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1 **Laboratory growth and genetic manipulation of *Eimeria tenella***

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16 **SIGNIFICANCE STATEMENT**

17
18 The availability of protocols supporting genetic complementation of *Eimeria tenella* has raised the
19 prospect of generating transgenic parasite lines which can function as vaccine vectors expressing and
20 delivering heterologous proteins from other *Eimeria* species, but also from other pathogens of
21 veterinary or zoonotic significance which can infect poultry. Current protocols can also be used to
22 expand biological understanding about the *Eimeria* species through reverse genetics.

24 **KEYWORDS**

25
26 *Eimeria tenella*; transfection; genetic manipulation; transgenic parasites; vaccine delivery vector

28 **ABSTRACT**

29
30 *Eimeria* is a genus of apicomplexan parasites that contains a large number of species, most of which are
31 absolutely host-specific. Seven species have been recognised to infect chickens. Infection of susceptible
32 chickens results in an intestinal disease called coccidiosis, characterised by mucoid or haemorrhagic
33 enteritis, and associated with an impaired feed conversion or mortality in severe cases. Intensive farming
34 practices have increased the significance of coccidiosis since parasite transmission is favoured by high-

35 density housing of large numbers of susceptible chickens. Routine chemoprophylaxis and/or
36 vaccination with live parasite vaccines provides effective control for *Eimeria*, although the emergence
37 of drug resistance and the relative cost and production capacity of current vaccine lines can prove
38 limiting. As pressure to reduce drug use in livestock production intensifies novel vaccination strategies
39 are needed. Development of effective protocols supporting genetic complementation of *Eimeria* species
40 has until recently been hampered by their inability to replicate efficiently *in vitro*. Now, the availability
41 of such protocols has raised the prospect of generating transgenic parasite lines that function as vaccine
42 vectors to express and deliver heterologous antigens. For example, this technology has the potential to
43 streamline the production of live anticoccidial vaccines through the generation of parasite lines that co-
44 express immunoprotective antigens derived from multiple *Eimeria* species. In this paper we describe
45 detailed protocols for genetic manipulation, laboratory growth and *in vivo* propagation of *Eimeria*
46 *tenella* parasites which will encourage future work from other researchers to expand biological
47 understanding of *Eimeria* through reverse genetics.

48

49 **INTRODUCTION**

50

51 Coccidiosis is a common disease caused by apicomplexan parasites of the genus *Eimeria*. To date, more
52 than 1,200 *Eimeria* species have been described infecting birds, mammals, reptiles or amphibians, most
53 with absolute host-specificity. Coccidiosis is a major cause of poor performance and mortality in
54 domestic livestock, with greatest economic significance in production of poultry. Seven *Eimeria* species
55 can infect chickens and co-infection is common. Modern husbandry systems including large numbers
56 of chickens reared at high stocking densities provide an ideal environment for successful parasite
57 transmission (Chapman, 2014; Shirley, Smith, & Tomley, 2005). Current control options include dietary
58 supplementation with anticoccidial drugs and/or vaccination with live parasites, combined with careful
59 husbandry (Blake & Tomley, 2014). However, commercial availability of the existing live attenuated
60 vaccines is constrained by the capacity of their production, which still relies on the use of live chickens
61 (Williams, 1998). Further, the routine use of anticoccidial chemoprophylaxis is increasingly coming under
62 public and legislative spotlights due to the potential, albeit not proven, presence of drug residues in the
63 food chain and environment, and widespread resistance caused by intensive drug use (Chapman, 1997;
64 Jenkins, Parker, & Ritter, 2017).

65 The availability of techniques to transfect exogenous DNA has expanded knowledge on the biology of
66 many apicomplexan parasites such as *Toxoplasma gondii* and some *Plasmodium* species, allowing the
67 dissection of specific gene functions. This has been made possible thanks to the availability of effective
68 *in vitro* systems to obtain and select specific parasite populations, but also knowledge of relevant

69 regulatory DNA sequences (Sibley, Messina, & Niesman, 1994; Soldati & Boothroyd, 1993). In contrast,
70 for many years the inability of *Eimeria* species to efficiently complete their lifecycle *in vitro* and a lack of
71 knowledge about regulatory DNA sequences obstructed development of effective protocols for stable
72 transfection (Kelleher & Tomley, 1998). *Eimeria* species feature an oral-faecal homoxenous lifecycle that
73 involves three phases of replication: schizogony (or merogony), gametogony and sporogony (or
74 sporulation). Sporulated oocysts are the infective transmission stage, containing four sporocysts with
75 two sporozoites in each. When ingested, the sporozoites are released and penetrate epithelial cells of
76 the intestine, where they replicate asexually through several rounds of schizogony, each producing
77 numerous merozoites. This is followed by gametogony, in which merozoites develop into macro and
78 microgamonts and produce macro and microgametes (sexual replication). Fertilisation of
79 macrogametes by biflagellated microgametes results in the production of oocysts (zygotes) that are
80 excreted in the faeces. Once in the environment, unsporulated oocysts undergo sporogony and form
81 sporocysts containing infective sporozoites.

82 Current advances in genetic manipulation of sporozoites from different *Eimeria* species have now
83 allowed the generation of stable populations expressing specific selectable markers (see Figure 1 for
84 outline) (Clark et al., 2008; Yan et al., 2009). Access to high-throughput next-generation genomic and
85 transcriptomic sequencing data for *Eimeria* parasites can now be exploited towards the identification of
86 specific promoters capable of inducing low to high transcription levels of transfected gene(s) in a
87 constitutive or stage-specific manner (Marugan-Hernandez, Long, Blake, Crouch, & Tomley, 2017; Reid
88 et al., 2014). The development of these protocols has prompted the idea to utilise *Eimeria* parasites (e.g.
89 *Eimeria tenella*) to express immunoprotective antigens from other *Eimeria* species (e.g. antigen A from
90 *Eimeria maxima* and antigen B from *Eimeria necatrix*) as live vaccines, thus inducing immunity against
91 the vector (*E. tenella*) and the antigen donors (*E. maxima* and *E. necatrix*). This approach could streamline
92 commercial vaccine formulations from up to eight *Eimeria* species/strains, due to the lack of cross-
93 protective immunity, to just a few lines expressing relevant antigens from all other species (Blake &
94 Tomley, 2014). The toolbox would also allow expression of additional antigens directed against other
95 pathogens which affect poultry, even modifying their trafficking within the parasite to target antigen
96 exposure to the host immune system (Clark et al., 2012; Marugan-Hernandez et al., 2016; Marugan-
97 Hernandez et al., 2017; Pastor-Fernandez et al., 2018; Tang et al., 2017).

98 Herein we discuss the current limitations of *in vitro* and *in vivo* propagation, as well as transfection when
99 applied to *Eimeria* spp. and the key factors determining transfection success including plasmid
100 development and methods to improve rates of DNA integration. We provide detailed protocols for
101 genetic manipulation, laboratory growth and *in vivo* propagation of *Eimeria tenella* parasites which can
102 potentially be used to expand knowledge about *Eimeria* spp. through reverse genetics.

103 **STRATEGIC PLANNING**

104

105 **Parasite species/strain selection**

106

107 Of the seven *Eimeria* species that infect chickens, *E. tenella* is most effective at invading, replicating and
108 developing *in vitro* in a range of primary cells and established cell lines (most notably the *E. tenella*
109 Wisconsin strain) (Doran, 1974). Nevertheless, efficient *in vitro* development is still limited to the early
110 asexual stages, failing to support efficient production of oocysts (Bussi re et al., 2018). In addition, *E.*
111 *tenella* preferentially replicates in the caeca, which simplifies its harvest from caecal scrapes instead of
112 faecal samples (Eckert J., 1995). This approach also reduces the amount of clinical waste generated
113 during parasite maintenance and simplifies adherence to relevant regulations regarding genetically
114 modified organisms.

115

116 **Chickens and Facilities**

117

118 High quality *Eimeria* oocysts can only be obtained by *in vivo* passage using coccidia-free chickens.
119 However, different chicken breeds/strains display different susceptibility to infection by each *Eimeria*
120 species. We recommend to perform initial studies to determine the oocyst/sporozoite dose that
121 provides maximum oocyst yield with minimum pathology for each species of interest (Bumstead &
122 Millard, 1992; Smith, Hesketh, Archer, & Shirley, 2002). The use of chickens from a specific pathogen
123 free (SPF) flock is not necessary as long as chicks are reared under coccidia-free conditions from hatch
124 onwards. Food rations should be bought from suppliers of high quality diets for laboratory animals, and
125 always free of anticoccidial drugs unless required for selection of resistance (the use of medicated feed
126 is common within the poultry industry). National and international animal welfare regulations on
127 housing, husbandry and care of animals should be considered.

128

129 **Safety Concerns**

130

131 *Eimeria* parasites are enzootic wherever chickens are farmed, ranked in the lowest risk group by the
132 Department for the environment, fisheries and rural affairs (Defra, UK), and not zoonotic. However, while
133 biological containment is not required it is beneficial to work under containment level 2 conditions,
134 including lab coats and disposable gloves, to maintain biological purity of parasite lines. Access to
135 isolated ventilation systems that allow fumigation with ammonia is beneficial.

136 Generation of transgenic parasite lines expressing foreign proteins and/or resistance to medically
137 important drugs should be carefully managed, since these parasites should not be released to the

138 environment. Once transfected, parasites must be considered as genetically modified organisms (GMO),
139 and therefore, relevant local and national regulations must be reviewed and followed including waste
140 disposal.

141

142 **Handling of Infectious Oocysts**

143 Sporulated oocysts can be stored in PBS (see Reagents & Solutions), water, or 2% potassium dichromate
144 at ~4 °C, ideally in glass, and used for up to six months.

145

146 **Development of constructs for transfection**

147

148 Success of *Eimeria* transfection relies on the design of appropriate constructs, the use of specific
149 electroporation protocols, and the purification of fresh and viable parasites. Transfection constructs are
150 modifications of commercially available plasmids that are subsequently complemented with a variety of
151 elements which may encode regulatory sequences, the coding sequence(s) of interest, fluorescent
152 reporters, drug markers, etc. To date all published transfection plasmids support ampicillin selection,
153 indicating they can be easily propagated using competent *Escherichia coli* cells, and purified using a
154 broad range of commercial kits (from miniprep to gigaprep, depending on the size of the bacterial
155 culture and corresponding plasmid yield).

156

157 **a) Selection of regulatory regions to promote heterologous expression**

158 A number of 5' and 3' endogenous regulatory regions have been successfully identified from *Eimeria*
159 genomes and used to drive expression of different exogenous genes (normally fluorescent reporters),
160 most commonly in *E. tenella*. These include promoters from constitutively expressed genes, such as
161 actin, beta tubulin and histone H4, but also other regulatory regions from genes expressed during
162 specific stages of the parasite lifecycle such as those encoding microneme proteins (MIC) 1, 2, 3 and 5,
163 and the surface antigen (SAG) 13 from the zoite stages, as well as the Gam56 protein from the sexual
164 stages (Clark et al., 2008; Hanig, Entzeroth, & Kurth, 2012; Hao, Liu, Zhou, Li, & Suo, 2007; Kelleher &
165 Tomley, 1998; Kurth & Entzeroth, 2009; Marugan-Hernandez et al., 2016; Marugan-Hernandez et al.,
166 2017; Tang et al., 2017). Thus, the choice of promoter can determine whether the transgene is expressed
167 throughout the entire parasite lifecycle, or is restricted to specific lifecycle stages. Interestingly,
168 promoters from *T. gondii* involved in the expression of the *Tgtubulin* and *Tgsag1* genes have also been
169 used effectively to induce the expression of fluorescent reporters in transfected *E. tenella* parasites (Zou
170 et al., 2009), and the same has been described for the *Etactin*, *Etmic1* and *Etgam56* promoters in
171 transfected *E. nieschulzi* parasites (Hanig et al., 2012; Kurth & Entzeroth, 2009). Recent transcriptome
172 analysis of *E. tenella* has made available the relative transcript abundance from different stages of its

173 lifecycle, demonstrating that some of these genes are transcribed at lower or higher levels relative to
174 others (Reid et al., 2014; Walker et al., 2015). These data can be now exploited to test new putative
175 promoter regions and induce higher levels of expression of the gene of interest in transgenic
176 populations.

177 For this purpose, we selected nine genes with constitutive or varied stage-specific expression that are
178 transcribed at high or medium levels to produce a flexible tool kit, cloned their putative 5' regulatory
179 regions, and tested their ability to induce higher levels of expression of the mCitrine reporter in *E. tenella*
180 transfected sporozoites (Table 1). Transfection with the promoter region *Et8*, which regulates expression
181 of the Translation Initiation Factor (TIF), induced the highest fluorescence levels in transgenic parasites,
182 which were comparable to those obtained in sporozoites transfected with mCitrine under the control of
183 the *Etmic1* promoter. For this reason, we selected this promoter to drive expression of foreign genes in
184 *E. tenella* (Marugan-Hernandez et al., 2016). Similarly, transfection with the promoter region *Et9* induced
185 high levels of fluorescence in transgenic sporozoites, and this was comparable to the levels achieved by
186 transfecting sporozoites with mCitrine under the control of the *Etactin* promoter (Table 1).

187

188 **b) Use of markers for selection of transgenic *Eimeria***

189 Transfected parasites can easily be identified and selected by the inclusion of reporter genes within
190 transfection constructs. The first report describing the transient transfection of *E. tenella* employed the
191 beta-galactosidase gene as a reporter (Kelleher & Tomley, 1998), but due to ease of use all subsequent
192 studies have opted to use fluorescent proteins. The expression of these reporters permits not only
193 assessment of the efficiency of transfection by simple microscopic visualization, but also allows the
194 selective isolation of transgenic (fluorescent) oocysts by flow-activated cell sorting (FACS) from progeny
195 individuals (those obtained after infecting chickens with transfected sporozoites) and verification of the
196 expression and localisation of a protein of interest when it is fused to a reporter protein (Clark et al.,
197 2008; Marugan-Hernandez et al., 2016; Marugan-Hernandez et al., 2017; Pastor-Fernandez et al., 2018;
198 Yan et al., 2009). Notably, transfection efficiency does not seem to be affected by the choice of different
199 fluorescent reporters (mCherry, tdTomato, mCitrine, YFPmYFP or AmCyan) in *E. tenella* (Clark et al.,
200 2008).

201 In addition to reporter proteins, the use of drug-resistance genes also provides an advantage for
202 positive selection of transgenic *Eimeria*. For example, pyrimethamine is a drug approved to treat
203 toxoplasmosis and some forms of malaria, but it is also effective against *Eimeria*. This compound inhibits
204 dihydrofolate reductase–thymidylate synthase (DHFR-TS), a key enzyme required for the synthesis of
205 DNA and proteins in protozoa. Earlier experiments carried out in *T. gondii* resulted in the generation of
206 the DHFR-TSm2m3 gene, a mutated form which codes for a version of the enzyme that is not inhibited
207 by pyrimethamine treatment (Donald & Roos, 1993). Transfection of the DHFR-TSm2m3 gene coupled

208 with subsequent dietary pyrimethamine supplementation has been proven to be an efficient way to
209 obtain stable transgenic populations of *Eimeria* parasites (Clark et al., 2008; Pastor-Fernandez et al.,
210 2018; Qin et al., 2016; Tang et al., 2017; Yan et al., 2009). Furthermore, dual selection approaches by
211 transfection with genes coding for a fluorescent reporter and pyrimethamine resistance (either in a
212 single or in two different plasmids) has been found to speed up the generation of stable populations
213 (Clark et al., 2008; Hanig et al., 2012). However, the generation of strains resistant to medically important
214 drugs should be carefully managed and limited to experimental uses, since these parasites should not
215 be released to the environment (see Safety Concerns).

216

217 **Methods for the optimisation of DNA integration**

218

219 **a) Restriction enzyme mediated integration**

220 Transfection efficiency in *Eimeria* spp. has been greatly improved by the use of restriction enzyme
221 mediated integration (REMI) techniques (Clark et al., 2008; Kurth & Entzeroth, 2009; Liu et al., 2008). This
222 method relies on linearisation of the transfection plasmid with a restriction enzyme combined with the
223 addition of the same enzyme to the transfection mix before shock, apparently improving plasmid
224 integration into the genome at open sites that have been generated by the endonuclease (Schiestl &
225 Petes, 1991). This results in a largely random and heterologous integration of the plasmid into the
226 parasite genome. Using *E. tenella* as a model, Liu and colleagues demonstrated that transfection
227 efficiency was considerably higher in sporozoites transfected with linearised plasmids (increased by
228 6,900 %) and PCR amplicons (increased by 2,490 %), compared to non-linearised plasmids. In addition,
229 they showed that co-transfection with the restriction enzyme used for plasmid digestion also increased
230 transfection efficiency by 215 % for linearised plasmids and 37 % for PCR amplicons (Liu et al., 2008).
231 Therefore, we always opt for the combination of linearised plasmids with their respective restriction
232 enzymes to obtain high frequency integration. Nevertheless, it has been shown that REMI performance
233 also depends on the starting amount of DNA used and the choice of restriction enzyme employed for
234 transfection, as discussed below.

235

236 a.1) Effect of plasmid concentration and size on transfection efficiency

237 To date, transfection of *E. tenella* sporozoites has been performed using a broad range of plasmid
238 concentrations (Clark et al., 2008; Liu et al., 2013; Yan et al., 2009). However, these studies have not
239 directly evaluated the effect of DNA starting concentration on transfection efficiency, which has now
240 been found to have a dose dependent effect. Experiments completed in our group with *E. tenella*
241 sporozoites transfected with 1.5 to 12 µg of the p*Eten*REPORTER plasmid that confers green
242 fluorescence to the transfected parasites (p5'UTR-EtMIC1_mCitrine_3'UTR-Actin, from (Clark et al.,

243 2008)) have shown that the greater the concentration of DNA, the higher the percentage of fluorescent
244 oocysts generated after transfection and *in vivo* passage (Figure 2).

245 We previously described a negative correlation between plasmid size and efficiency of transfection:
246 increasing the size of a given plasmid by 33–50% resulted in a decreased efficiency of transient
247 transfection between 10- and 25-fold. Since FACS enrichment of progeny oocysts has been shown to
248 be efficacious for selection of transfected lines, constructs containing a single fluorescent reporter is
249 adequate for many studies and reduces the impact of construct size (Clark et al., 2008).

250

251 a.2) Effect of restriction enzyme choice on transfection efficiency

252 The first examples of REMI using *Saccharomyces cerevisiae* and *Dictyostelium* spp. hypothesized that the
253 mechanism of transgene integration was based on a simple ligation-repair model (Kuspa & Loomis,
254 1992; Schiestl & Petes, 1991). However, a later study reported that transfection efficiency of *T. gondii*
255 was dependent on the restriction enzyme used, and that the same enzyme does not need to be used
256 for linearisation and electroporation (Black, Seeber, Soldati, Kim, & Boothroyd, 1995). In order to assess
257 this in *Eimeria* spp., we analysed *in silico* the p*Eten*REPORTER plasmid (Clark et al., 2008) to find single
258 restriction sites not present within promoter or coding regions, and selected *PsiI* and *ScaI* enzymes for
259 REMI. *In silico* digestions of the *E. tenella* genome showed a difference of 6.7% in the number of sites
260 per genome for both enzymes (*PsiI* = 7547; *ScaI* = 7075). Of these sites, there was a 20% reduction of
261 *PsiI* in predicted coding regions compared to *ScaI* (*PsiI* = 1775, *ScaI* = 2220), which suggests a greater
262 risk of coding sequence disruption for *ScaI* compared to *PsiI*. Subsequently, *PsiI* and *ScaI* enzymes were
263 used to linearize the p*Eten*REPORTER plasmid and added to the transfection reaction prior to
264 electroporation (0.5 U/condition). A total of 5 µg and 3.5 µg of *PsiI* and *ScaI*-digested plasmids were
265 used to transfect freshly purified sporozoites. For all plasmid starting concentrations, *PsiI*-transfected
266 populations resulted in a higher proportion of transgenic parasites, a higher average fluorescence
267 intensity and greater parasite survival, supporting our *in silico* findings (Figure 3). These results highlight
268 the importance of careful selection and testing of restriction enzymes for transfection.

269

270 **b) Transfection technologies**

271 Effective transfection of *Eimeria* parasites has been achieved by electroporation (BTX or BioRad's
272 GenePulser™ systems) or nucleofection (AMAXA™ system, Lonza), utilising a wide range of conditions
273 and buffers. However, the switch from electroporation to nucleofection technology, which allows DNA
274 to enter directly into the nucleus, has improved the poor transfection rates reported earlier (Clark et al.,
275 2008; Hao et al., 2007; Kelleher & Tomley, 1998; Marugan-Hernandez et al., 2016). When compared to
276 BTX electroporation, use of the AMAXA™ Nucleofector II system improved efficiency of transient *E.*
277 *tenella* transfections up to 3.6-fold using a cytomix buffer and preset program U-33. This efficiency was

278 directly comparable with that noted for the closely related *T. gondii* (Clark et al., 2008). Due to the
279 availability of new and improved nucleofection systems, optimal conditions for *E. tenella* transfection
280 using the AMAXA™ 4D-Nucleofector™ System, the 16-well Nucleocuvette™ Strips, and a range of
281 transfection buffers have been standardised here (Table 2). The combination of P3 buffer and program
282 EO-114 successfully generated transfected sporozoites. Besides, the use of a 16-well strip system
283 provided ideal conditions for screening multiple constructs and transfection conditions at the same
284 time, using relatively small numbers of parasites.

285

286 **c) Stability of the transgenes**

287 The experiments carried out in our group using transgenic populations obtained by REMI transfection
288 and nucleofection, and successively propagated in chickens using FACS selection, have shown a dramatic
289 decrease in transgene insertion numbers after two passages (Figure 4A). We observed similar results in
290 parasites propagated in chickens using FACS and pyrimethamine selection, which were phenotypically
291 stable after three passages (100% fluorescent oocysts after caecal harvest) and displayed an average
292 insertion of 10-15 transgene copies per *E. tenella* genome (Clark et al., 2008). Interestingly, *in vivo*
293 propagation seemingly helped to stabilise these populations, since average transgene copy numbers
294 tend to decrease, whereas percentage of transgenic parasites tends to increase by successive passage
295 (Figure 4B). This suggests that there is a selection pressure against parasites expressing higher numbers
296 of transgenes, and therefore, more passages would be needed to obtain stable integration of exogenous
297 DNA.

298

299 **BASIC PROTOCOL 1: Cracking, hatching and purification of Eimeria tenella parasites**

300

301 The protective walls of the oocyst and sporocyst makes direct transfection impossible using current
302 technologies. To date, sporozoites present in these structures need to be released through physical,
303 chemical and enzymatic disruption (cracking and hatching), and subsequently purified using columns
304 based on anion exchange chromatography. Purified sporozoites are only viable for a short period of
305 time, as they are required to invade host cells to carry on with their cycle. Thus, the cracking and hatching
306 must be followed by transfection and subsequent *in vitro* (cell culture) or *in vivo* (chicken) infection. In
307 this protocol we describe the necessary steps to release sporozoites from *E. tenella* oocysts, and clean
308 them up from oocyst and sporocyst debris in readiness for transfection.

309

310

311

312 Materials

- 313 - Sporulated oocysts of *E. tenella* in H₂O or PBS (produced in-house, see BASIC PROTOCOL 3: *In vivo*
- 314 *propagation of transgenic E. tenella*).
- 315 - Sterile saturated salt solution (SSS, see Reagents & Solutions).
- 316 - Sterile phosphate buffered saline (PBS, see Reagents & Solutions).
- 317 - Sterile diethylaminoethyl cellulose (see Reagents & Solutions).
- 318 - Hatching solution (see Reagents & Solutions).
- 319 - 1M MgCl₂ solution in ultrapure water.
- 320 - Eluting buffer (PBS-1% glucose, see Reagents & Solutions).
- 321 - Ballotini SiLibeads® solid soda glass beads, 0.4-0.6 mm diameter (Catalogue No: 201-0465, VWR).
- 322 - 20 mL-disposable syringes (slip tip).
- 323 - Sterile Erlenmeyer flask (borosilicate glass, narrow neck, 250 ml, catalogue No: 1130/14D, Pyrex).
- 324 - Nylon wool fiber (Catalogue No: 18369, Polysciences Inc).
- 325 - Set of two pet slicker brushes with fine wire.
- 326 - Micropipettes, tips, pipette controller and serological pipettes.
- 327 - Swing bucket centrifuge.
- 328 - Vortex mixer.
- 329 - Optical microscope.
- 330 - Water bath.
- 331 - Small measuring cylinders or retort stands.
- 332 - Parafilm.
- 333 - Cell counting chambers (modified Fuchs Rosenthal, catalogue No: AC6000, Hawksley).

334

335 Procedure

- 336 1. Using a cell counting chamber (modified Fuchs Rosenthal), estimate the concentration of the oocyst
- 337 stock by loading 10 µl of the culture per chamber and counting them at 10X/10X magnification
- 338 under the optical microscope. If oocysts concentration is too high to count, dilute the stocks in H₂O
- 339 or PBS (1:10 to 1:100) until parasite numbers are adequate for an accurate count, correcting the
- 340 final concentration accordingly.
- 341 2. Pellet 10-50x10⁶ oocysts using a swing bucket centrifuge (750 x g for 10 min) in a 50 ml universal
- 342 tube, and re-suspend in 3 ml of PBS. If more parasites are required, prepare them in a different 50
- 343 ml universal tube.
- 344 3. Add the glass beads (1:1 proportion, oocysts suspension:beads) and crack parasites by vortexing
- 345 for 15 sec bursts until most of the oocysts have released their sporocysts. Check oocysts
- 346 microscopically between bursts to ensure sporocyst release. Repeat this process as many times as

347 necessary, aiming to get a good balance between breakage of oocyst walls and maintenance of
348 sporocyst integrity.

349 *If damaged sporocysts or released sporozoites can be seen, cracking should stop as excessive cracking*
350 *will reduce the final yield of purified sporozoites (see Figure 5A).*

351 *When very fresh oocysts are employed, wall cracking may be difficult. In these cases, resuspension of*
352 *oocysts in 1.2% sodium hypochlorite solution (prepared from 12% sodium hypochlorite solution,*
353 *Catalogue No: 301696S, VWR Chemicals) for 10 minutes, followed by three PBS washes by*
354 *centrifugation (750 x g for 10 min), may help to soften the oocyst walls and improve cracking*
355 *efficiency.*

356 4. Transfer the resulting sporocyst/oocyst/debris suspension to a 250 ml Erlenmeyer flask by washing
357 the beads by pipetting with 40 ml of hatching solution.

358 5. Incubate the sporocyst/oocyst/debris suspension for 30 min at 41 °C in a water bath, and check the
359 hatching progress microscopically.

360 6. Supplement hatching solution with 1 M MgCl₂ to a final concentration of 10 mM (500 µl 1 M MgCl₂
361 per 50 ml of hatching solution added).

362 7. Incubate the sporocyst/oocyst/debris suspension for an additional 1 h at 41 °C. Mix gently every 30
363 min and check hatching progress microscopically.

364 8. Prepare a suitable number of purification columns during the parasite excystation (Figure 5). One
365 20 ml syringe column is sufficient for up to ~50x10⁶ oocysts.

366 a. Use two pet slicker brushers to tease out small bunches of nylon wool and remove any knots
367 by brushing.

368 b. Fill the syringe barrel with the teased-out wool to a depth of 5 ml. Gently push the wool to the
369 bottom using the plunger.

370 *The use of non-teased out nylon wool and/or its excessive compression within the column may*
371 *result in lower recovery yields.*

372 c. Place the column on a measuring cylinder or in a retort stand and wash through nylon wool
373 with 10 ml of eluting buffer.

374 d. Before all the eluting buffer has run out, pour sterile DE-52 on top of the nylon wool up to a 10
375 ml depth and allow the excess fluid to drain out. Ensure that the column does not dry.

376 e. Wash column through with 20 ml of eluting buffer and plug syringe nozzle with parafilm. Make
377 sure that there is still a little excess of buffer in the column to prevent drying out.

378 9. Once hatching has finished, enumerate the total number of sporozoites using a cell counting
379 chamber.

- 380 10. Pellet sporozoites in a 50 ml universal tube using a swing bucket centrifuge (500 x g for 10 min),
381 remove hatching solution carefully using a serological pipette, and re-suspend pellet in 30 ml of
382 elution buffer. Check microscopically that the supernatant does not contain non-pelleted
383 sporozoites, try to recover them by centrifugation, and re-suspend pellet in elution buffer if
384 necessary.
- 385 11. Gently pour the sporozoite suspension into the top of a separation column, transfer column to a
386 new 50 ml universal tube, and unplug.
- 387 12. Collect at least 50 ml in different fractions from each column by topping up regularly with eluting
388 buffer. Since the wall debris tends to pellet and block the column, regularly use a 1 ml serological
389 pipette to gently stir the interphase between the DE-52 and the sporozoites suspension.
- 390 13. Monitor eluate microscopically by taking droplets on microscope slides from every fraction
391 recovered. Once sporozoite numbers are reduced, residual parasites can be 'pushed' through the
392 column by replacing the plunger and exerting pressure gently.
- 393 *Pushing too hard can result in debris and sporocysts coming through.*
394 *If contamination with DE-52, sporocysts, or any other debris is considered to be excessive the relevant*
395 *fraction can be passed through a fresh column or discarded.*
- 396 14. Pellet sporozoites in a 50 ml universal tube using a swing bucket centrifuge (500 x g for 10 min),
397 resuspend in fresh PBS and enumerate parasite numbers using a cell counting chamber. Compare
398 starting and final numbers of sporozoites to estimate percentage of recovery and identify issues
399 related to the column purification.
- 400 15. Once excysted, sporozoites should be handled with care, and their infectivity will drop after 1-2
401 hours.

402

403 **BASIC PROTOCOL 2: Transfection of *E. tenella* sporozoites**

404

405 This protocol describes the methods for successful transfection of freshly purified *E. tenella* sporozoites
406 using REMI and nucleofection approaches. Other relevant factors such as plasmid preparation are also
407 discussed. The protocol is optimized for transfection of 1×10^6 sporozoites with 12 μg of linearized
408 plasmid and 6 units of the chosen restriction enzyme. The Nucleocuvette™ Strips allow up to 16
409 independent transfections in a single assay, so different constructs, restriction enzymes, numbers of
410 parasites, and DNA starting concentrations can be assessed to optimize specific needs in a single
411 experiment.

412

413

414

415 Materials

- 416 - Freshly hatched *E. tenella* sporozoites (see *BASIC PROTOCOL 1: Cracking, hatching and purification*
417 *of Eimeria tenella parasites*).
- 418 - Linearized transfection constructs (see *Development of constructs for transfection* and *Effect of*
419 *plasmid concentration and size on transfection efficiency*).
- 420 - Selected restriction enzyme for REMI (see *Effect of restriction enzymes on transfection efficiency*).
- 421 - Micro-volume Spectrophotometer.
- 422 - Amaxa™ 4D-Nucleofector™ (core and X units for nucleofection in suspensions, catalogue No: AAF-
423 1002B and AAF-1002X, Lonza).
- 424 - Amaxa™ P3 Primary Cell 4D-Nucleofector™ X Kit (includes transfection buffer and 16-well
425 Nucleocuvette™ Strips, Catalogue No: V4XP-3032, Lonza) (see *Transfection technologies*).
- 426 - CO₂ incubator.
- 427 - Roswell Park Memorial Institute (RPMI)-1640 medium with L-glutamine, sodium bicarbonate and
428 phenol red (Catalogue No: R8758, Sigma-Aldrich).
- 429 - 0.4% Trypan Blue solution (Catalogue No: T8154, Sigma-Aldrich).
- 430 - Flat bottom 96-well cell culture plate (Catalogue No: 266120, Nunc).
- 431 - Micropipettes, tips, pipette controller and serological pipettes.
- 432 - Cell counting chambers (modified Fuchs Rosenthal).

433

434 Procedure

- 435 1. Linearized plasmids for REMI should be ready in advance to avoid unnecessary delay in transfection
436 and consequent loss of sporozoite viability:
- 437 a. Plasmids are commonly propagated using *E. coli* competent cells (e.g. XL1-Blue strain, from
438 Agilent), purified using column-based kits (e.g. Plasmid Midi Kit from QIAGEN, for up to 100 µg
439 of plasmid DNA), quantified using a spectrophotometer, and digested overnight using
440 restriction enzymes that cut them in a single site upstream of the expression cassette (e.g. *Pst*I,
441 digesting in the region upstream the *EtMic1* promoter fused to the mCitrine reporter gene as
442 in Clark et al., 2008).
- 443 b. In order to concentrate the plasmid and avoid interference of digestion buffer with transfection
444 buffer, digested plasmids need to be cleaned up by precipitation following these steps:
- 445 i. Add to the digestion mix 1/10 volumes of 3M sodium acetate and 3 volumes of
446 absolute ethanol.
- 447 ii. Incubate sample at -20 °C for 30 min to 16 hours. Then, centrifuge at 14,000 x g for 30
448 min at 4 °C.
- 449 iii. Discard supernatant and wash pellet with 70% (v/v) ethanol.

- 450 iv. Centrifuge at 14,000 x g for 30 min at 4 °C.
- 451 v. Discard supernatant and re-suspend pellet with transfection buffer (see below). The
452 final volume will depend on the amount of plasmid subjected to precipitation, but the
453 ideal concentration is 2-4 µg/µl.
- 454 2. Pellet freshly purified sporozoites in a 50 ml universal tube using a swing bucket centrifuge (500 x
455 g for 10 min), discard eluting buffer, and re-suspend parasites in the appropriate volume of P3
456 buffer (20 µl per 1x10⁶ sporozoites).
- 457 *In most cases, the use of fresh oocyst stocks and an adequate cracking, hatching and column*
458 *purification will yield the best transfection efficiencies. In fact, we have previously shown an inverse*
459 *correlation between parasite age and transfection efficiency (Clark et al., 2008).*
- 460 3. Add 1x10⁶ sporozoites, 12 µg of the transfection construct(s) and 6 units of the restriction enzyme
461 P3 buffer per well (final volume should not exceed 25 µl). If more than one plasmid is to be
462 transfected, consider Avogadro's constant to transfect equal copies of both plasmids (1 bp =
463 660x10⁶ µg/mole; 1 mole = 6.023x10²³ copies).
- 464 4. Dispense the appropriate volume into each well of the Nucleocuvette™ Strips, select the preset EO-
465 114 program from the Amaxa™ 4D-Nucleofector™ and start the nucleofection as indicated by the
466 manufacturer.
- 467 *Nucleofection conditions and buffer composition are part of Lonza's intellectual property, and*
468 *therefore these cannot be modified. Other factors affecting transfection success are discussed in detail*
469 *in the Methods for the optimisation of DNA integration section.*
- 470 *Inclusion of mock transfected parasites (electroshocked with no DNA), and parasites transfected with*
471 *previously tested plasmids can be useful to validate the nucleofection and determine the source of the*
472 *issue, if any.*
- 473 *Once transfected, sporozoites are considered as genetically modified organisms (GMO), and therefore,*
474 *relevant legislations need to be reviewed and followed.*
- 475 5. Once shocked, add 80 µl of RPMI-1640 medium per well, and let the parasites stand for 15 min.
- 476 6. Estimate survival rate by Trypan Blue exclusion using the cell counting chamber. Non-viable
477 parasites whose membrane permeability is altered will take up the dye, turning blue. In our
478 experience, survival rates ranging from 10 to 20% are considered adequate.
- 479 7. Use the transfected parasites instantly for further *in vivo* or *in vitro* experiments. Alternatively,
480 sporozoites can be transferred to 96-well plates in RPMI-1640 medium (~100 µl/well), left for 24-
481 48 h in an incubator (41 °C, 5% CO₂) to assess transfection efficiency under a fluorescence
482 microscope.

483

484 **BASIC PROTOCOL 3: In vitro propagation of *E. tenella***

485

486 *Eimeria tenella* cannot complete its lifecycle *in vitro* efficiently, where its growth is confined to the early
487 asexual stages of the parasite. Infection of cultured cells with purified sporozoites results in the
488 development of first generation merozoites, but further progression to the second and third generation
489 of merozoites and the subsequent gametogony is rarely observed in most systems. Moreover, it has
490 been described that some strains (e.g. Wisconsin) are better adapted to cell culture and their replication
491 is higher under this system (Doran, 1974). Earlier studies have shown that many different epithelial cell
492 lines support development of *E. tenella*, but Madin-Darby bovine kidney (MDBK) cells appear to be best
493 suited (Tierney & Mulcahy, 2003). The present protocol describes the cultivation of *E. tenella* asexual
494 stages in MDBK cells in order to assess transfection success by analysis of reporter gene expression.

495

496 Materials

- 497 - MDBK (NBL-1) cells (Catalogue No: CCL-22, ATCC).
498 - Freshly hatched sporozoites (see BASIC PROTOCOL 1: *Cracking, hatching and purification of Eimeria*
499 *tenella* parasites section).
500 - Advanced Dulbecco's Modified Eagle Medium (DMEM) (Catalogue No: 12491023, Gibco)
501 supplemented with penicillin and streptomycin (100 U/ml each) (Catalogue No: 15140122, Gibco).
502 - 0.025% Trypsin/0.01% EDTA solution (Catalogue No: R001100, Gibco).
503 - Foetal bovine serum (FBS), heat inactivated.
504 - Laminar flow hood.
505 - CO₂ incubator.
506 - Cell culture 24-well plates (Catalogue No: 140685, Nunc) and T75 flasks (Catalogue No: 156499,
507 Nunc).
508 - Micropipettes, sterile tips, pipette controller and serological pipettes.

509

510 Procedure

- 511 1. MDBK cells can be passaged twice a week, using a subcultivation ratio of 1:2 to 1:4, and a 0.025%
512 Trypsin/0.01% EDTA solution to detach adherent cells from the vessel.
513 2. Dispense cells into 24 well culture plates (0.3x10⁶ cells per well in 500 µl of advanced DMEM-2%
514 FBS) and incubate at 41 °C, 5% CO₂ for at least 2 hours before infection.

515 *Recently settled and near confluent monolayers improve invasion rates and are critical for the*
516 *experiment success. It is important to ensure that the cells are in an optimal condition before the*
517 *experiment by tracking the cultures a few days in advance.*

- 518 3. Prepare transfected sporozoites in a suspension of 0.3×10^6 parasites per ml in advanced DMEM-2%
519 FBS. Do not keep sporozoites on ice, as this will reduce the degree of invasion achieved.
520 *A multiplicity of infection (MOI) 1:1 is recommended to follow the parasite lifecycle, but this can be*
521 *modified based on the experimental requirements. Bear in mind that the use of a MOI of 4:1 may*
522 *result in the destruction of the cell monolayer in less than 24 h.*
- 523 4. Carefully remove medium from cells and add 1 ml of sporozoite suspension per well.
- 524 5. Allow sporozoites to infect the monolayer at 41 °C, 5% CO₂ for 2-4 h, by which time maximum
525 invasion will have occurred. Carefully remove medium and replace with fresh advanced DMEM-2%
526 FBS.
527 *If parasites are left invading for longer than 4 hours these could overload the monolayer causing its*
528 *destruction within 24 hours.*
- 529 6. Incubate plates at 41 °C for 24 for the observation of invaded sporozoites, or for 48 hours for the
530 observation of schizogony and merozoite formation/release.
- 531 7. The presence of fluorescent reporters in transgenic parasites can be followed and quantified by
532 fluorescence microscopy (Figure 6).
533 *Analysis of fluorescence can be directly done in fresh or fixed monolayers on 24-well plates using an*
534 *inverted microscope (up to 40x). If higher magnification is needed (up to 100x), cells can be seeded*
535 *onto rounded coverslips placed in 24-well plates, fixed, and placed on microscope slides with mounting*
536 *medium.*

537

538 **BASIC PROTOCOL 4: In vivo propagation of transgenic *E. tenella***

539

540 *In vivo* infection with transfected sporozoites results in the propagation of mainly wild-type parasites
541 that did not integrate the coding sequence (construct) of interest, but also a small percentage of
542 sporozoites that integrated the DNA construct and will generate transgenic oocysts during *in vivo*
543 propagation (Figure 1). If selection markers such as drug resistance genes and/or fluorescent reporters
544 are used, transgenic oocysts can be easily enriched by dietary drug supplementation and/or flow
545 cytometry (see *Use of markers for selection of transgenic Eimeria*), and subsequently propagated *in vivo*
546 (see *Stability of the transgenes*). This protocol describes the amplification of transgenic *E. tenella*
547 populations in chickens, involving cloacal dosing, caecal harvest and sporulation of progeny oocysts,
548 selection of transgenic parasites, and stabilization of transgenic populations.

549

550 Materials

- 551 - Transfected sporozoites.

- 552 - Coccidia-free chickens, 3-4 weeks old. All experiments must be approved by the appropriate local
- 553 or national ethical review boards.
- 554 - Floor wire cages, ideally previously fumigated with ammonia.
- 555 - Water bath.
- 556 - Swing bucket centrifuge.
- 557 - Orbital shaker.
- 558 - Flow cytometer.
- 559 - 1 ml syringes.
- 560 - Fine tipped Pasteur pipettes.
- 561 - Silicon tubing (~4 cm length, 2 mm diameter).
- 562 - 40 µm cell strainers (Catalogue No: 431750, Corning).
- 563 - Dissecting tools.
- 564 - Micropipettes, sterile tips, pipette controller and serological pipettes.
- 565 - Cell counting chambers (modified Fuchs Rosenthal, catalogue No: AC6000, Hawksley).
- 566 - Sterile baffled Erlenmeyer flask (borosilicate glass, narrow neck, 1,000 ml, catalogue No: 1134/12,
- 567 Pyrex).
- 568 - Centrifuge bottles.
- 569 - Parafilm.
- 570 - Distilled water.
- 571 - Pyrimethamine (Catalogue No: 46706, Sigma-Aldrich).
- 572 - Sterile phosphate buffered saline (PBS), pH 8 (see Reagents and solutions).
- 573 - Trypsin from porcine pancreas (Catalogue No: T4799, Sigma-Aldrich).
- 574 - Sterile 2% potassium dichromate (supplied at 6% dilute down to 2% for use).
- 575 - Sterile saturated salt solution (SSS) (see Reagents and solutions).
- 576 - 1.2% sodium hypochlorite solution (from 12% stock solution, catalogue No: 301696S, VWR
- 577 Chemicals).

578

579 Procedure

- 580 1. Withdraw feed from cages 3 h before cloacal dosing to prevent transgenic sporozoites being
- 581 expelled with droppings.
- 582 2. Gently dose birds with transfected sporozoites via the cloaca (up to 150,000 live parasites per bird,
- 583 diluted in up to 500 µl of RPMI-1640). A dosing catheter can be crafted with a 1 ml syringe coupled
- 584 to silicone tubing, using the cut off end of a fine tipped Pasteur pipette as an adaptor between the
- 585 syringe and the silicone tubing. Once dosed, chicken feed can be replaced in cages.

586 *Freshly transfected sporozoites are administered to chickens via the cloaca. This prevents degradation*
587 *of the sporozoites in the proventriculus, as they are not protected by the oocyst wall. When a parasite*
588 *suspension is dripped onto the cloacal lips, it shows a typical sucking movement that uptakes the fluid,*
589 *resulting in an effective lower intestinal infection.*

590 3. If the DHFR-TSm2m3 gene has been included in the transfected construct, supplement the diet with
591 150 ppm of pyrimethamine 24 h after dosing, and keep feeding all chickens with this for 6 days. To
592 ensure homogeneous distribution of the drug, feed can be ground and mixed using a mixing
593 paddle.

594 4. One week after infection, cull chickens and remove the caeca using dissecting tools.

595 5. Carefully cut each caecum open longitudinally. Holding the tip of each caecum with one glass
596 microscope slide, use a second slide to carefully scrape away the caecal contents, the mucosal and
597 the deeper muscle layers (Figure 7A). Place the scrapings in a 50 ml tube containing PBS or distilled
598 water (1:1 proportion).

599 6. Add trypsin to 1.5% (w/v) of the total volume to break down any connective tissue. Incubate in a
600 water bath at 41 °C for ~90 min, swirling the tube frequently.

601 *An adequate scraping technique and trypsin digestion will improve oocyst yields, as the parasites will*
602 *not be trapped within caecal tissues and will be easier to recover.*

603 7. Centrifuge homogenate (750 x g for 10 minutes), and discard supernatant. Then, wash the resulting
604 pellet twice using distilled water and re-pellet by centrifugation. Check microscopically all
605 supernatants to confirm that oocysts are not being lost during washes.

606 8. To start oocyst sporulation, enumerate the number of oocysts using a cell counting chamber and
607 dilute them with 2% (w/v) potassium dichromate to a final concentration of 0.1-0.25x10⁶ oocysts
608 per ml using a baffled flask of at least twice the culture volume.

609 *Since sporulation depends on aeration, the use of a baffled flask of at least twice the culture volume*
610 *is highly desirable. We do not recommend the use of air pumps coupled to serological pipettes, as this*
611 *system increases the chances to cross-contaminate adjacent cultures.*

612 9. Seal the flask with parafilm, pierce it using micropipette tips to ensure aeration, clamp the flask on
613 an orbital shaker, and shake it at 50-100 rpm for 72-96 h at 26-28 °C.

614 *Although oocyst walls are relatively resistant, if shaking is too vigorous during sporulation it may*
615 *result in oocyst breakage, and therefore, loss of viable parasites.*

616 10. During sporulation check oocysts microscopically to estimate sporulation rate using a counting
617 chamber. Rates over 85% are expected.

618 11. Once sporulation has finished, pour the oocyst suspension into centrifuge bottles or 50 ml tubes
619 and centrifuge (750 x g for 10 minutes). Carefully discard the supernatant using a serological pipette
620 and check microscopically that all oocysts have been pelleted.

621 *We always recommend the use of swing rotor centrifuges to pellet oocysts and serological pipettes or*
622 *vacuum pumps to discard supernatants. When experiencing difficulties to pellet oocysts, it is advisable*
623 *to dilute the sample with distilled water to reduce residual flotation.*

624 *Consider current GMO regulations for waste disposal. Aim to autoclave all vessels and solutions that*
625 *have been in contact with transgenic parasites before disposal.*

626 12. Wash the oocyst pellet three times with distilled water by centrifugation (750 x g for 10 minutes),
627 checking microscopically that all oocysts have been pelleted from all supernatants. Aim to
628 constantly reduce the volume.

629 13. Re-suspend the pellet in 5-10 ml of 1.2% sodium hypochlorite solution in a 50 ml tube. Treat for 5-
630 10 minutes and swirl intermittently.

631 14. Top up the vessel with distilled water, mix and centrifuge to pellet the oocysts (750 x g for 10
632 minutes). Carefully discard the supernatant using a serological pipette and check microscopically
633 that all oocysts have been pelleted.

634 15. Re-suspend the pellet in SSS thoroughly using a Pasteur pipette and leaving no lumps.

635 16. Fill the 50 ml tube to 40 ml with SSS and overlay with distilled water by gently running the water
636 down the side of the tube with a Pasteur pipette to 45 ml. Then centrifuge at 750 g for 10 minutes.

637 17. Collect the parasites at the interface between the salt and water phases with a Pasteur pipette
638 (Figure 7B). Dispense into a suitable tube and add at least double the volume of water. After the
639 oocyst layer has been removed, check the pellet at the bottom of the tube microscopically for any
640 remaining oocysts. If a significant number are observed, then repeat the salt flotation as in step 14.

641 18. Centrifuge recovered oocysts at 750 x g for 10 minutes, discard supernatant and re-suspend in
642 water. Repeat this two more times, checking microscopically that all oocysts have been pelleted
643 from all supernatants.

644 19. After the final wash, re-suspend the oocysts in a suitable volume of distilled water and enumerate
645 using a cell counting chamber (see BASIC PROTOCOL 1: *Cracking, hatching and purification of*
646 *Eimeria tenella parasites*).

647 20. Once purified, oocysts can be stored at 4 °C in a universal tube (preferably made of glass) and used
648 for up to 6 months.

649 *We have observed that oocysts viability declines progressively over the time as shown by the decrease*
650 *in subsequent oocyst output when old parasite stocks are used to dose new batches of birds. In these*
651 *cases, infection doses need to be increased 2-fold or more to achieve similar oocyst yields. However,*
652 *this must be done with caution, as it could result in the induction of severe caecal lesions and the*
653 *death of the animal. When trying to refresh valuable oocysts older than 6 months, one should aim to*
654 *recover low numbers of fresher parasites than can be subsequently passaged in new birds using the*

655 *recommended dose (4,000 oocysts/bird) to obtain substantial amounts of parasites without inducing*
656 *severe pathology.*

- 657 21. If a fluorescent reporter has been transfected, recovered oocysts:
- 658 a. Can be analysed by fluorescent microscopy.
 - 659 b. Can be submitted to cracking, hatching and purification as described above in order to:
 - 660 i. analyse the localisation of the fluorescent tag within the sporocyst and/or sporozoite,
 - 661 or
 - 662 ii. infect MDBK cells and let them develop to first generation merozoites to track
 - 663 transgene behaviour on different parasite stages.
 - 664 c. Can be sorted by flow cytometry. FACS settings will depend on the equipment available, the
 - 665 reporter transfected, the experimental needs, etc. In general, pre-filtering the parasites through
 - 666 40 µm cell strainers is highly recommended. Sorting oocysts at relatively high pressure (40 psi)
 - 667 using 70 to 100 µm nozzles does not seem to impact their viability, yielding recovery rates up
 - 668 to 96%. Gate out strategies to discard cell debris (by cell complexity analysis) and to select
 - 669 fluorescent parasites at specific thresholds are also recommended (Figure 8).
- 670 22. In order to increase the ratio of transgenic/wild type parasites, and stabilise transgenic populations,
- 671 sorted parasites should be used to infect successive new batches of coccidia-free birds employing
- 672 doses up to 4,000 oocysts per bird by oral gavage, and progeny oocysts can be recovered and
- 673 selected again as detailed above.

674

675 **REAGENTS AND SOLUTIONS**

676

677 Sterile saturated salt solution (SSS, 6.4 M NaCl)

- 678 1. Mix 375 g of NaCl with 1 litre of boiling ultrapure water and leave overnight at room
- 679 temperature.
- 680 2. The day after check that the specific gravity is within the expected range (1.18 - 1.20) using a
- 681 hydrometer.
- 682 3. Autoclave the solution (121 °C, 15 min). The presence of undissolved salt is normal, this helps
- 683 to maintain saturation of the solution.

684 Sterile phosphate buffered saline (PBS)

- 685 1. Prepare a solution containing 95 mM Na₂HPO₄, 6.5 mM NaH₂PO₄ and 72 mM NaCl in ultrapure
- 686 water.
- 687 2. Adjust pH to 8 and autoclave (121 °C, 15 min).
- 688 3. This buffer can also be prepared as a 4X stock solution and autoclaved (121 °C, 15 min).

689

690 Sterile diethylaminoethyl cellulose

- 691 1. Mix DE-52 (Whatman pre-swollen microgranular anion exchange; Catalogue No: 4057-050) with
692 sterile 1X PBS (~75 ml per gram) and allow to settle for 30-60 min.
- 693 2. Discard supernatant to remove small particles, add more sterile PBS, mix and allow to settle for
694 30-60 min.
- 695 3. Discard supernatant to remove small particles, add more sterile PBS (~25 ml per gram), mix and
696 adjust to pH 8.0 with 5% (w/v) H₃PO₄.
- 697 4. Allow DE-52 to settle overnight at 4 °C.
- 698 5. Remove supernatant, leaving a small amount of PBS on top of the DE-52, and autoclave solution
699 (121 °C, 15 min).

700 Hatching Solution

- 701 1. Mix 9.8 g Hanks' Balanced Salts (Catalogue No: H6136, Sigma-Aldrich), 10 g sodium
702 taurocholate hydrate (Catalogue No: 86339, Sigma-Aldrich), and 2.5 g trypsin from porcine
703 pancreas (Catalogue No: T4799, Sigma-Aldrich) in 950 mL of ultrapure water.
- 704 2. Adjust to pH 8.0, make up to 1000 ml, aliquot in 50 ml universal tubes, and store at -20 °C. If
705 required, this solution can be sterile filtered using 0.2 µm membranes prior to freezing.

706 Eluting buffer (PBS-1% glucose)

- 707 1. Dissolve 1 g of glucose per 100 mL of sterile PBS pH 8. This buffer can be prepared as a 10X
708 stock solution (10 g of glucose per 100 mL of sterile PBS pH 8), filtered using 0.2 µm membranes,
709 and stored at 4 °C for several months.

710

711

712 **COMMENTARY: PREVIOUS CONSIDERATIONS**

713

714 **Selection of *Eimeria* species**

715

716 The first successful transient complementation of *Eimeria* spp. was described in *E. tenella* sporozoites
717 using the beta-galactosidase reporter (Kelleher & Tomley, 1998). Almost ten years later, two studies
718 described the stable complementation of the same species with specific fluorescent reporters (Clark et
719 al., 2008; Yan et al., 2009). In all examples, the choice of *E. tenella* was not arbitrary, since it is the species
720 most capable of invading, replicating and developing *in vitro* in a range of primary cells and established
721 cell lines (most notably the *E. tenella* Wisconsin strain) (Doran, 1974). Nevertheless, *E. tenella in vitro*
722 development is largely limited to the early asexual stages, and fails to support efficient sexual replication
723 and the subsequent production of oocysts, meaning that parasite propagation is only feasible through
724 controlled passage using live animals (Bussi ere et al., 2018). In addition, of the *Eimeria* species which

725 can infect the chicken, *E. tenella* preferentially replicates in the caeca, simplifying its harvest from caecal
726 scrapes instead of faecal samples (Eckert J., 1995). This approach also reduces the amount of clinical
727 waste generated, and simplifies adherence to relevant regulations regarding genetically modified
728 organisms. Transgenic *E. tenella* parasites can still induce severe haemorrhagic lesions if uncontrolled
729 doses are administered to chickens. Thus, the use of highly prolific and less pathogenic species such as
730 *Eimeria acervulina* for genetic complementation would be highly beneficial (Zou et al., 2009). To date, a
731 number of studies have shown evidence of successful transfection not only in most of the *Eimeria*
732 species affecting poultry (*E. acervulina*, *E. maxima*, *E. mitis*, *E. praecox* in addition to *E. tenella*), but also
733 in other *Eimeria* species from rats and rabbits (*E. nieschulzi*, *E. intestinalis*) (Blake et al., 2011; Kurth &
734 Entzeroth, 2009; Qin et al., 2014; Shi et al., 2016; Zou et al., 2009), which suggests that most *Eimeria*
735 species would be suitable for transfection following the protocols hereby described.

736

737 **Use of targeting signals to modify transprotein delivery**

738

739 The use of *Eimeria* species parasites as live replicating vectors has been proposed as a method to
740 express pathogen-derived antigens, protect them from gastric digestion, and deliver them directly to
741 the gastrointestinal system. However, a number of studies carried out with transgenic *Leishmania major*,
742 *Trypanosoma cruzi*, *T. gondii*, and *E. tenella* have shown that the nature of the immune response elicited
743 by such delivery differs depending on the subcellular localisation of the expressed foreign antigen
744 (Bertholet et al., 2005; Garg, Nunes, & Tarleton, 1997; Gregg et al., 2011; Huang et al., 2011; Kwok et al.,
745 2003; Pepper, Dzierszynski, Crawford, Hunter, & Roos, 2004). Initial work carried out with stable
746 populations of transgenic *Eimeria* described that the transgenes were mostly expressed, and therefore
747 retained, in the sporozoite cytosol (Clark et al., 2008). Nevertheless, recent studies have successfully
748 exploited the inclusion of specific delivery signals within transfection constructs to modify transgene
749 trafficking, hypothetically improving antigen exposure to the host immune system. Delivery sequences
750 which have been tested in *Eimeria* spp. include: (i) the signal peptide from the *T. gondii* dense granule
751 protein 8 (GRA8), the repetitive interspersed family protein in *Plasmodium falciparum*, and the signal
752 peptide from the *E. tenella* SAG1 protein, targeting the transprotein to the parasitophorous vacuole
753 membrane (Liu et al., 2008; Shi, Yan, Ren, Liu, & Suo, 2009); (ii) the nuclear localization sequence (NLS)
754 from the *E. tenella* histone H4 protein, which tags a protein for import into the cell nucleus (Liu et al.,
755 2008); (iii) the signal peptide from the *E. tenella* MIC1 protein, which targets the transprotein into the
756 micronemes (Huang et al., 2011); (iv) the signal peptide from the *E. tenella* MIC2 protein, which induces
757 the secretion of the transfected protein into the sporocyst cavity after oocyst sporulation (Marugan-
758 Hernandez et al., 2017); and (v) the glycoposphatidylinositol (GPI) anchor sequence from the *E. tenella*

759 SAG1 protein, that induces the anchorage of the protein onto the sporozoite surface (Marugan-
760 Hernandez et al., 2017).

761

762 **Present limitations for transfection in *Eimeria* parasites**

763

764 Current techniques available for *Eimeria* spp. transfection still rely on random, non-homologous
765 insertion of plasmid DNA, which entails two main limitations. Firstly, DNA integration through REMI (see
766 *Restriction enzyme mediated integration*) results in extremely high rates of insertion, reducing the
767 likelihood to obtain stable populations. Secondly, non-directed transfections are likely to disrupt
768 essential regions of the genome, increasing the mortality rate of the transfectants. In addition, the
769 inefficiency of *in vitro* cultivation to propagate *Eimeria* spp. obstructs generation of genetically
770 homogeneous (clonal) lines, and consequently yields highly variable results (Qin et al., 2014).
771 Furthermore, while the sexual stages of the *Eimeria* lifecycle take place *in vivo*, genetic segregation and
772 recombination occur during oocyst sporulation *ex vivo*, remote from selectable markers such as drug
773 selection or enrichment for fluorescent reporter proteins. This results in transgene loss due to cross-
774 fertilisation between transgenic and non-transgenic parents (Clark et al., 2008). The occurrence of this
775 phenomenon is clear when parasites are transfected for the first time with fluorescent reporters, used
776 to infect birds, and analysed under fluorescence microscopy after harvest, when it is possible to observe
777 progeny oocysts carrying one, two, three, and/or four fluorescent sporocysts (Kurth & Entzeroth, 2009).
778 However, stabilization of parasite populations by successive *in vivo* passage seemingly contributes to
779 reduce this effect as discussed in *Stability of the transgenes* section. The generation of KU80 knockout
780 mutant strains (Fox, Ristuccia, Gigley, & Bzik, 2009; Huynh & Carruthers, 2009) and development of the
781 CRISPR/Cas9 system in *Toxoplasma gondii* (Shen, Brown, Lee, & Sibley, 2014; Sidik, Hackett, Tran,
782 Westwood, & Lourido, 2014) have significantly improved homologous integration efficiency and
783 disruption of targeted genes in this parasite system, but unfortunately these technologies are not yet
784 available for *Eimeria*. Nevertheless, and despite all these inconveniences, the protocols described here
785 have proven to be of value for generating stable transgenic populations of *Eimeria* spp. (Clark et al.,
786 2008; Yan et al., 2009).

787

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789

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798 LITERATURE CITED

799

800 Bertholet, S., Debrabant, A., Afrin, F., Caler, E., Mendez, S., Tabbara, K. S., . . . Sacks, D. L. (2005). Antigen requirements for efficient priming
801 of CD8+ T cells by Leishmania major-infected dendritic cells. *Infect Immun*, *73*(10), 6620-6628. doi:10.1128/IAI.73.10.6620-
802 6628.2005

803 Black, M., Seeber, F., Soldati, D., Kim, K., & Boothroyd, J. C. (1995). Restriction enzyme-mediated integration elevates transformation
804 frequency and enables co-transfection of *Toxoplasma gondii*. *Mol Biochem Parasitol*, *74*(1), 55-63. Retrieved from
805 <http://www.ncbi.nlm.nih.gov/pubmed/8719245>

806 Blake, D. P., Billington, K. J., Copestake, S. L., Oakes, R. D., Quail, M. A., Wan, K. L., . . . Smith, A. L. (2011). Genetic mapping identifies novel
807 highly protective antigens for an apicomplexan parasite. *PLoS Pathog*, *7*(2), e1001279. doi:10.1371/journal.ppat.1001279

808 Blake, D. P., & Tomley, F. M. (2014). Securing poultry production from the ever-present *Eimeria* challenge. *Trends Parasitol*, *30*(1), 12-19.
809 doi:10.1016/j.pt.2013.10.003

810 Bumstead, N., & Millard, B. J. (1992). Variation in susceptibility of inbred lines of chickens to seven species of *Eimeria*. *Parasitology*, *104* (Pt
811 3), 407-413. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/1386419>

812 Chapman, H. D. (1997). Biochemical, genetic and applied aspects of drug resistance in *Eimeria* parasites of the fowl. *Avian Pathol*, *26*(2), 221-
813 244. doi:10.1080/03079459708419208

814 Chapman, H. D. (2014). Milestones in avian coccidiosis research: a review. *Poult Sci*, *93*(3), 501-511. doi:10.3382/ps.2013-03634

815 Clark, J. D., Billington, K., Bumstead, J. M., Oakes, R. D., Soon, P. E., Sopp, P., . . . Blake, D. P. (2008). A toolbox facilitating stable transfection
816 of *Eimeria* species. *Mol Biochem Parasitol*, *162*(1), 77-86. doi:10.1016/j.molbiopara.2008.07.006

817 Clark, J. D., Oakes, R. D., Redhead, K., Crouch, C. F., Francis, M. J., Tomley, F. M., & Blake, D. P. (2012). *Eimeria* species parasites as novel
818 vaccine delivery vectors: anti-Campylobacter jejuni protective immunity induced by *Eimeria tenella*-delivered CjaA. *Vaccine*,
819 *30*(16), 2683-2688. doi:10.1016/j.vaccine.2012.02.002

820 Donald, R. G., & Roos, D. S. (1993). Stable molecular transformation of *Toxoplasma gondii*: a selectable dihydrofolate reductase-thymidylate
821 synthase marker based on drug-resistance mutations in malaria. *Proc Natl Acad Sci U S A*, *90*(24), 11703-11707. Retrieved from
822 <http://www.ncbi.nlm.nih.gov/pubmed/8265612>

823 Doran, D. J. V., J. M.; Augustine, P. C. (1974). *Eimeria tenella*: An in vivo and in vitro Comparison of the Wisconsin, Weybridge, and Beltsville
824 Strains. *Proc Helminthol Soc Wash*, *41*, 77-80.

825 Eckert J., B. R., Shirley M.W., Coudert P. (1995). Guidelines on Techniques in Coccidiosis Research. *Luxembourg: Office for Official Publications*
826 *of the European Communities*.

827 Fox, B. A., Ristuccia, J. G., Gigley, J. P., & Bzik, D. J. (2009). Efficient gene replacements in *Toxoplasma gondii* strains deficient for
828 nonhomologous end joining. *Eukaryot Cell*, *8*(4), 520-529. doi:10.1128/EC.00357-08

829 Garg, N., Nunes, M. P., & Tarleton, R. L. (1997). Delivery by *Trypanosoma cruzi* of proteins into the MHC class I antigen processing and
830 presentation pathway. *J Immunol*, *158*(7), 3293-3302. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9120286>

831 Gregg, B., Dzierszinski, F., Tait, E., Jordan, K. A., Hunter, C. A., & Roos, D. S. (2011). Subcellular antigen location influences T-cell activation
832 during acute infection with *Toxoplasma gondii*. *PLoS One*, *6*(7), e22936. doi:10.1371/journal.pone.0022936

833 Hanig, S., Entzeroth, R., & Kurth, M. (2012). Chimeric fluorescent reporter as a tool for generation of transgenic *Eimeria* (Apicomplexa,
834 Coccidia) strains with stage specific reporter gene expression. *Parasitol Int*, *61*(3), 391-398. doi:10.1016/j.parint.2012.01.010

835 Hao, L., Liu, X., Zhou, X., Li, J., & Suo, X. (2007). Transient transfection of *Eimeria tenella* using yellow or red fluorescent protein as a marker.
836 *Mol Biochem Parasitol*, *153*(2), 213-215. doi:10.1016/j.molbiopara.2007.02.005

837 Huang, X., Zou, J., Xu, H., Ding, Y., Yin, G., Liu, X., & Suo, X. (2011). Transgenic *Eimeria tenella* expressing enhanced yellow fluorescent protein
838 targeted to different cellular compartments stimulated dichotomic immune responses in chickens. *J Immunol*, *187*(7), 3595-3602.
839 doi:10.4049/jimmunol.1100043

840 Huynh, M. H., & Carruthers, V. B. (2009). Tagging of endogenous genes in a *Toxoplasma gondii* strain lacking Ku80. *Eukaryot Cell*, 8(4), 530-
841 539. doi:10.1128/EC.00358-08

842 Jenkins, M. C., Parker, C., & Ritter, D. (2017). Eimeria Oocyst Concentrations and Species Composition in Litter from Commercial Broiler
843 Farms During Anticoccidial Drug or Live Eimeria Oocyst Vaccine Control Programs. *Avian Dis*, 61(2), 214-220. doi:10.1637/11578-
844 010317-Reg.1

845 Kelleher, M., & Tomley, F. M. (1998). Transient expression of beta-galactosidase in differentiating sporozoites of *Eimeria tenella*. *Mol*
846 *Biochem Parasitol*, 97(1-2), 21-31. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9879884>

847 Kurth, M., & Entzeroth, R. (2009). Reporter gene expression in cell culture stages and oocysts of *Eimeria nieschulzi* (Coccidia, Apicomplexa).
848 *Parasitol Res*, 104(2), 303-310. doi:10.1007/s00436-008-1192-0

849 Kuspa, A., & Loomis, W. F. (1992). Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid
850 DNA. *Proc Natl Acad Sci U S A*, 89(18), 8803-8807. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/1326764>

851 Kwok, L. Y., Lutjen, S., Soltek, S., Soldati, D., Busch, D., Deckert, M., & Schluter, D. (2003). The induction and kinetics of antigen-specific CD8
852 T cells are defined by the stage specificity and compartmentalization of the antigen in murine toxoplasmosis. *J Immunol*, 170(4),
853 1949-1957. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12574363>

854 Liu, X., Shi, T., Ren, H., Su, H., Yan, W., & Suo, X. (2008). Restriction enzyme-mediated transfection improved transfection efficiency in vitro
855 in Apicomplexan parasite *Eimeria tenella*. *Mol Biochem Parasitol*, 161(1), 72-75. doi:10.1016/j.molbiopara.2008.06.006

856 Liu, X., Zou, J., Yin, G., Su, H., Huang, X., Li, J., . . . Suo, X. (2013). Development of transgenic lines of *Eimeria tenella* expressing M2e-enhanced
857 yellow fluorescent protein (M2e-EYFP). *Vet Parasitol*, 193(1-3), 1-7. doi:10.1016/j.vetpar.2012.12.019

858 Marugan-Hernandez, V., Cockle, C., Macdonald, S., Pegg, E., Crouch, C., Blake, D. P., & Tomley, F. M. (2016). Viral proteins expressed in the
859 protozoan parasite *Eimeria tenella* are detected by the chicken immune system. *Parasit Vectors*, 9, 463. doi:10.1186/s13071-
860 016-1756-2

861 Marugan-Hernandez, V., Long, E., Blake, D., Crouch, C., & Tomley, F. (2017). *Eimeria tenella* protein trafficking: differential regulation of
862 secretion versus surface tethering during the life cycle. *Sci Rep*, 7(1), 4557. doi:10.1038/s41598-017-04049-1

863 Pastor-Fernandez, I., Kim, S., Billington, K., Bumstead, J., Marugan-Hernandez, V., Kuster, T., . . . Tomley, F. M. (2018). Development of cross-
864 protective *Eimeria*-vectored vaccines based on apical membrane antigens. *Int J Parasitol*, 48(7), 505-518.
865 doi:10.1016/j.ijpara.2018.01.003

866 Pepper, M., Dzierszynski, F., Crawford, A., Hunter, C. A., & Roos, D. (2004). Development of a system to study CD4+T-cell responses to
867 transgenic ovalbumin-expressing *Toxoplasma gondii* during toxoplasmosis. *Infect Immun*, 72(12), 7240-7246.
868 doi:10.1128/IAI.72.12.7240-7246.2004

869 Qin, M., Liu, X. Y., Tang, X. M., Suo, J. X., Tao, G. R., & Suo, X. (2014). Transfection of *Eimeria mitis* with yellow fluorescent protein as reporter
870 and the endogenous development of the transgenic parasite. *PLoS One*, 9(12), e114188. doi:10.1371/journal.pone.0114188

871 Qin, M., Tang, X., Yin, G., Liu, X., Suo, J., Tao, G., . . . Suo, X. (2016). Chicken IgY Fc expressed by *Eimeria mitis* enhances the immunogenicity
872 of *E. mitis*. *Parasit Vectors*, 9, 164. doi:10.1186/s13071-016-1451-3

873 Reid, A. J., Blake, D. P., Ansari, H. R., Billington, K., Browne, H. P., Bryant, J., . . . Pain, A. (2014). Genomic analysis of the causative agents of
874 coccidiosis in domestic chickens. *Genome Res*, 24(10), 1676-1685. doi:10.1101/gr.168955.113

875 Schiestl, R. H., & Petes, T. D. (1991). Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*. *Proc Natl Acad*
876 *Sci U S A*, 88(17), 7585-7589. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/1881899>

877 Shen, B., Brown, K. M., Lee, T. D., & Sibley, L. D. (2014). Efficient gene disruption in diverse strains of *Toxoplasma gondii* using CRISPR/CAS9.
878 *MBio*, 5(3), e01114-01114. doi:10.1128/mBio.01114-14

879 Shi, T., Tao, G., Bao, G., Suo, J., Hao, L., Fu, Y., & Suo, X. (2016). Stable Transfection of *Eimeria intestinalis* and Investigation of Its Life Cycle,
880 Reproduction and Immunogenicity. *Front Microbiol*, 7, 807. doi:10.3389/fmicb.2016.00807

881 Shi, T., Yan, W., Ren, H., Liu, X., & Suo, X. (2009). Dynamic development of parasitophorous vacuole of *Eimeria tenella* transfected with the
882 yellow fluorescent protein gene fused to different signal sequences from apicomplexan parasites. *Parasitol Res*, 104(2), 315-320.
883 doi:10.1007/s00436-008-1194-y

884 Shirley, M. W., Smith, A. L., & Tomley, F. M. (2005). The biology of avian *Eimeria* with an emphasis on their control by vaccination. *Adv*
885 *Parasitol*, 60, 285-330. doi:10.1016/S0065-308X(05)60005-X

886 Sibley, L. D., Messina, M., & Niesman, I. R. (1994). Stable DNA transformation in the obligate intracellular parasite *Toxoplasma gondii* by
887 complementation of tryptophan auxotrophy. *Proc Natl Acad Sci U S A*, *91*(12), 5508-5512. Retrieved from
888 <http://www.ncbi.nlm.nih.gov/pubmed/8202518>

889 Sidik, S. M., Hackett, C. G., Tran, F., Westwood, N. J., & Lourido, S. (2014). Efficient genome engineering of *Toxoplasma gondii* using
890 CRISPR/Cas9. *PLoS One*, *9*(6), e100450. doi:10.1371/journal.pone.0100450

891 Smith, A. L., Hesketh, P., Archer, A., & Shirley, M. W. (2002). Antigenic diversity in *Eimeria maxima* and the influence of host genetics and
892 immunization schedule on cross-protective immunity. *Infect Immun*, *70*(5), 2472-2479. Retrieved from
893 <http://www.ncbi.nlm.nih.gov/pubmed/11953384>

894 Soldati, D., & Boothroyd, J. C. (1993). Transient transfection and expression in the obligate intracellular parasite *Toxoplasma gondii*. *Science*,
895 *260*(5106), 349-352. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/8469986>

896 Tang, X., Liu, X., Yin, G., Suo, J., Tao, G., Zhang, S., & Suo, X. (2017). A Novel Vaccine Delivery Model of the Apicomplexan *Eimeria tenella*
897 Expressing *Eimeria maxima* Antigen Protects Chickens against Infection of the Two Parasites. *Front Immunol*, *8*, 1982.
898 doi:10.3389/fimmu.2017.01982

899 Tierney, J., & Mulcahy, G. (2003). Comparative development of *Eimeria tenella* (Apicomplexa) in host cells in vitro. *Parasitol Res*, *90*(4), 301-
900 304. doi:10.1007/s00436-003-0846-1

901 Walker, R. A., Sharman, P. A., Miller, C. M., Lippuner, C., Okoniewski, M., Eichenberger, R. M., . . . Smith, N. C. (2015). RNA Seq analysis of
902 the *Eimeria tenella* gametocyte transcriptome reveals clues about the molecular basis for sexual reproduction and oocyst
903 biogenesis. *BMC Genomics*, *16*, 94. doi:10.1186/s12864-015-1298-6

904 Williams, R. B. (1998). Epidemiological aspects of the use of live anticoccidial vaccines for chickens. *Int J Parasitol*, *28*(7), 1089-1098.
905 Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9724880>

906 Yan, W., Liu, X., Shi, T., Hao, L., Tomley, F. M., & Suo, X. (2009). Stable transfection of *Eimeria tenella*: constitutive expression of the YFP-YFP
907 molecule throughout the life cycle. *Int J Parasitol*, *39*(1), 109-117. doi:10.1016/j.ijpara.2008.06.013

908 Zou, J., Liu, X., Shi, T., Huang, X., Wang, H., Hao, L., . . . Suo, X. (2009). Transfection of *Eimeria* and *Toxoplasma* using heterologous regulatory
909 sequences. *Int J Parasitol*, *39*(11), 1189-1193. doi:10.1016/j.ijpara.2009.03.006

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929 **TABLES**

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931 **Table 1.** Putative promoter regions of *E. tenella* selected to test their ability to induce specific or higher
 932 levels of expression of foreign genes transfected in *E. tenella* sporozoites. Accession numbers
 933 (<http://www.toxodb.org>), relative transcript abundances, stage-specific regulations, ortholog/paralog
 934 products (compared to *Toxoplasma gondii* and *Neospora caninum*), and levels of mCitrine expression
 935 after transfection of sporozoites are displayed.

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| Promoter region | Accession number (ToxoDB) | Expression level (mRNA) | Expression regulation | Product | Fluorescence levels (mCitrine) |
|-----------------|---------------------------|-------------------------|--------------------------------|--|--------------------------------|
| <i>Et1</i> | ETH_00000210 | High | Constitutive | Heat shock protein 70, related / RNA recognition motif domain-containing protein, putative | + |
| <i>Et2</i> | ETH_00004225 | High | Sporulated oocyst & sporozoite | No homologous | + |
| <i>Et3</i> | ETH_00004795 | Medium | Constitutive | No homologous | + |
| <i>Et4</i> | ETH_00004955 | High | Sporozoite & merozoite | No homologous | + |
| <i>Et5</i> | ETH_00009335 | Medium | Constitutive | Zinc finger (C3HC4 RING finger) protein, putative | - |
| <i>Et6</i> | ETH_00009460 | High | Constitutive | Putative pyruvate dehydrogenase (lipoamide) kinase | + |
| <i>Et7</i> | ETH_00010410 | Medium | Constitutive | Hypothetical protein | - |
| <i>Et8</i> | ETH_00025365 | High | Constitutive | Putative translation initiation factor SUI1 | +++ |
| <i>Et9</i> | ETH_00031740 | High | Constitutive | No homologous | ++ |

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948 **Table 2.** The set of programmes assessed for optimization of transfection in *E. tenella* using the Amaxa™
 949 4D-Nucleofector™ System. Transfection was carried out using 1x10⁶ freshly purified sporozoites, 5 µg
 950 of plasmid DNA carrying the mCitrine reporter, and 0.5 U of the *ScaI* restriction enzyme per well, and
 951 assessed by visual confirmation of sporozoite fluorescence. **X**: shock failure. **✓**: shock successful. **NF**: no
 952 fluorescent sporozoites observed. **F**: fluorescent sporozoites observed.

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| AMAXA™ 4D Program | Cytomix buffer | P1 buffer | P3 buffer | P4 buffer |
|------------------------------|-----------------------|------------------|--------------------|------------------|
| EO-115 | X , NF | ✓, NF | ✓, NF | ✓, NF |
| FI-115 | X , NF | ✓, NF | ✓, NF | ✓, NF |
| FP-167 | X , NF | ✓, NF | ✓, NF | ✓, NF |
| FP-158 | X , NF | Not tested | ✓, NF | Not tested |
| FB-158 | X , NF | Not tested | X , NF | Not tested |
| EZ-158 | X , NF | ✓, NF | ✓, NF | ✓, NF |
| FI-158 | X , NF | Not tested | SF, NF | Not tested |
| ES-100 | Not tested | ✓, NF | ✓, NF | ✓, NF |
| FF-158 | Not tested | ✓, NF | ✓, NF | ✓, NF |
| ER-115 | Not tested | ✓, NF | ✓, NF | ✓, NF |
| EX-115 | Not tested | ✓, NF | ✓, NF | ✓, NF |
| <u>EO-114</u> | Not tested | ✓, NF | <u>✓. F</u> | ✓, NF |
| FB-115 | Not tested | X , NF | ✓, NF | ✓, NF |
| FA-115 | Not tested | ✓, NF | ✓, NF | ✓, NF |

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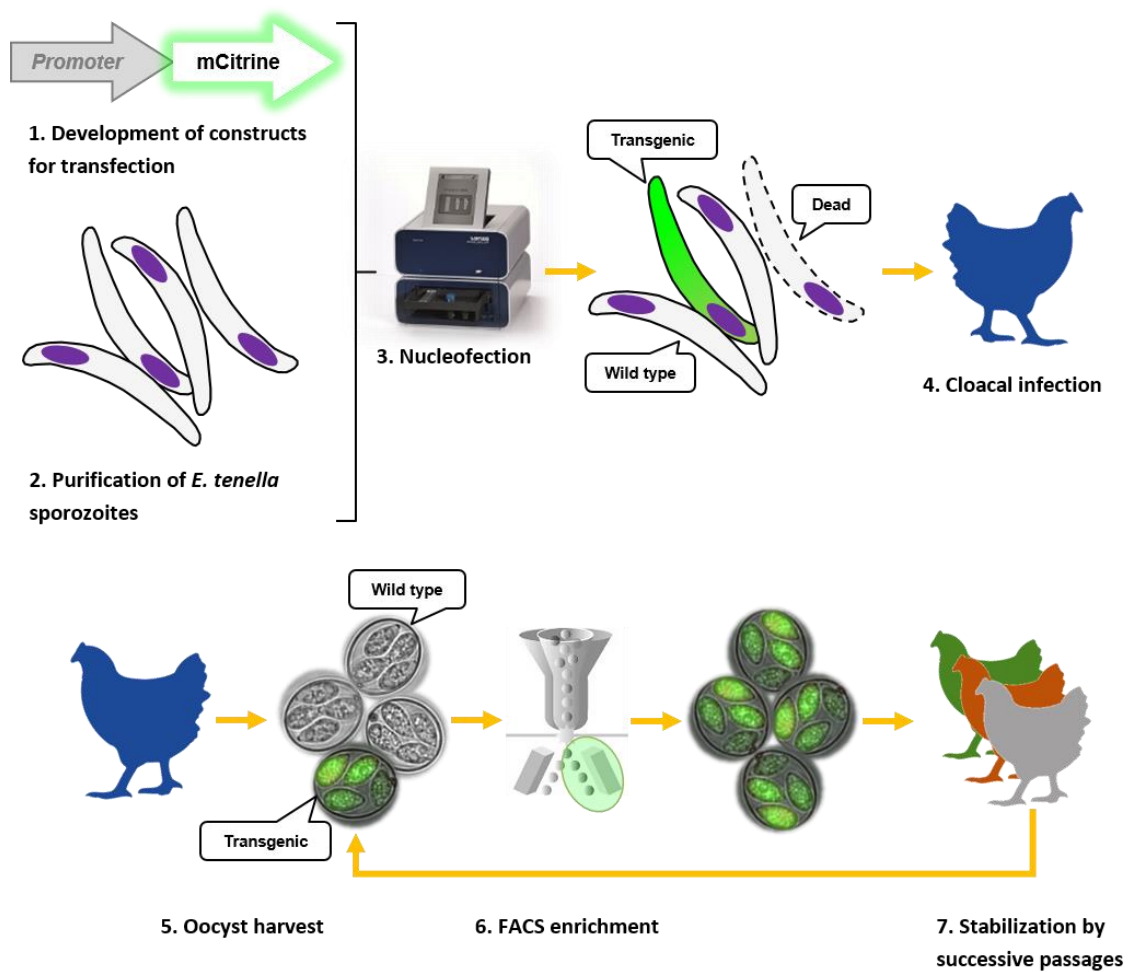
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965 **FIGURES AND FIGURE LEGENDS**

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967 **Figure 1.** Strategy for genetic complementation of *Eimeria tenella* parasites. Sporozoites are purified
968 from oocysts, transfected with the construct of interest (e. g. including mCitrine, a fluorescent reporter)
969 using nucleofection systems and immediately used to infect coccidia-free chickens via the cloaca. A
970 week after infection, progeny oocysts can be harvested from the caeca of infected chickens, sporulated,
971 and subjected to fluorescence-activated cell sorting (FACS) to select those parasites expressing the
972 fluorescent reporter. Enriched populations of fluorescent parasites can then be used to infect new
973 batches of coccidia-free chickens by oral gavage, thereby stabilizing the population.

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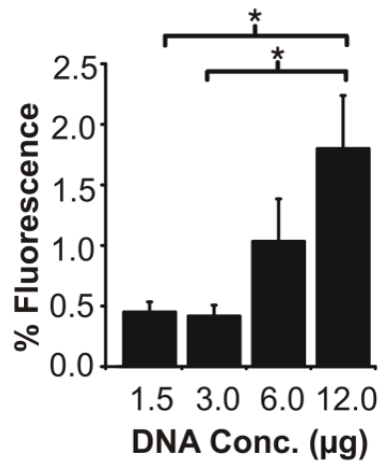
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982 **Figure 2.** Effect of p*Eten*REPORTER plasmid concentration on percentage of fluorescent oocysts
983 recovered after *in vivo* passage using coccidia-free chickens. Groups marked with * were significantly
984 different ($P < 0.05$; Fisher's exact test).

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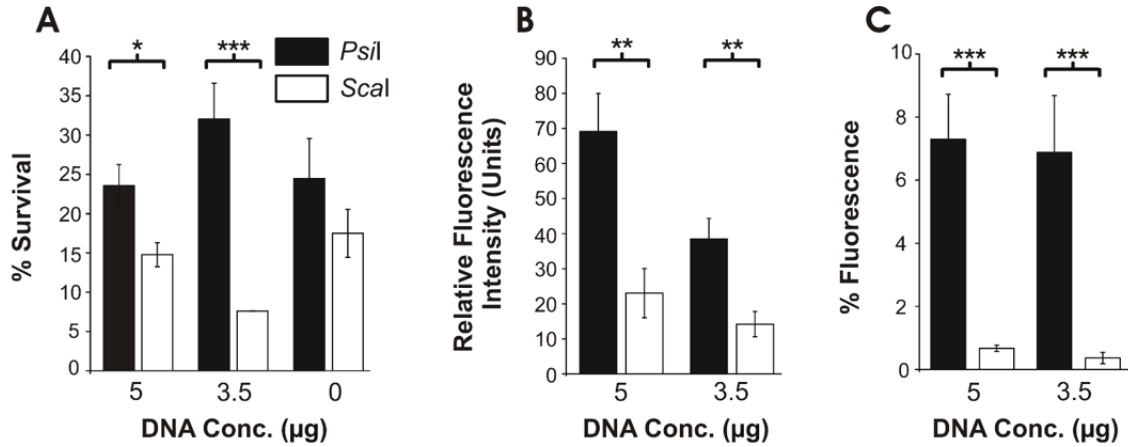
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1003 **Figure 3.** Effect of *PsiI* and *ScaI* restriction enzymes for REMI using either 5.0 or 3.5 μg of digested
 1004 plasmid DNA. **A:** Percentage survival of sporozoites immediately after transfection by Trypan blue
 1005 exclusion. **B:** Relative fluorescence intensity of sporozoites 24 h after transfection. **C:** Percentage of
 1006 fluorescent sporozoites 24 h after transfection. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (one way ANOVA
 1007 test).

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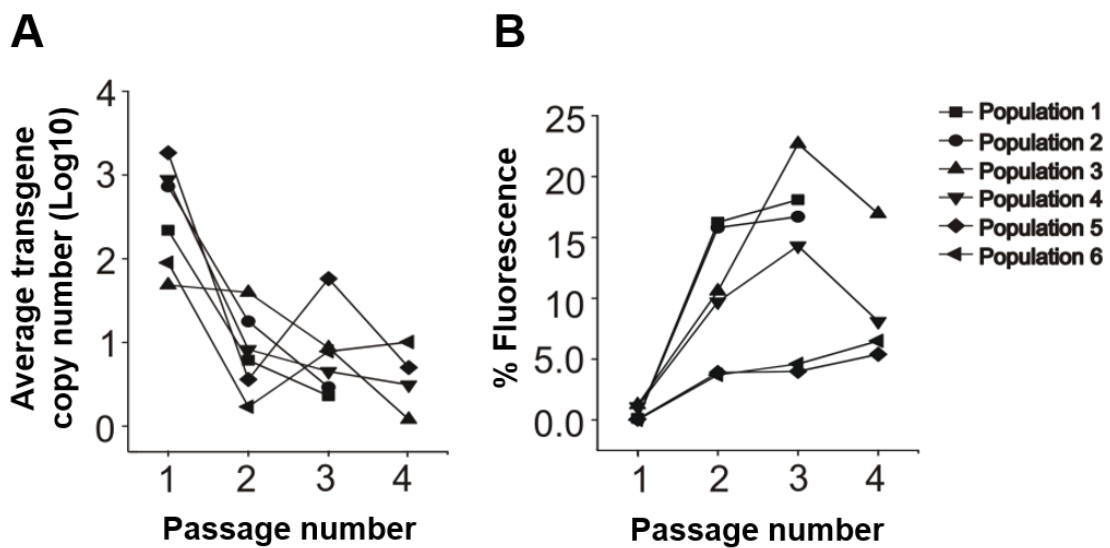
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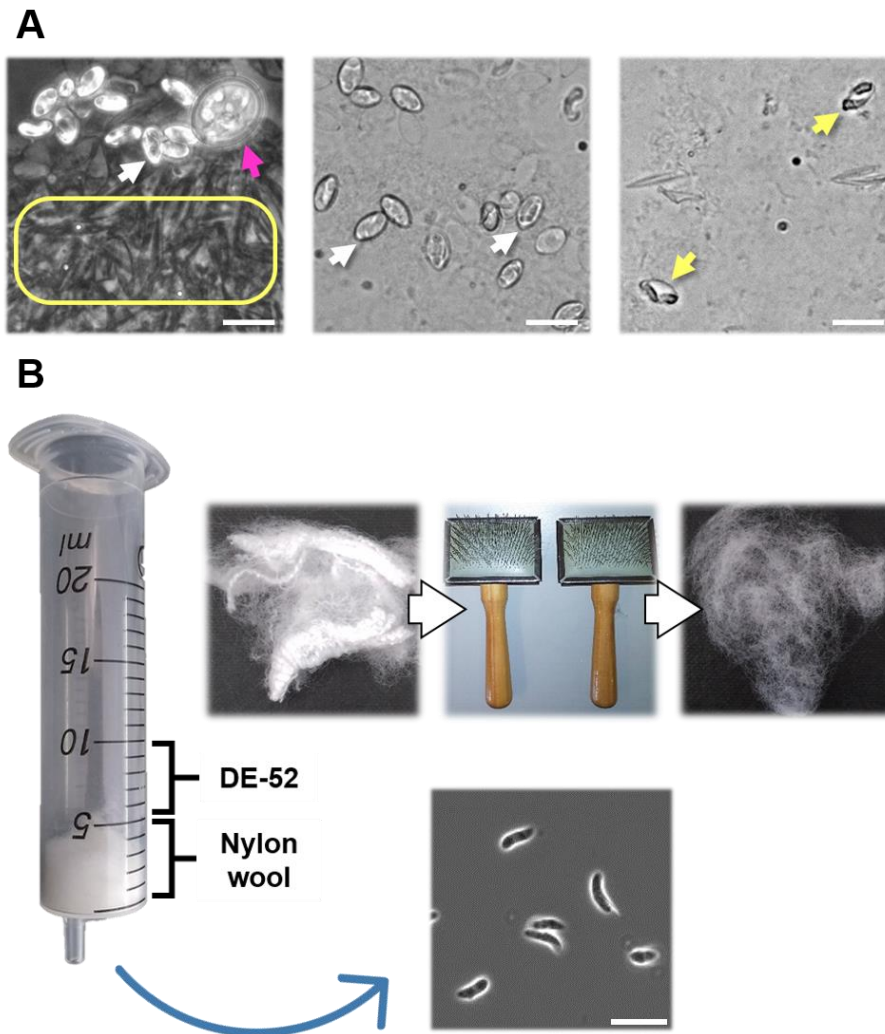
1030 **Figure 4.** Effect of repeated *in vivo* passage on transgenic *E. tenella* populations. Six different batches
 1031 of sporozoites were transfected with 12 µg of the same *ScaI*-digested construct (pCIT-CjaA plasmid
 1032 containing the mCitrine sequence and an additional cassette coding for the *Campylobacter jejuni*
 1033 antigen A, from Clark et al., 2012) and the *ScaI* endonuclease using the EO-114 program (AMAXA™ 4D-
 1034 Nucleofector™ System). Transgenic parasites were independently propagated up to four times in
 1035 coccidia-free birds using FACS-enriched populations between passages. **A:** Average transgene copy
 1036 number from the six different populations as determined by quantitative PCR using *mCitrine* as target
 1037 and the *Et5S* gene as internal control (Log10 scale). **B:** Percentage of fluorescent parasites out of the
 1038 total harvested parasites per passage as visualised under fluorescence microscopy.
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1051 **Figure 5. A:** Photomicrographs of oocysts cracked using glass beads. The yellow box delimits an area
1052 full of oocyst shells. The pink arrow points to a non-cracked oocyst, white arrows indicate released and
1053 unaltered sporocysts, and the yellow arrows show damaged sporocysts due to excessive cracking. Bars
1054 represent 20 μm . **B:** Representation of a column for purification of sporozoites using teased out nylon
1055 wool and DE-52 cellulose, and the appearance of purified sporozoites under the microscope (bar
1056 represents 20 μm).

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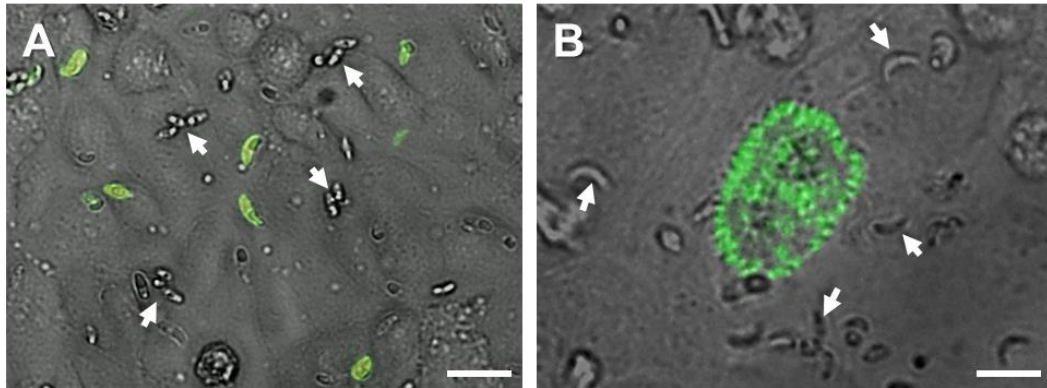
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1063 **Figure 6.** *In vitro* culture of transgenic *Eimeria tenella* Wisconsin parasites expressing the mCitrine
1064 reporter in MDBK cells. **A:** intracellular sporozoites after 24 h of infection. Only those successfully
1065 transfected show green fluorescence. **B:** late development schizonts showing green fluorescence 48 h
1066 after infection. In both cases sporozoites that did not integrate the mCitrine reporter can still be
1067 visualized (white arrows). Bars represent 20 μ m.

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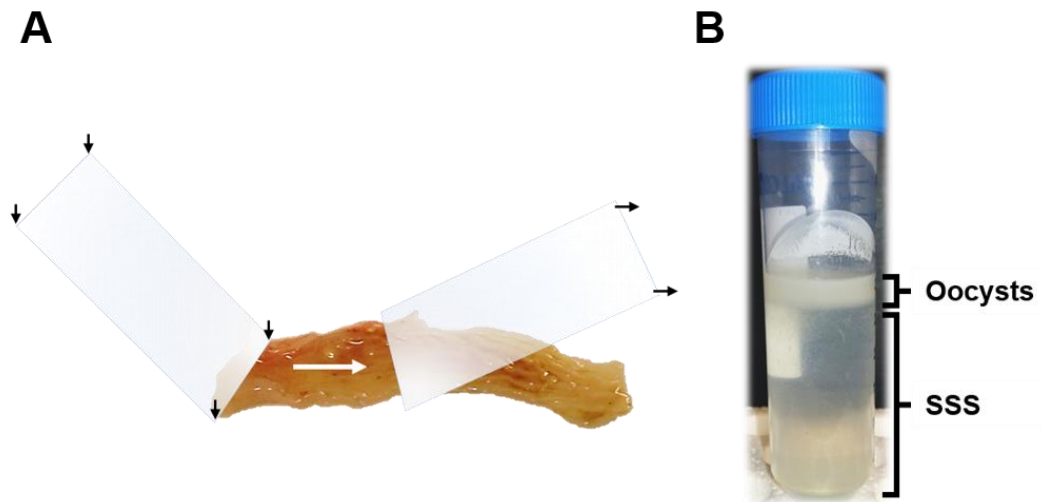
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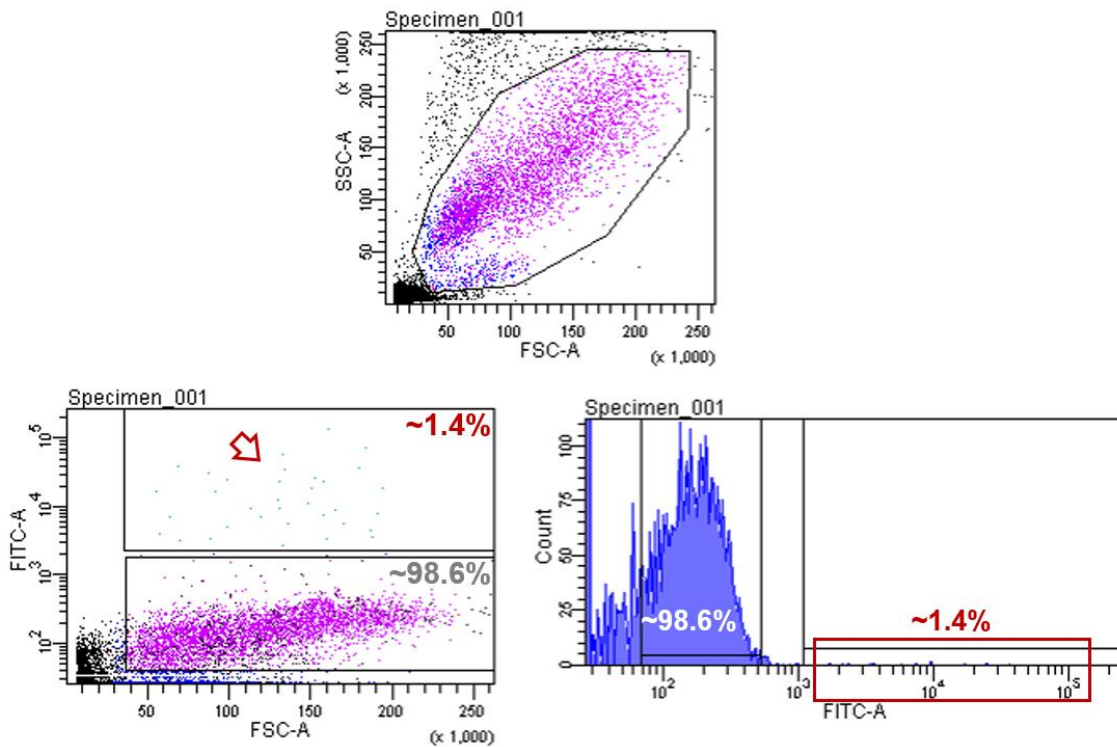
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1090 **Figure 7. A:** Schematic representation of caeca scraping technique. **B:** Flotation of *E. tenella* oocysts
1091 after sodium hypochlorite treatment using saturated salt solution (SSS). Oocysts are present at the
1092 interface between the SSS and water phases.
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1116 **Figure 8.** Flow cytometry analysis of transgenic *E. tenella* parasites expressing the mCitrine reporter
1117 after first *in vivo* passage. **Top:** exclusion of cell debris by SSC-A/FSC-A gating. **Bottom:** selection of
1118 FITC-positive parasites for sorting. Red arrow and box: transgenic parasites expressing mCitrine (note
1119 that they represent a minority among the whole population, ~1.4%). SSC-A: side scattered area; FSC-A:
1120 forward scattered area; FITC-A: Fluorescein isothiocyanate area.
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