supplementary
 676 Methods). **?TROUBLESHOOTING**
 677 **■ PAUSE POINT** Purified DNA can be stored at -20°C.
 678 42. Proceed as described in Steps 6-16 to clone the 5' homology arm in the digested vector.
 679 43. Digest the vector obtained in the previous step using NotI as described in Step 4 to clone
 680 the 3'HR in the previously obtained vector, following Steps 5-16.

681
 682 **H3 Mutagenesis of viruses by recombination with the insertion of a resistance cassette ● TIMING 5-7 days**
 683 **(8-10 hs hands-on time)**
 684

- 685 44. Linearize 6 μ g of the vector produced in Step 43 by digesting it overnight with appropriate
 686 restriction enzymes (HindIII/EcoRI and NotI, See Figures 1, 2) as follows
 687

	Amount	Final Concentration
Vector	6 μ g	120 ng/ μ l
Appropriate NEB buffer	5 μ l	1x
Enzyme 1	20 U	0.4 U/ μ l
Enzyme 2	20 U	0.4 U/ μ l
Nuclease-free water	To 50 μ l	

- 688
 689 45. Analyze digestion efficiency by running 1 μ l of the digested plasmid using standard agarose
 690 gel electrophoresis (135V, 20 min). Purify the rest of the digested plasmid with Monarch[®]
 691 PCR & DNA Cleanup Kit according to the manufacturer's instructions (Supplementary
 692 Methods).
 693 **▲ CRITICAL STEP** It is not necessary to separate the two fragments of the plasmid obtained after
 694 restriction digestion, for transfection.
 695 **▲ CRITICAL STEP** Split the sample in two and purify using two columns to avoid saturation and
 696 DNA loss during purification.
 697 **?TROUBLESHOOTING**
 698 46. Transfect wt *A. castellanii* with the linearized vector as described in Steps 17-27.
 699 **▲ CRITICAL STEP** Include as a negative control a well with cells transfected with no DNA. samples
 700 must be collected in parallel in order to assess the specificity of genotyping.
 701 47. Infect transfected cells with a high MOI of virus. Gently rock the plate to homogenize.
 702 **▲ CRITICAL STEP** The aim is to infect every transfected cell. For genetic manipulation of
 703 Mimiviridae, a MOI of 10 is sufficient, for pandoravirus or mollivirus, a MOI of 50-100 is preferable.
 704 48. Incubate 1 h at 32 °C.
 705 49. Remove the supernatant to wash away non-internalized viruses and replace with 2 ml
 706 PYG.
 707 50. Incubate overnight at 32 °C.

708 **■ PAUSE POINT** Viruses can be kept in the incubator for several days (typically 2-3 days), in which
709 case, seal the plates with parafilm to avoid evaporation of the culture media. If more time is
710 needed, perform the incubation at room temperature.

711 51. In 25 cm² flasks, prepare one culture per assay, including the negative control. Inoculate
712 5 x 10⁵ *A. castellanii* cells in 5 ml PYG (2 x 10⁴ cells / cm²), and incubate 24 hs at 32 °C, to
713 obtain healthy amoebas in a monolayer for the next round of infection.

714 52. Recover each well into 2 separate 1.5 ml microtubes (1 ml/tube). Centrifuge one of the
715 tubes at maximum speed (~16,000 g) for 20 min at room temperature to pellet the viruses.
716 Remove the supernatant by pipetting/aspiration and keep the pellet for genotyping.
717 Proceed to PCR genotyping to assess the successful integration of the genomic
718 modification (see Steps 97-102).

719 **▲ CRITICAL STEP** At this point, recombination events should be independent of the phenotype
720 associated with the modification of the genome and thus, recombinant DNA should be identified
721 by genotyping. Lack of amplification using primers P1+P3 (Figure 4) indicates inefficient
722 transfection or problems with PCR amplification.

723 **■ PAUSE POINT** Pellets can be stored at -20 °C for at least one month to proceed later to
724 genotyping.

725 **?-TROUBLESHOOTING**

726 53. Infect a 25 cm² flask of confluent cells produced in step 51 with the contents of the second
727 tube obtained during step 52.

728 54. Incubate 1 h at 32 °C.

729 **▲ CRITICAL STEP:** Incubation for 1 h without a selection marker allows the expression of the
730 resistance cassette by the virus.

731 55. Wash twice with 1 ml of PYG to eliminate extracellular viruses. Proceed carefully to avoid
732 detaching cells from the surface.

733 56. Add 5 ml of PYG containing nourseothricin 100 µg.ml⁻¹ or geneticin 100 µg.ml⁻¹ according
734 to the selection cassette introduced in the viral genome.

735 57. Incubate overnight at 32 °C.

736 **■ PAUSE POINT** Viruses can be kept in the incubator for several days (typically 2-3 days). If more
737 time is needed, perform the incubation at room temperature.

738 58. Prepare cultures of amoebas as described in step 51.

739 59. Recover the contents of the flasks in 15 ml tubes and centrifuge at maximum speed
740 (depending on the centrifuge 6,800 g for 45 min (swinging buckets) or 16,000 g for 20 min
741 (fixed rotor)) at room temperature to pellet the viruses. Discard the supernatants and
742 leave only a small volume to resuspend the pellets.

743 **▲ CRITICAL STEP** Centrifugation is necessary to remove the selection marker and allow a new
744 round of viral infection.

745 60. For each assay, infect one 25 cm² flask of confluent cells produced in step 58 with the
746 content of a tube obtained during step 59.

747 61. Repeat steps 54-57

748 62. Prepare cultures of amoebas as described in step 53.

749 63. Recover the viruses as described in step 59.

750 64. Repeat steps 54-55

751 65. Replace medium with 5 ml of PYG without selection.

- 752 66. Incubate overnight at 32 °C.
 753 ■ **PAUSE POINT** Viruses can be kept in the incubator for several days (typically 2-3 days). If more
 754 time is needed, perform the incubation at room temperature.
 755 67. Control viral production by observing the culture using an inverted microscope
 756 magnification x400.
 757 ▲ **CRITICAL STEP** At this step, significant differences between the samples and the negative
 758 control are expected. Viruses and cell lysis should be easily recognized in the samples while less
 759 abundant in the negative control.
 760 ▲ **CRITICAL STEP** Long incubation of the culture will decrease the differences between the samples
 761 and the negative control. This will not affect the overall success of the experiment.
 762 ?-TROUBLESHOOTING
 763 68. Recover the contents of the flasks in 15 ml tubes. Transfer 100 µl in one 1.5 ml microtube
 764 for the last round of enrichment in mutant viruses. Keep the remaining suspension at 4 °C.
 765 (OPTIONAL) Transfer 1 ml in a 1.5 ml tube and centrifuge at maximum speed and room
 766 temperature. Proceed to genotyping (step 97).
 767 69. Prepare cultures of amoebas as described in step 51.
 768 70. Infect a 25 cm² flask produced in step 68 with 5-100 µl of viruses according to the amount
 769 of viral particle present in the culture.
 770 71. Repeat steps 54-57
 771 ■ **PAUSE POINT** Viruses can be kept in the incubator for several days (typically 2-3 days). If more
 772 time is needed, perform the incubation at room temperature.
 773 72. Control viral production with an inverted microscope magnification x 400 to evaluate the
 774 quantity of produced viruses. Differences between samples and negative control are
 775 expected.
 776 73. Recover the contents of the flasks in 15 ml tubes.
 777 74. Transfer 1 ml in a 1.5 ml tube and centrifuge at maximum speed at room temperature for
 778 genotyping of the virus pellet (steps 97-102). Eliminate the supernatant. Keep the
 779 remaining suspension at 4 °C.
 780 ?-TROUBLESHOOTING
 781 75. Proceed to cloning to isolate recombinant viruses (steps 76-91)

783 H2 CLONING AND GENOTYPING OF VIRUSES AND AMOEBAS

784 H3 Cloning of viruses ● TIMING 5-7 days (3-5 h hands-on time)

- 786
 787 76. In one 25 cm² flask, inoculate 5 x 10⁵ wt amoebas in 5 ml PYG (2 x 10⁴ cells / cm²), and
 788 incubate 24 h at 32 °C, to obtain a monolayer of healthy amoebas.
 789 77. Recover cells from step 76
 790 78. Determine their concentration using a hemocytometer.
 791 79. In a 6-well dish, add 1.5 x 10⁵ wt cells per well, one well per assay. Let the amoebas adhere
 792 to the bottom, 5 minutes is usually enough with fresh cells.

- 793 80. Infect the cells prepared in step 77 with 2-10 μl of viral suspension, i.e. add 2 μl if the
794 apparent quantity of viruses is high, to 10 μl if few viruses are visible in steps vi and 72.
795 Gently rock the plate. Incubate 1 h at 32 °C.
- 796 **▲ CRITICAL STEP** In order to isolate/clone the viruses, the MOI should be less than 1. This way,
797 each cell will likely be infected by a single virus. Bear in mind that the proportion of infectious
798 particles of mimivirus are higher than pandoravirus or mollivirus. Thus, particle numbers should
799 be adapted for each different virus. Moreover, gene modification might affect infectivity and
800 consequently, the optimal number of viruses must be addressed empirically.
- 801 81. Wash 5-10 times with 1 ml of PYG to eliminate the maximum of extracellular viruses.
- 802 82. Collect the viruses from the wells by gently pipetting up and down into a 1.5 ml
803 microtubes.
- 804 83. Determine the cell concentration with a hemocytometer and dilute the sample with PYG
805 to adjust the concentration to ~ 1 amoeba/ μl of PYG.
- 806 **▲ CRITICAL STEP** Control the concentration by depositing 5 drops of 1 μl in an empty well of the
807 6-well dish and observing with an inverted microscope magnification x100 or less. There should
808 be 0-3 amoeba(s)/drop. Adjust the dilution if necessary.
- 809 84. Dilute the suspension in PYG 10 times to have 1 amoeba / 10 μl , which is easier to handle
810 with a multichannel pipet.
- 811 85. Prepare one 96 well dish per viral strain. In each well, add 100-1,000 of wt amoebas in 200
812 μL PYG. **▲ CRITICAL STEP** It is recommended to add 100 amoebas/well for assays with
813 pandoravirus and mollivirus, as their infectious cycle is long and amoebas tend to
814 overgrow the viruses. For experiments with mimivirus or megavirus, 1000 cells per well is
815 appropriate. No control is needed at this point.
- 816 86. Add 10 μl of diluted infected amoebas from step 84 to each well of the plate prepared in
817 step 85 using a multichannel pipette. Seal the plates to avoid evaporation, with parafilm
818 or adhesive seals.
- 819 87. Incubate at 32 °C for at least 2 days (generally 3 to 4 days).
- 820 88. Every well with an infection contains a potential recombinant virus. Observe each well of
821 the 96 well plates with an inverted microscope magnification x 400 to detect the
822 production of viruses.
- 823 **▲ CRITICAL STEP** If all wells appear infected, repeat the procedure from step 80 infecting with a
824 lower quantity of viruses. When all wells are lysed, it usually indicates infection with multiple
825 viruses.
- 826 **▲ CRITICAL STEP** Lysis induction depends not only on the type of virus (pandoravirus and
827 mollivirus tend to induce a lower degree of cell lysis), but also on the gene knocked out (and the
828 phenotype associated with that deletion). Concordantly, the infection should be carefully assessed
829 in each well to identify the presence or absence of viruses.
- 830 89. Mix the wells by pipetting vigorously up and down and transfer 100 μl of viral/cell
831 suspension from ~ 8 wells into a 1.5 ml microtube. Keep the plates sealed at 4 °C.
- 832 **▲ CRITICAL STEP** Label tubes and wells picked from the 96 wells plate. Upon identification of
833 recombinant and clonal viruses, the remaining 100 μl left in the wells will be used to amplify the
834 viruses.

- 835 90. Centrifuge the microtubes of step 89 at maximum speed (~16,000 g) for 20 min at room
 836 temperature and use the pellets for genotyping (see steps 97-102).
 837 91. Once mutant clones are identified, use the remaining content of the wells to infect 25 cm²
 838 flasks containing monolayers of amoebas. At this step and the next ones, no selection with
 839 nourseothricin is needed. Incubate at 32 °C until an acceptable quantity of viruses and cell
 840 lysis are observable. Then, proceed to the purification of viral particles³⁵ and phenotyping
 841 (see Supplementary Methods).
 842

843 **H3 Cloning of amoebas ● TIMING 5-7 days (2 h hands on time)**

- 844
 845 92. Collect the cells prepared in Step 36Biv, either by scraping or by gently pipetting up and
 846 down.
 847 93. Repeat Steps 83-86.
 848 94. Incubate at 32 °C until growth can be observed in the wells.
 849 ▲ **CRITICAL STEP** Seal the plates with parafilm or adhesive seals to avoid evaporation of the culture
 850 media.
 851 95. Repeat Step 89.
 852 96. When clones carrying the desired mutation are identified, use the remaining content of
 853 the wells to inoculate 25 cm² flasks containing 5 ml of PYG. At this step, and the next ones,
 854 no selection is needed. Incubate at 32 °C until cells have sufficiently grown. Then, proceed
 855 to phenotyping.
 856

857 **H3 Genotyping of viruses and amoebas ● TIMING 4 days (3 hs hands-on time)**

- 858
 859 97. Design primers for genotyping according to [Figure 4](#). Different sets of primers should allow
 860 the identification of recombinant and wild-type locus. To facilitate PCR amplification,
 861 design primers to amplify DNA products no longer than 2 kb in size.
 862 ▲ **CRITICAL STEP** Calculate the annealing temperature using the NEB Tm Calculator
 863 (<https://tmcalsculator.neb.com/#!/main>). Preferentially, design primers with annealing
 864 temperatures between 55 and 60°C.
 865 98. Resuspend the pellets of cultures in 20 µl to 100 µl DNase-RNase free water depending on
 866 the size of the pellet.
 867 ▲ **CRITICAL STEP** Include a sample of parental amoebas or virus (wild-type) as control for PCR
 868 specific amplification.
 869 99. Use samples prepared in step 98 as direct templates for PCR amplification with Terra™
 870 PCR Direct Red Dye Premix (TaKaRa) according to the following reaction mixture:

	Amount	Final Concentration
Nuclease-free water	To 15 µl	
2X Terra PCR Direct Red Dye Premix	7.5 µl	1x
10 µM Forward Primer	0.45 µl	0.3 µM
10 µM Reverse Primer	0.45 µl	0.3 µM

Template	1 µl	---
----------	------	-----

871
872 100. Perform PCR following the cycling conditions below, and analyze the products by
873 migrating the PCR reaction using standard agarose gel electrophoresis conditions (135V,
874 20 min).

Cycle number	Denature	Anneal	Extend	Final
1	98°C, 2 min			
2-28	98°C, 10 sec	50-60°C, 15 sec	68°C, 1-2 min	
29				10°C, Hold

875 **?TROUBLESHOOTING** See Table 3

876 101. Check the sequence of the mutations with the help of a company performing standard
877 Sanger sequencing, according to their recommendations.

878 **▲ CRITICAL STEP** Next-Generation Sequencing is an efficient way of analyzing all the alleles of *A.*
879 *castellanii* in a single experiment. If primers are barcoded, multiple clones of amoebas can be
880 analyzed in a single run of sequencing.

881 102. Select the clones carrying the desired genomic modifications (See Figure 4 and Anticipated
882 results section), and proceed to amplification, purification using the protocol previously
883 described in ³⁵ (in case of viruses) and phenotyping (Step 109).

884 **▲ CRITICAL STEP** In case Terra amplifications do not lead to conclusive results, purify the gDNA of
885 the samples using PureLink® Genomic DNA Kit (Invitrogen) or Wizard® Genomic DNA Purification
886 Kit (Promega) and elute in the minimal possible volume according to manufacturers' instructions.
887 Then, proceed to genotyping using Phusion™ High-Fidelity DNA Polymerase (see steps 1-2 for PCR
888 conditions).

889

890 **H3** Vector preparation for trans-complementation ● **TIMING 5 days**

891 103. Prepare a PCR mix as described in step 1 using the appropriate template, most probably
892 gDNA (10 to 50 ng per 50-µl reaction volume) from the studied virus or *A. castellanii*.

893 104. Perform PCR following the cycling conditions below, and analyze the products by running
894 1 µl of the PCR reaction using standard gel electrophoresis (135V, 20 min).

895

Cycle number	Denature	Anneal	Extend	Final
1	98°C, 30 sec			
2-30	98°C, 10 sec	45-72°C, 30 sec	72°C, 30 sec/kb	
31			72°C, 5 min	
32				10°C, Hold

896 **▲ CRITICAL STEP** Calculate the annealing temperature using the NEB Tm Calculator
897 (<https://tmcalsculator.neb.com/#!/main>) without considering the 15 bp corresponding to
898 the receptor vector and used for In-Fusion® recombination

899 105. Analyze and purify PCR reaction as described in step 3.

900 **?TROUBLESHOOTING**

901 106. Linearize at least 1 µg of the desired vector (Table 1) by digesting it with one or two
902 restriction enzymes at 37°C between 3 hours to overnight following the reaction:

	Amount	Final Concentration
--	--------	---------------------

Vector	1 µg	50 ng/µl
NEB 3.1 or NEB CutSmart buffer	2 µl	1x
Restriction enzyme(s)	20 U (or 10U + 10U)	1 U/µl (or 0.5 U/µl of each)
Nuclease-free water	To 20 µl	

903

904 107. Repeat steps 5-16. **?TROUBLESHOOTING**

905 108. Proceed to transfection and selection of amoebas as described in steps 17-37

906

907 **H3 Viral phenotyping TIMING: variable**

908 109. Refer to Supplementary Methods for viral particle quantification¹⁵, infectious dose
 909 determination^{15,36}, DNA replication measurements¹⁵, Immunofluorescence assays
 910 (adapted from³⁷) or western blotting (adapted from³⁸).

911 **H1 ANTICIPATED RESULTS**

912 Successful examples of genome manipulation are shown in Figure 4. Genotyping and sequencing of PCR
 913 products must be performed in parallel with a negative control (amplified from parental virus/amoebas)
 914 to demonstrate specificity of the PCR reactions. Other examples of gene manipulation can be found in our
 915 publications^{15,18}. We typically obtain recombinant viruses or amoebas in 3 weeks or one month,
 916 respectively), with low rates of unsuccessful attempts (less than 5%). Importantly, in our experience,
 917 essential genes are still difficult to modify and further effort to develop conditional systems for gene
 918 depletion will be essential to efficiently target the variety of genes encoded by both *A. castellanii* and GVs.

919 **H1 TIMING**

920 Steps 1-16, Vector preparation for CRISPR/Cas9 expression: 5 days.

921 Steps 17-27, Transfection in *A. castellanii*: 2 days.

922 Steps 28-35, Selection of amoebas stably expressing a transgene such as cas9 and gRNAs: 7-10 days.

923 Step 36A, Mutagenesis of viruses with CRISPR/Cas9: 7-10 days.

924 Step 36B, Mutagenesis of amoebas using CRISPR/Cas9: 3 weeks.

925 Steps 37-43, Vector preparation for homologous recombination experiments: 10 days.

926 Steps 44-75, Mutagenesis of viruses by recombination with the insertion of a resistance cassette: 5-7 days.

927 Steps 76-91, Cloning of viruses: 5-7 days.

928 Steps 92-96, Cloning of amoebas: 5-7 days.

929 Steps 97-102, Genotyping of viruses and amoebas: 4 days

930 Steps 103-108, Vector preparation for trans-complementation: 5 days

931 Step 109, Viral phenotyping: variable

932

933 **H1 TROUBLESHOOTING**

934 Troubleshooting advice can be found in [Table 3](#).

935

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1030 **H1 Data availability**

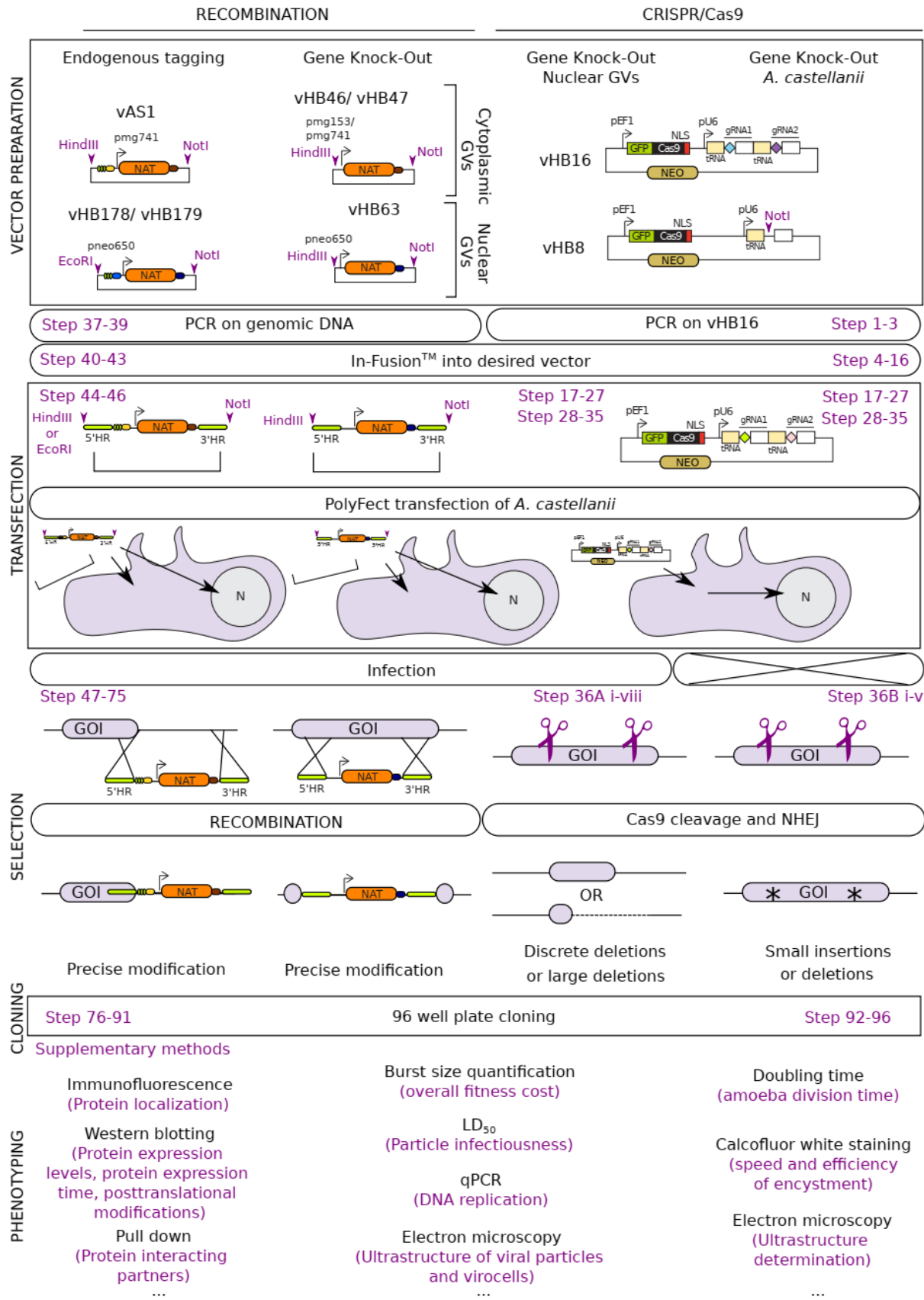
1031 All data supporting this paper are included within the article in the form of figures and tables. Raw data
1032 associated with Figure 4 are provided in the primary articles^{15,18}. All plasmids were deposited in Addgene
1033 (reference number indicated in Table 1).

1034 **H1 Author Contributions Statement**

1035 H.B. Writing of the original draft. N.P. Writing of the original draft. A.S. Writing of the original draft. C.A.
1036 Project administration, funding acquisition and writing of the original draft.

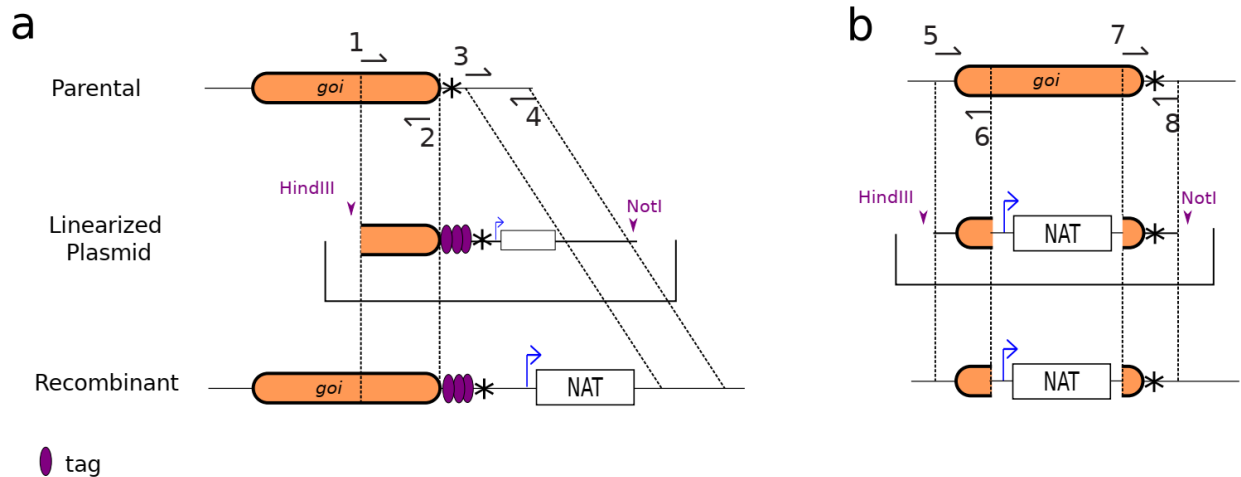
1037 **H1 Competing interests**

1038 The authors declare no competing interests as defined by Nature Research, or other interests that might
1039 be perceived to influence the interpretation of the article



1041 **Figure 1. Schematic representation of the overall methodology for genetic manipulation of *A. castellanii***
 1042 **and GVs.**

1043 A general workflow of the Procedure is shown. Genome modification by recombination or CRISPR/Cas9
 1044 are depicted, including vector preparation, transfection, selection, cloning, and phenotyping. The nature
 1045 of the modification expected on the genomes of *A. castellanii* or GVs is also indicated. vAS1, vHB46, and
 1046 vH47 were developed for gene editing in cytoplasmic GVs (mimivirus, megavirus). vHB178, vHB179, and
 1047 vHB63 were developed for gene editing of nuclear GVs (pandoravirus, mollivirus) (Table 1). pmg741,
 1048 pmg153 are the promoters from the *Megavirus chilensis* early genes *mg741* and *mg153*. They are efficient
 1049 in Mimivirus as well. pneo650 is the promoter from *Pandoravirus neocaledonia pneo_cds_650* early gene.
 1050 NAT: Nourseothricin N-Acetyl Transferase conferring resistance to nourseothricin. NEO: Neomycin
 1051 phosphotransferase conferring resistance to geneticin. GOI: Gene Of Interest. N: nucleus of *A. castellanii*.
 1052 pU6: type III promoter from U6 gene of *A. castellanii* driving the transcription of small RNAs such as tRNAs.
 1053 Green ellipses in vectors vAS1, vHB178, and 179: HA or FLAG tags. The yellow box in vAS1, green box in
 1054 vHB178 and 179, and boxes in 3' position of NAT: 3' UTRs. vHB16 template to produce a DNA sequence
 1055 containing 2 consecutive gRNAs that will be cloned into vHB8 digested with NotI. NLS: nuclear localization
 1056 signal. GFP: green fluorescent protein. HR: homologous recombination. NHEJ: Non-homologous end
 1057 joining. Restriction enzymes are shown in purple with purple arrowheads indicating the cut site.

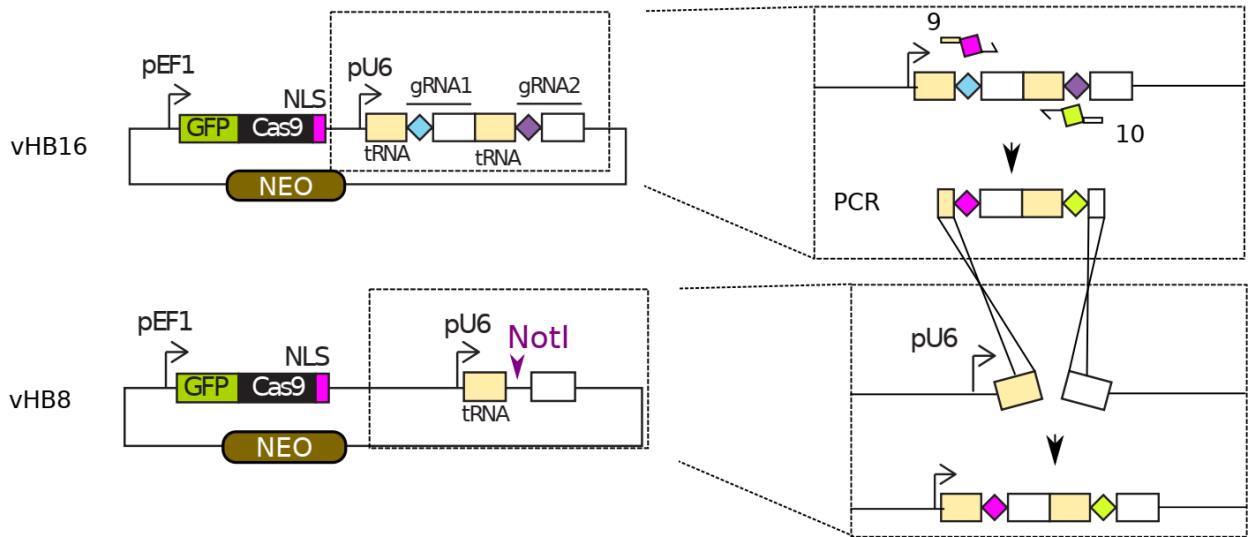


1058

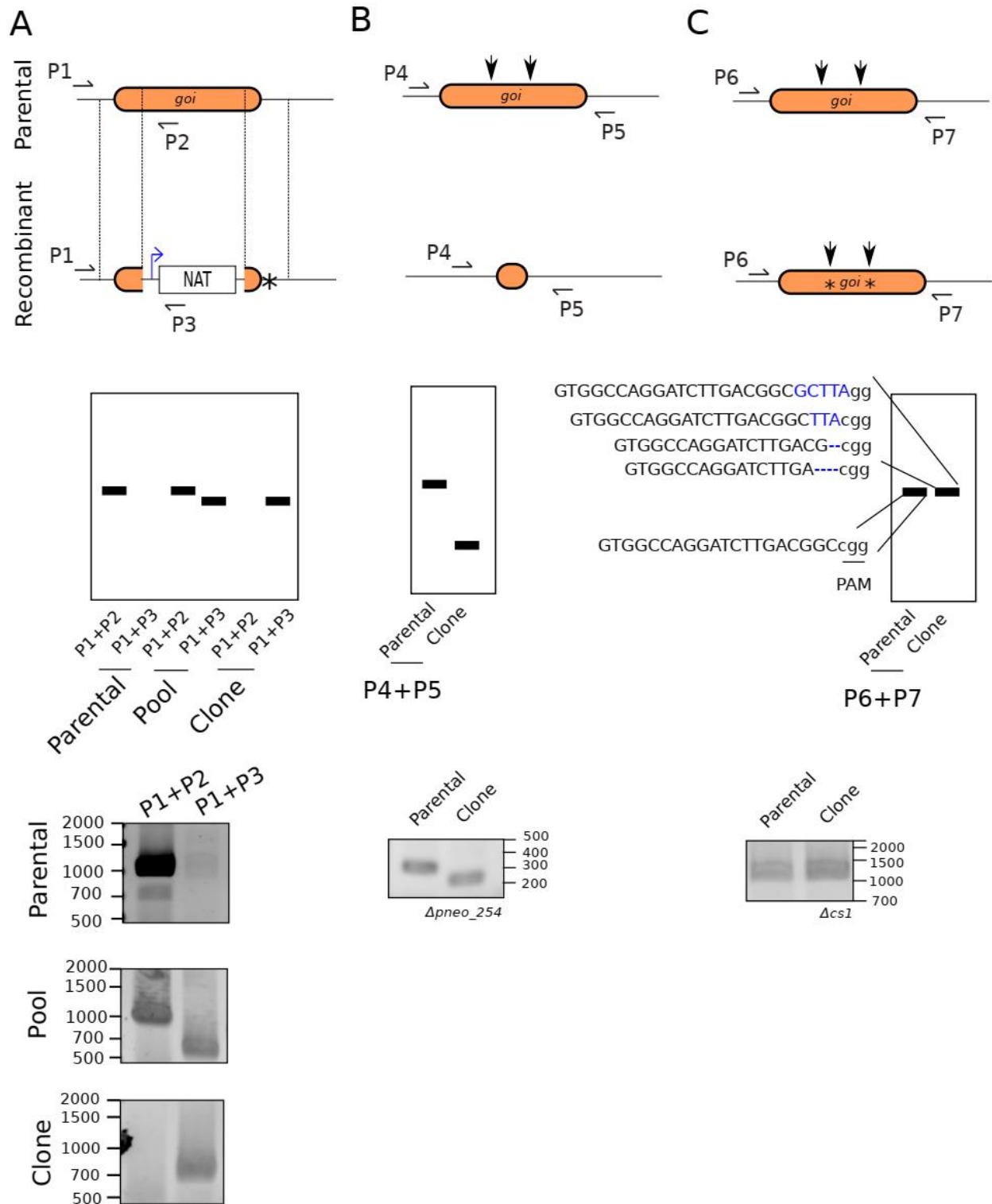
1059 **Figure 2. Design of homology arms for gene modification by homologous recombination.**

- 1060 a. Schematic representation of the strategy to endogenously tag a gene using recombination.
 1061 Example primer sequences, labelled 1-4, are shown in Table 2. 500bp at the end of the gene of
 1062 interest (*goi*), excluding the stop codon, and 500bp at the 3' of the gene are amplified by PCR and
 1063 used as homology arms for recombination. Homology arms are cloned into a vector containing the
 1064 epitope tag and a drug-resistance cassette for the selection of recombinant viruses. Homologous
 1065 recombination allows the insertion of the linearized cassette into the locus of the *goi*. Asterisks
 1066 denote stop codons and dashed line delimitate areas of homologous recombination.
- 1067 b. Schematic representation of the strategy for gene knockout using recombination as an example.
 1068 Example primer sequences, labeled 5-8, are shown in Table 2. 500bp at each extremity of the *goi*
 1069 are amplified by PCR and cloned into a vector containing a drug resistance cassette. Homologous

1070 recombination allows the insertion of the linearized cassette into the targeted locus. Asterisks
 1071 denotate stop codons and dashed line delimitate areas of homologous recombination.
 1072



1073
 1074 **Figure 3. Cloning of polycistronic DNA sequences for gRNAs expression.**
 1075 Schematic representation of the strategy to clone 2 gRNA sequences in the plasmid vHB8 encoding
 1076 CRISPR/Cas9. Primers I and J containing the new gRNA sequences (coloured diamonds) are used for PCR
 1077 amplification of a tracrRNA and tRNA, using vHB16 as a template (upper panel). The resulting product is
 1078 then cloned into vHB8 digested with NotI which encodes a single tRNA and tracrRNA (lower panel). vHB8
 1079 does not contain any gRNA but rather a NotI site instead, allowing the insertion of the PCR product
 1080 between the tRNA and tracrRNA.



1081

1082 **Figure 4. Design of primers for genotyping.**

1083 A. Design of primers and genotyping for modification of GVs by homologous recombination. Primers
 1084 should be designed outside of the homology arms to detect recombination at the expected locus.
 1085 'Pool' indicates suspension of viruses after drug selection but prior to cloning (Step 75). 'Clone'

1086 indicates a homogeneous population after successful cloning (Step 91). Primers P1 and P2 allow
 1087 to detect unmodified locus while primers P1 and P3 would allow amplifying the recombinant locus.
 1088 Analyzing the presence of recombinant viruses right after transfection (Step 66) is highly
 1089 recommended and both P1+P2 and P1+P3 are expected to display positive amplification. As an
 1090 example, genotyping of the insertion of a second copy of *mcp* into the genome of *Mollivirus*
 1091 *sibericum* is shown¹⁵.

1092 B. Design of primers and genotyping for modification of nuclear GVs by CRISPR/Cas9. Primers should
 1093 be designed flanking the regions targeted by gRNAs (black arrows). Clonal viruses that display a
 1094 smaller PCR amplification product should be preferentially selected. The absence of PCR product
 1095 indicates larger deletions including one or both of the primer annealing sequences. As an example,
 1096 genotyping of the knockout of *pneo_254* in *Pandoravirus neocaledonia* is shown¹⁵

1097 C. Design of primers and genotyping for modification of *A. castellanii* by CRISPR/Cas9. Primers should
 1098 be designed flanking the regions targeted by gRNAs (black arrows). *A. castellanii* tends to repair
 1099 double-strand breaks by small indels and thus, shifts in PCR product size are rarely observed.
 1100 Sequencing of PCR products is necessary for the analysis of recombinant cells. Importantly, since
 1101 *A. castellanii* is a polyploid organism, multiple recombinant alleles are expected for each PCR
 1102 amplification. As an example, genotyping of the knockout of the *cellulose synthase 1* of *A.*
 1103 *castellanii* is shown¹⁵

1104

1105 **Table 1. Vectors**

Identifier	Genotype	Resistance cassette	Comments	Addgene ID
PAM1	pTFIID-GFP	Geneticin	Expression in <i>A. castellanii</i> (weak promoter)	198740
PAM2	pGAPDH-GFP	Geneticin	Expression in <i>A. castellanii</i> (strong promoter)	198741
PAM3	pTPBF-GFP	Geneticin	Expression in <i>A. castellanii</i> (medium promoter)	198742
PAM10	pEF1-GFP	Geneticin	Expression in <i>A. castellanii</i> (very strong promoter)	198743
PAM22 (vc241)	pEF1-GFP	Nourseothricin	Expression in <i>A. castellanii</i> (very strong promoter)	193453
PAM24	pGAPDH-GFP	Nourseothricin	Expression in <i>A. castellanii</i> (strong promoter)	198744
vAS1	3xHA-3'UTRmg105-pmg153-NAT	Nourseothricin	3xHA endogenous tagging in mimivirus and megavirus	198745
vHB8	pEFI-Cas9-NLS, pU6-tRNA-NotI-tracrRNA	Geneticin	Expression of nuclear Cas9 and gRNAs in amoebas (for cloning)	193454
vHB16	pEFI-Cas9-NLS, pU6-2x(tRNA-gRNA-tracrRNA)	Geneticin	Expression of nuclear Cas9 and gRNAs in amoebas (template for PCR)	193455
vHB46	pmg153-NAT	Nourseothricin	Vector for knockout in mimivirus/megavirus	198746

vHB47	pmg741-NAT	Nourseothricin	Vector for knockout in mimivirus/megavirus	198747
vHB63	pneo650-NAT	Nourseothricin	Vector for knockout in pandoravirus/mollivirus	198748
vHB178	3xHA-pneo650-NAT	Nourseothricin	3xHA endogenous tagging in pandoravirus and mollivirus	193457
vHB179	3xFLAG-pneo650-NAT	Nourseothricin	3xFLAG endogenous tagging in pandoravirus and mollivirus	193458

1106

1107 **Table 2. Primers**

Primer	Sequence	Comments
1	CTTTTGCAAAA <u>AAGCTT</u> NNNNNNNNNNNNNNNNNNNNNNNN	Contains the 5' homology arm for cloning in vector vAS1 HindIII. Forward
2	GAACATCGTATGGGTANNNNNNNNNNNNNNNNNNNNNNNNN	Contains the 5' homology arm for cloning in vector vAS1 HindIII. Reverse
3	AAATAGTCCTTTAGANNNNNNNNNNNNNNNNNNNNNNNNN	Contains the 3' homology arm for cloning in vector vAS1 NotI. Forward
4	CTTATCGCT <u>GCGGCCG</u> CNNNNNNNNNNNNNNNNNNNNNNNN	Contains the 3' homology arm for cloning in vector vAS1 NotI. Reverse
5	CTTTTGCAAAA <u>AAGCTT</u> NNNNNNNNNNNNNNNNNNNNNNNN	Contains the 5' homology arm for cloning in vector vHB47 HindIII. Forward
6	CTAATATTTTAAGCTNNNNNNNNNNNNNNNNNNNNNNNN	Contains the 5' homology arm for cloning in vector vHB47 HindIII. Reverse
7	TCCTTTAGAGCGCCNNNNNNNNNNNNNNNNNNNNNNNN	Contains the 3' homology arm for cloning in vector vHB47 NotI. Forward
8	CTTATCGCT <u>GCGGCCG</u> CNNNNNNNNNNNNNNNNNNNNNNNN	Contains the homology arm for cloning in vector vHB47 NotI. Reverse
9	TCCCCATACTGGCCANNNNNNNNNNNNNNNNNNNNNNGT TTTAGAGCTAGAAATAGC	Contains homology arms to insert into vHB8 NotI. N region depicts the sequence of the gRNA #1. Forward
10	TTCTAGCTCTAAAACNNNNNNNNNNNNNNNNNNNNNTG GCCAGTATGGGG	Contains homology arms to insert into vHB8 NotI. N region depicts the sequence of gRNA #2. Reverse
11	GTTGTTCCGGGTCGACTCTAG	Primer used for colony screening of vHB8 plasmids

1108 Restriction sites are indicated with underlined text.

1109

1110 **Table 3. Troubleshooting Table**

Step	Problem	Possible reason	Solution
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5, 41,107	Multiple bands are visible on the electrophoresis gel	Incomplete digestion of the vector	Perform digestion for a longer period of time or slice a single band of interest from an agarose gel and purify it with a DNA gel extraction kit (e.g. QIAEX II Gel Extraction Kit from Qiagen)
3, 38, 105	Multiple bands are visible on the electrophoresis gel	PCR conditions or primers do not allow specific amplification	Modify the PCR conditions or slice the band from the gel with the correct size and purify with a DNA gel extraction kit (e.g. QIAEX II Gel Extraction Kit from Qiagen)
<u>45</u>	<u>More than two bands are visible on the electrophoresis gel.</u>	<u>Incomplete digestion of the vector</u>	<u>Purify the plasmid as described in Step 45 and repeat digestion from Step 44.</u>
<u>321</u>	Low transfection efficiency	<ul style="list-style-type: none"> i) Cells are not in good enough condition ii) PolyFect was not homogenized before transfection and its concentration is not sufficient iii) PolyFect efficiency has decreased due to poor conservation, or expiration date 	<ul style="list-style-type: none"> i) Make sure to use cells that are healthy, adherent, preferentially with low passage rate. ii) Make sure to homogenize the PolyFect solution by inverting or gently tapping the tube (no vortexing). iii) Try a new batch of PolyFect.
52	Absence of amplification using primers P1+P3 (Figure 4)	<ul style="list-style-type: none"> i) Problems of amplification with Terra PCR ii) Absence of recombinant viruses in the population 	<ul style="list-style-type: none"> i) Proceed to troubleshooting for step 100 ii) Repeat transfection. If the lack of recombinant viruses persists, new vectors should be prepared with different homology arms.
67	Similar high production of viruses in samples and negative control	<ul style="list-style-type: none"> i) Too long incubation time of viruses ii) Inefficient drug selection 	<ul style="list-style-type: none"> i) Use low MOI for Step 84 ii) Replace the stock of the selection drug or increase its concentration. Confirm the lack of contamination of wild-type viruses (parental) and negative control by PCR amplification with primers

			annealing on the selection cassette.
67	Apparent lack of viruses in samples	i) Modification introduced is lethal or highly fitness costing ii) Low inoculum of viruses	i) Proceed to genotyping (steps 115-120) to determine the presence or absence of recombinant viruses ii) Use high MOI for Step 84
74	Lack of amplification using primers P1+P3 (Figure 4)	i) Inefficient TERRA amplification ii) Modification introduced is lethal	i) Proceed to troubleshooting 118. ii) New vectors should be prepared with different homology arms in order to exclude dominant negative effects. If still unsuccessful, repeat transfection and selection using trans-complemented amoebas (Steps 120-125). If trans-complementation also fails, RNA silencing would be a more appropriate tool for the study of the gene of interest.
100	Lack of PCR amplification or inconclusive results.	TERRA polymerase is designed to amplify PCR fragments from unpurified templates without the need for extracting genomic DNA, thereby saving time and money. However, the results may not be conclusive, noisy or some PCR product untenable.	Perform genomic DNA extraction of leftover samples (as described in supplementary protocol 34-49), followed by PCR amplification with Phusion DNA polymerase (ThermoFisher Scientific).

1111

1112 **H1 Related links**

1113 **Key references where this protocol has been used:**

1114 Liu, Y. et al. Virus-encoded histone doublets are essential and form nucleosome-like structures. Cell 184,
1115 4237-4250 e4219 (2021). <https://doi.org:10.1016/j.cell.2021.06.032>

1116 Bisio, H. *et al.* Evolution of giant pandoravirus revealed by CRISPR/Cas9. *Nat Commun* **14**, 428 (2023).
1117 <https://doi.org/10.1038/s41467-023-36145-4>

1118 Alempic, J-M. *et al.* No fitness impact of the knockout of the two main components of mimivirus genomic
1119 fiber and fibril layer. *bioRxiv*, 2023.04.28.538727 (2023). <https://doi.org/10.1101/2023.04.28.538727>

1120