TITLE: Laboratory Growth and Genetic Manipulation of Eimeria tenella

AUTHORS: Pastor-Fernández, I; Pegg, E; Macdonald, S E; Tomley, F M; Blake, D P; Marugán-Hernández, V

JOURNAL: Current Protocols in Microbiology

PUBLISHER: Wiley

PUBLICATION DATE: 27 February 2019 (online)

DOI: https://doi.org/10.1002/cpmc.81

Laboratory growth and genetic manipulation of *Eimeria tenella*

Iván Pastor-Fernández[a], Elaine Pegg[a], Sarah E. Macdonald[a], Fiona M. Tomley[a], Damer P. Blake[a], Virginia Marugán-Hernández[a*]

[a] *Department of Pathobiology and Population Sciences, Royal Veterinary College, University of London, Hertfordshire, United Kingdom.*

[*] *Corresponding author:* vhernandez@rvc.ac.uk

Iván Pastor-Fernández: ipastorfernandez@rvc.ac.uk

Elaine Pegg: peggee@tcd.ie

Sarah E. Macdonald: smacdonald@rvc.ac.uk

Fiona M. Tomley: ftomley@rvc.ac.uk

Damer P. Blake: dblake@rvc.ac.uk

Virginia Marugán-Hernández: vhernandez@rvc.ac.uk

SIGNIFICANCE STATEMENT

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

KEYWORDS

Eimeria tenella; transfection; genetic manipulation; transgenic parasites; vaccine delivery vector

ABSTRACT

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

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

INTRODUCTION

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

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

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

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

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

STRATEGIC PLANNING

Parasite species/strain selection

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

Chickens and Facilities

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

Safety Concerns

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

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

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

Handling of Infectious Oocysts

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

Development of constructs for transfection

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

a) Selection of regulatory regions to promote heterologous expression

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

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

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

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

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

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

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

Methods for the optimisation of DNA integration

a) Restriction enzyme mediated integration

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

<u>a.1) Effect of plasmid concentration and size on transfection efficiency</u>

To date, transfection of *E. tenella* sporozoites has been performed using a broad range of plasmid concentrations (Clark et al., 2008; Liu et al., 2013; Yan et al., 2009). However, these studies have not directly evaluated the effect of DNA starting concentration on transfection efficiency, which has now been found to have a dose dependent effect. Experiments completed in our group with *E. tenella* sporozoites transfected with 1.5 to 12 µg of the p*Eten*REPORTER plasmid that confers green fluorescence to the transfected parasites (p5'UTR-Et*MIC1_mCitrine*_3'UTR-*Actin*, from (Clark et al.,

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

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

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

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

b) Transfection technologies

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

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

c) Stability of the transgenes

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

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

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

Materials

- Sporulated oocysts of *E. tenella* in H_2O or PBS (produced in-house, see BASIC PROTOCOL 3: *In vivo propagation of transgenic E. tenella*).
- Sterile saturated salt solution (SSS, see Reagents & Solutions).
- Sterile phosphate buffered saline (PBS, see Reagents & Solutions).
- Sterile diethylaminoethyl cellulose (see Reagents & Solutions).
- Hatching solution (see Reagents & Solutions).
- 1M $MgCl_2$ solution in ultrapure water.
- Eluting buffer (PBS-1% glucose, see Reagents & Solutions).
- Ballotini SiLibeads® solid soda glass beads, 0.4-0.6 mm diameter (Catalogue No: 201-0465, VWR).
- 20 mL-disposable syringes (slip tip).
- Sterile Erlenmeyer flask (borosilicate glass, narrow neck, 250 ml, catalogue No: 1130/14D, Pyrex).
- Nylon wool fiber (Catalogue No: 18369, Polysciences Inc).
- Set of two pet slicker brushes with fine wire.
- Micropipettes, tips, pipette controller and serological pipettes.
- Swing bucket centrifuge.
- Vortex mixer.
- Optical microscope.
- Water bath.
- Small measuring cylinders or retort stands.
- Parafilm.
- Cell counting chambers (modified Fuchs Rosenthal, catalogue No: AC6000, Hawksley).

Procedure

1. Using a cell counting chamber (modified Fuchs Rosenthal), estimate the concentration of the oocyst stock by loading 10 µl of the culture per chamber and counting them at 10X/10X magnification under the optical microscope. If oocysts concentration is too high to count, dilute the stocks in H_2O or PBS (1:10 to 1:100) until parasite numbers are adequate for an accurate count, correcting the final concentration accordingly.
2. Pellet 10-50x10^6 oocysts using a swing bucket centrifuge (750 x g for 10 min) in a 50 ml universal tube, and re-suspend in 3 ml of PBS. If more parasites are required, prepare them in a different 50 ml universal tube.
3. Add the glass beads (1:1 proportion, oocysts suspension:beads) and crack parasites by vortexing for 15 sec bursts until most of the oocysts have released their sporocysts. Check oocysts microscopically between bursts to ensure sporocyst release. Repeat this process as many times as

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

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

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

4. Transfer the resulting sporocyst/oocyst/debris suspension to a 250 ml Erlenmeyer flask by washing the beads by pipetting with 40 ml of hatching solution.
5. Incubate the sporocyst/oocyst/debris suspension for 30 min at 41 °C in a water bath, and check the hatching progress microscopically.
6. Supplement hatching solution with 1 M $MgCl_2$ to a final concentration of 10 mM (500 µl 1 M $MgCl_2$ per 50 ml of hatching solution added).
7. Incubate the sporocyst/oocyst/debris suspension for an additional 1 h at 41 °C. Mix gently every 30 min and check hatching progress microscopically.
8. Prepare a suitable number of purification columns during the parasite excystation (Figure 5). One 20 ml syringe column is sufficient for up to ~$50x10^6$ oocysts.
    a. Use two pet slicker brushers to tease out small bunches of nylon wool and remove any knots by brushing.
    b. Fill the syringe barrel with the teased-out wool to a depth of 5 ml. Gently push the wool to the bottom using the plunger.

    The use of non-teased out nylon wool and/or its excessive compression within the column may result in lower recovery yields.
    c. Place the column on a measuring cylinder or in a retort stand and wash through nylon wool with 10 ml of eluting buffer.
    d. Before all the eluting buffer has run out, pour sterile DE-52 on top of the nylon wool up to a 10 ml depth and allow the excess fluid to drain out. Ensure that the column does not dry.
    e. Wash column through with 20 ml of eluting buffer and plug syringe nozzle with parafilm. Make sure that there is still a little excess of buffer in the column to prevent drying out.
9. Once hatching has finished, enumerate the total number of sporozoites using a cell counting chamber.

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

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

This protocol describes the methods for successful transfection of freshly purified *E. tenella* sporozoites using REMI and nucleofection approaches. Other relevant factors such as plasmid preparation are also discussed. The protocol is optimized for transfection of $1x10^6$ sporozoites with 12 µg of linearized plasmid and 6 units of the chosen restriction enzyme. The Nucleocuvette™ Strips allow up to 16 independent transfections in a single assay, so different constructs, restriction enzymes, numbers of parasites, and DNA starting concentrations can be assessed to optimize specific needs in a single experiment.

Materials

- Freshly hatched *E. tenella* sporozoites (see *BASIC PROTOCOL 1: Cracking, hatching and purification of Eimeria tenella parasites*).
- Linearized transfection constructs (see *Development of constructs for transfection* and *Effect of plasmid concentration and size on transfection efficiency*).
- Selected restriction enzyme for REMI (see *Effect of restriction enzymes on transfection efficiency*).
- Micro-volume Spectrophotometer.
- Amaxa™ 4D-Nucleofector™ (core and X units for nucleofection in suspensions, catalogue No: AAF-1002B and AAF-1002X, Lonza).
- Amaxa™ P3 Primary Cell 4D-Nucleofector™ X Kit (includes transfection buffer and 16-well Nucleocuvette™ Strips, Catalogue No: V4XP-3032, Lonza) (see *Transfection technologies*).
- CO_2 incubator.
- Roswell Park Memorial Institute (RPMI)-1640 medium with L-glutamine, sodium bicarbonate and phenol red (Catalogue No: R8758, Sigma-Aldrich).
- 0.4% Trypan Blue solution (Catalogue No: T8154, Sigma-Aldrich).
- Flat bottom 96-well cell culture plate (Catalogue No: 266120, Nunc).
- Micropipettes, tips, pipette controller and serological pipettes.
- Cell counting chambers (modified Fuchs Rosenthal).

Procedure

1. Linearized plasmids for REMI should be ready in advance to avoid unnecessary delay in transfection and consequent loss of sporozoite viability:
    a. Plasmids are commonly propagated using *E. coli* competent cells (e.g. XL1-Blue strain, from Agilent), purified using column-based kits (e.g. Plasmid Midi Kit from QIAGEN, for up to 100 µg of plasmid DNA), quantified using a spectrophotometer, and digested overnight using restriction enzymes that cut them in a single site upstream of the expression cassette (e.g. *PsiI*, digesting in the region upstream the Et*Mic1* promoter fused to the mCitrine reporter gene as in Clark et al., 2008).
    b. In order to concentrate the plasmid and avoid interference of digestion buffer with transfection buffer, digested plasmids need to be cleaned up by precipitation following these steps:
        i. Add to the digestion mix 1/10 volumes of 3M sodium acetate and 3 volumes of absolute ethanol.
        ii. Incubate sample at -20 ºC for 30 min to 16 hours. Then, centrifuge at 14,000 x g for 30 min at 4 ºC.
        iii. Discard supernatant and wash pellet with 70% (v/v) ethanol.

iv. Centrifuge at 14,000 x g for 30 min at 4 ºC.

v. Discard supernatant and re-suspend pellet with transfection buffer (see below). The final volume will depend on the amount of plasmid subjected to precipitation, but the ideal concentration is 2-4 µg/µl.

2. Pellet freshly purified sporozoites in a 50 ml universal tube using a swing bucket centrifuge (500 x g for 10 min), discard eluting buffer, and re-suspend parasites in the appropriate volume of P3 buffer (20 µl per $1x10^6$ sporozoites).

   In most cases, the use of fresh oocyst stocks and an adequate cracking, hatching and column purification will yield the best transfection efficiencies. In fact, we have previously shown an inverse correlation between parasite age and transfection efficiency (Clark et al., 2008).

3. Add $1x10^6$ sporozoites, 12 µg of the transfection construct(s) and 6 units of the restriction enzyme P3 buffer per well (final volume should not exceed 25 µl). If more than one plasmid is to be transfected, consider Avogadro's constant to transfect equal copies of both plasmids (1 bp = $660x10^6$ µg/mole; 1 mole = $6.023x10^{23}$ copies).

4. Dispense the appropriate volume into each well of the Nucleocuvette™ Strips, select the preset EO-114 program from the Amaxa™ 4D-Nucleofector™ and start the nucleofection as indicated by the manufacturer.

   Nucleofection conditions and buffer composition are part of Lonza's intellectual property, and therefore these cannot be modified. Other factors affecting transfection success are discussed in detail in the Methods for the optimisation of DNA integration *section.*

   Inclusion of mock transfected parasites (electroshocked with no DNA), and parasites transfected with previously tested plasmids can be useful to validate the nucleofection and determine the source of the issue, if any.

   Once transfected, sporozoites are considered as genetically modified organisms (GMO), and therefore, relevant legislations need to be reviewed and followed.

5. Once shocked, add 80 µl of RPMI-1640 medium per well, and let the parasites stand for 15 min.

6. Estimate survival rate by Trypan Blue exclusion using the cell counting chamber. Non-viable parasites whose membrane permeability is altered will take up the dye, turning blue. In our experience, survival rates ranging from 10 to 20% are considered adequate.

7. Use the transfected parasites instantly for further *in vivo* or *in vitro* experiments. Alternatively, sporozoites can be transferred to 96-well plates in RPMI-1640 medium (~100 µl/well), left for 24-48 h in an incubator (41 ºC, 5% CO_2) to assess transfection efficiency under a fluorescence microscope.

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

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

Materials

- MDBK (NBL-1) cells (Catalogue No: CCL-22, ATCC).
- Freshly hatched sporozoites (see BASIC PROTOCOL 1: *Cracking, hatching and purification of Eimeria tenella parasites* section).
- Advanced Dulbecco's Modified Eagle Medium (DMEM) (Catalogue No: 12491023, Gibco) supplemented with penicillin and streptomycin (100 U/ml each) (Catalogue No: 15140122, Gibco).
- 0.025% Trypsin/0.01% EDTA solution (Catalogue No: R001100, Gibco).
- Foetal bovine serum (FBS), heat inactivated.
- Laminar flow hood.
- CO_2 incubator.
- Cell culture 24-well plates (Catalogue No: 140685, Nunc) and T75 flasks (Catalogue No: 156499, Nunc).
- Micropipettes, sterile tips, pipette controller and serological pipettes.

Procedure

1. MDBK cells can be passaged twice a week, using a subcultivation ratio of 1:2 to 1:4, and a 0.025% Trypsin/0.01% EDTA solution to detach adherent cells from the vessel.
2. Dispense cells into 24 well culture plates ($0.3x10^6$ cells per well in 500 µl of advanced DMEM-2% FBS) and incubate at 41 ºC, 5% CO_2 for at least 2 hours before infection.
   Recently settled and near confluent monolayers improve invasion rates and are critical for the experiment success. It is important to ensure that the cells are in an optimal condition before the experiment by tracking the cultures a few days in advance.

3. Prepare transfected sporozoites in a suspension of $0.3x10^6$ parasites per ml in advanced DMEM-2% FBS. Do not keep sporozoites on ice, as this will reduce the degree of invasion achieved.
   A multiplicity of infection (MOI) 1:1 is recommended to follow the parasite lifecycle, but this can be modified based on the experimental requirements. Bear in mind that the use of a MOI of 4:1 may result in the destruction of the cell monolayer in less than 24 h.
4. Carefully remove medium from cells and add 1 ml of sporozoite suspension per well.
5. Allow sporozoites to infect the monolayer at 41 ºC, 5% CO_2 for 2-4 h, by which time maximum invasion will have occurred. Carefully remove medium and replace with fresh advanced DMEM-2% FBS.
   If parasites are left invading for longer than 4 hours these could overload the monolayer causing its destruction within 24 hours.
6. Incubate plates at 41 ºC for 24 for the observation of invaded sporozoites, or for 48 hours for the observation of schizogony and merozoite formation/release.
7. The presence of fluorescent reporters in transgenic parasites can be followed and quantified by fluorescence microscopy (Figure 6).
   Analysis of fluorescence can be directly done in fresh or fixed monolayers on 24-well plates using an inverted microscope (up to 40x). If higher magnification is needed (up to 100x), cells can be seeded onto rounded coverslips placed in 24-well plates, fixed, and placed on microscope slides with mounting medium.

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

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

<u>Materials</u>

- Transfected sporozoites.

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

Procedure

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

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

3. If the DHFR-TSm2m3 gene has been included in the transfected construct, supplement the diet with 150 ppm of pyrimethamine 24 h after dosing, and keep feeding all chickens with this for 6 days. To ensure homogeneous distribution of the drug, feed can be ground and mixed using a mixing paddle.
4. One week after infection, cull chickens and remove the caeca using dissecting tools.
5. Carefully cut each caecum open longitudinally. Holding the tip of each caecum with one glass microscope slide, use a second slide to carefully scrape away the caecal contents, the mucosal and the deeper muscle layers (Figure 7A). Place the scrapings in a 50 ml tube containing PBS or distilled water (1:1 proportion).
6. Add trypsin to 1.5% (w/v) of the total volume to break down any connective tissue. Incubate in a water bath at 41 ºC for ~90 min, swirling the tube frequently.

   An adequate scraping technique and trypsin digestion will improve oocyst yields, as the parasites will not be trapped within caecal tissues and will be easier to recover.
7. Centrifuge homogenate (750 x g for 10 minutes), and discard supernatant. Then, wash the resulting pellet twice using distilled water and re-pellet by centrifugation. Check microscopically all supernatants to confirm that oocysts are not being lost during washes.
8. To start oocyst sporulation, enumerate the number of oocysts using a cell counting chamber and dilute them with 2% (w/v) potassium dichromate to a final concentration of 0.1-0.25x10^6 oocysts per ml using a baffled flask of at least twice the culture volume.

   Since sporulation depends on aeration, the use of a baffled flask of at least twice the culture volume is highly desirable. We do not recommend the use of air pumps coupled to serological pipettes, as this system increases the chances to cross-contaminate adjacent cultures.
9. Seal the flask with parafilm, pierce it using micropipette tips to ensure aeration, clamp the flask on an orbital shaker, and shake it at 50-100 rpm for 72-96 h at 26-28 ºC.

   Although oocyst walls are relatively resistant, if shaking is too vigorous during sporulation it may result in oocyst breakage, and therefore, loss of viable parasites.
10. During sporulation check oocysts microscopically to estimate sporulation rate using a counting chamber. Rates over 85% are expected.
11. Once sporulation has finished, pour the oocyst suspension into centrifuge bottles or 50 ml tubes and centrifuge (750 x g for 10 minutes). Carefully discard the supernatant using a serological pipette and check microscopically that all oocysts have been pelleted.

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

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

12. Wash the oocyst pellet three times with distilled water by centrifugation (750 x g for 10 minutes), checking microscopically that all oocysts have been pelleted from all supernatants. Aim to constantly reduce the volume.
13. Re-suspend the pellet in 5-10 ml of 1.2% sodium hypochlorite solution in a 50 ml tube. Treat for 5-10 minutes and swirl intermittently.
14. Top up the vessel with distilled water, mix and centrifuge to pellet the oocysts (750 x g for 10 minutes). Carefully discard the supernatant using a serological pipette and check microscopically that all oocysts have been pelleted.
15. Re-suspend the pellet in SSS thoroughly using a Pasteur pipette and leaving no lumps.
16. Fill the 50 ml tube to 40 ml with SSS and overlay with distilled water by gently running the water down the side of the tube with a Pasteur pipette to 45 ml. Then centrifuge at 750 g for 10 minutes.
17. Collect the parasites at the interface between the salt and water phases with a Pasteur pipette (Figure 7B). Dispense into a suitable tube and add at least double the volume of water. After the oocyst layer has been removed, check the pellet at the bottom of the tube microscopically for any remaining oocysts. If a significant number are observed, then repeat the salt flotation as in step 14.
18. Centrifuge recovered oocysts at 750 x g for 10 minutes, discard supernatant and re-suspend in water. Repeat this two more times, checking microscopically that all oocysts have been pelleted from all supernatants.
19. After the final wash, re-suspend the oocysts in a suitable volume of distilled water and enumerate using a cell counting chamber (see BASIC PROTOCOL 1: *Cracking, hatching and purification of* Eimeria tenella *parasites*).
20. Once purified, oocysts can be stored at 4 °C in a universal tube (preferably made of glass) and used for up to 6 months.

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

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

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

REAGENTS AND SOLUTIONS

Sterile saturated salt solution (SSS, 6.4 M NaCl)

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

Sterile phosphate buffered saline (PBS)

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

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 ºC.
698 5. Remove supernatant, leaving a small amount of PBS on top of the DE-52, and autoclave solution
699 (121 ºC, 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 ºC. 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 ºC 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 (Bussière 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, Dzierszinski, 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 glycophosphatidylinositol (GPI) anchor sequence from the *E. tenella*

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

Present limitations for transfection in *Eimeria* parasites

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

ACKNOWLEDGMENTS

This manuscript has been assigned the reference PPS_01830 by the Royal Veterinary College, UK. This work was partially funded by an Industrial Partnership Award (IPA) from the Biotechnology and Biological Sciences Research Council (BBSRC) in partnership with MSD Animal Health under the reference BB/H020195/1, and by the SAPHIR project from the European Union's Horizon 2020

Programme for research, technological development and demonstration under the Grant Agreement n°633184. This publication reflects the views only of the authors, and not the European Commission (EC). The EC is not liable for any use that may be made of the information contained herein.

LITERATURE CITED

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

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

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 highly protective antigens for an apicomplexan parasite. *PLoS Pathog, 7*(2), e1001279. doi:10.1371/journal.ppat.1001279

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

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

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

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

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 of Eimeria species. *Mol Biochem Parasitol, 162*(1), 77-86. doi:10.1016/j.molbiopara.2008.07.006

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 vaccine delivery vectors: anti-Campylobacter jejuni protective immunity induced by Eimeria tenella-delivered CjaA. *Vaccine, 30*(16), 2683-2688. doi:10.1016/j.vaccine.2012.02.002

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

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 Strains. *Proc Helminthol Soc Wash, 41*, 77-80.

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

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

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

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

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

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. *Mol Biochem Parasitol, 153*(2), 213-215. doi:10.1016/j.molbiopara.2007.02.005

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

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

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

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

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

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

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

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

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 yellow fluorescent protein (M2e-EYFP). *Vet Parasitol, 193*(1-3), 1-7. doi:10.1016/j.vetpar.2012.12.019

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

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

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

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

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 and the endogenous development of the transgenic parasite. *PLoS One, 9*(12), e114188. doi:10.1371/journal.pone.0114188

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 of E. mitis. *Parasit Vectors, 9*, 164. doi:10.1186/s13071-016-1451-3

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 coccidiosis in domestic chickens. *Genome Res, 24*(10), 1676-1685. doi:10.1101/gr.168955.113

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

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

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, Reproduction and Immunogenicity. *Front Microbiol, 7*, 807. doi:10.3389/fmicb.2016.00807

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

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

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

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

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

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

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 Expressing Eimeria maxima Antigen Protects Chickens against Infection of the Two Parasites. *Front Immunol, 8*, 1982. doi:10.3389/fimmu.2017.01982

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

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

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

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 molecule throughout the life cycle. *Int J Parasitol, 39*(1), 109-117. doi:10.1016/j.ijpara.2008.06.013

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

TABLES

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

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

Table 2. The set of programmes assessed for optimization of transfection in *E. tenella* using the Amaxa™ 4D-Nucleofector™ System. Transfection was carried out using $1x10^6$ freshly purified sporozoites, 5 µg of plasmid DNA carrying the mCitrine reporter, and 0.5 U of the *Sca*I restriction enzyme per well, and assessed by visual confirmation of sporozoite fluorescence. ✕: shock failure. ✓**:** shock successful. **NF:** no fluorescent sporozoites observed. **F:** fluorescent sporozoites observed.

AMAXA™ 4D Program	**Cytomix buffer**	**P1 buffer**	**P3 buffer**	**P4 buffer**
EO-115	✕, NF	✓, NF	✓, NF	✓, NF
FI-115	✕, NF	✓, NF	✓, NF	✓, NF
FP-167	✕, NF	✓, NF	✓, NF	✓, NF
FP-158	✕, NF	Not tested	✓, NF	Not tested
FB-158	✕, NF	Not tested	✕, NF	Not tested
EZ-158	✕, NF	✓, NF	✓, NF	✓, NF
FI-158	✕, 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
EO-114	Not tested	✓, NF	**✓, F**	✓, NF
FB-115	Not tested	✕, NF	✓, NF	✓, NF
FA-115	Not tested	✓, NF	✓, NF	✓, NF

FIGURES AND FIGURE LEGENDS

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

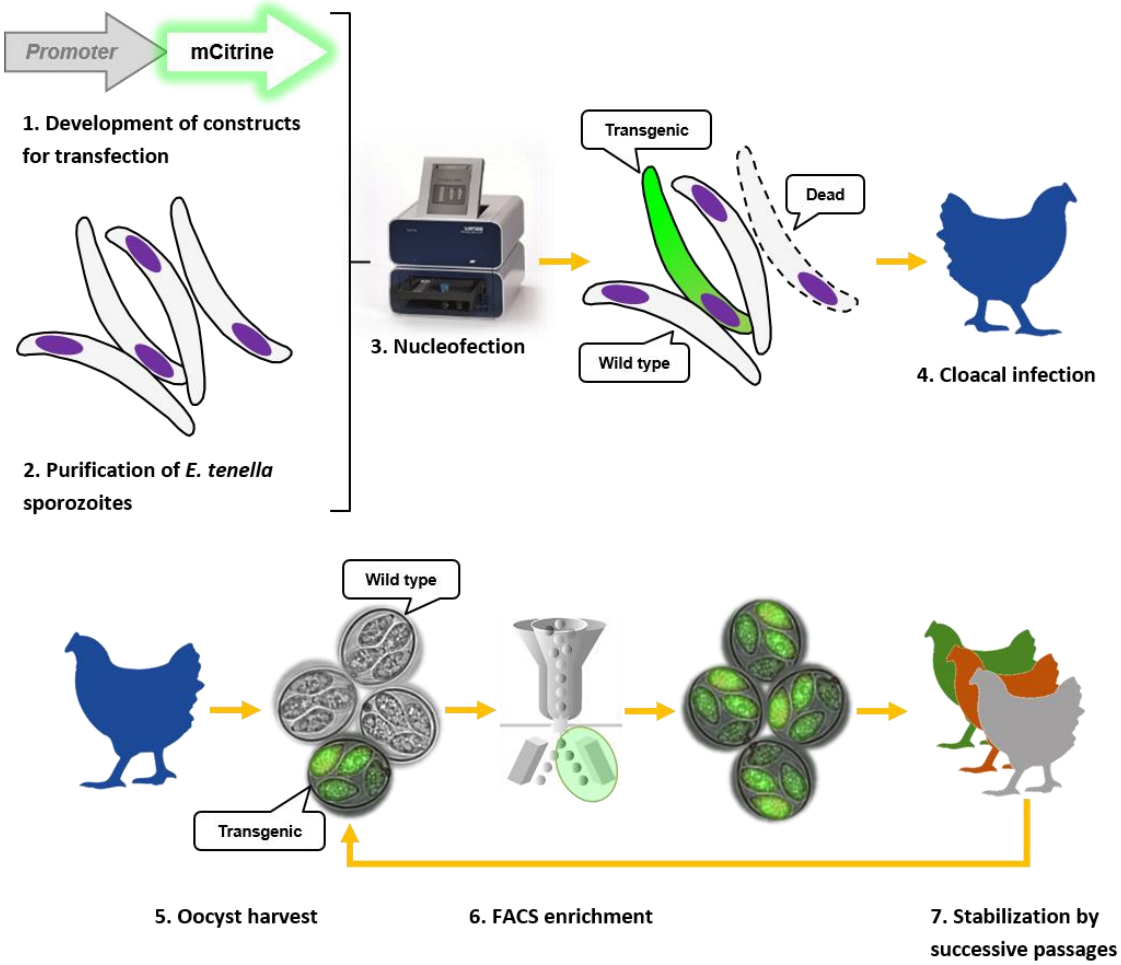

Figure 2. Effect of p*Eten*REPORTER plasmid concentration on percentage of fluorescent oocysts recovered after *in vivo* passage using coccidia-free chickens. Groups marked with ***** were significantly different ($P<0.05$; Fisher's exact test).

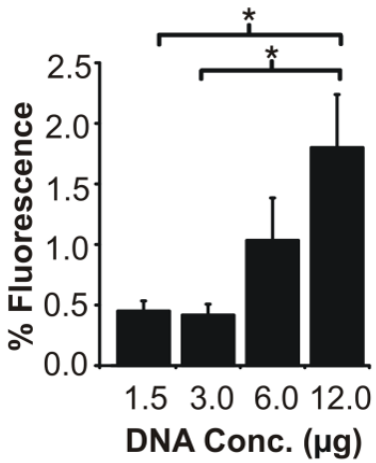

Figure 3. Effect of *PsiI* and *ScaI* restriction enzymes for REMI using either 5.0 or 3.5 µg of digested plasmid DNA. **A:** Percentage survival of sporozoites immediately after transfection by Trypan blue exclusion. **B:** Relative fluorescence intensity of sporozoites 24 h after transfection. **C:**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

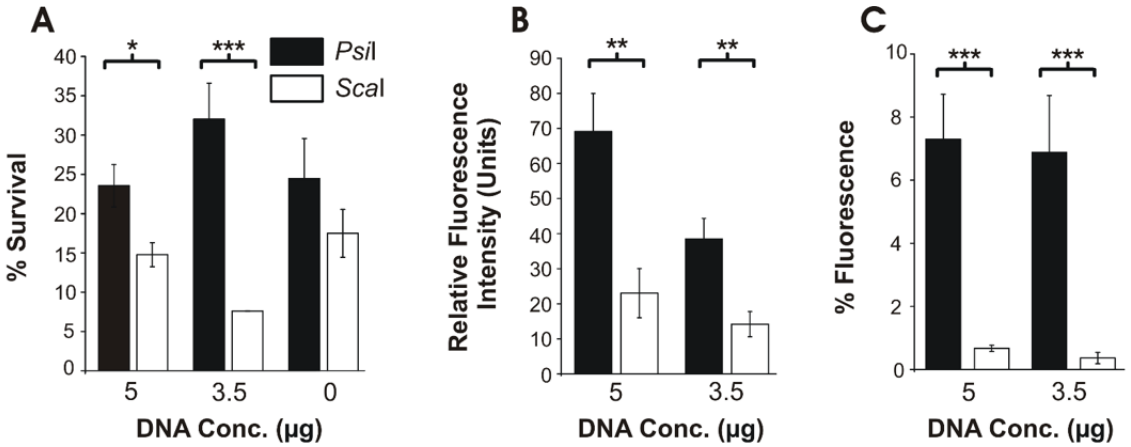

Figure 4. Effect of repeated *in vivo* passage on transgenic *E. tenella* populations. Six different batches of sporozoites were transfected with 12 µg of the same *Sca*I-digested construct (pCIT-CjaA plasmid containing the mCitrine sequence and an additional cassette coding for the *Campylobacter jejuni* antigen A, from Clark et al., 2012) and the *Sca*I endonuclease using the EO-114 program (AMAXA™ 4D-Nucleofector™ System). Transgenic parasites were independently propagated up to four times in coccidia-free birds using FACS-enriched populations between passages. **A:** Average transgene copy number from the six different populations as determined by quantitative PCR using *mCitrine* as target and the *Et5S* gene as internal control (Log10 scale). **B:** Percentage of fluorescent parasites out of the total harvested parasites per passage as visualised under fluorescence microscopy.

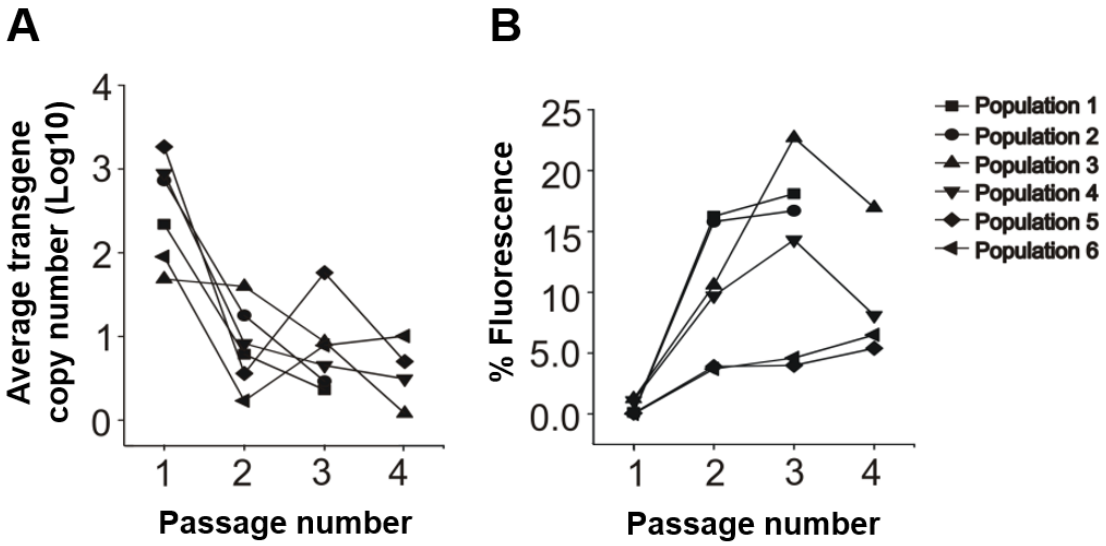

Figure 5. A: Photomicrographs of oocysts cracked using glass beads. The yellow box delimits an area full of oocyst shells. The pink arrow points to a non-cracked oocyst, white arrows indicate released and unaltered sporocysts, and the yellow arrows show damaged sporocysts due to excessive cracking. Bars represent 20 µm. **B:** Representation of a column for purification of sporozoites using teased out nylon wool and DE-52 cellulose, and the appearance of purified sporozoites under the microscope (bar represents 20 µm).

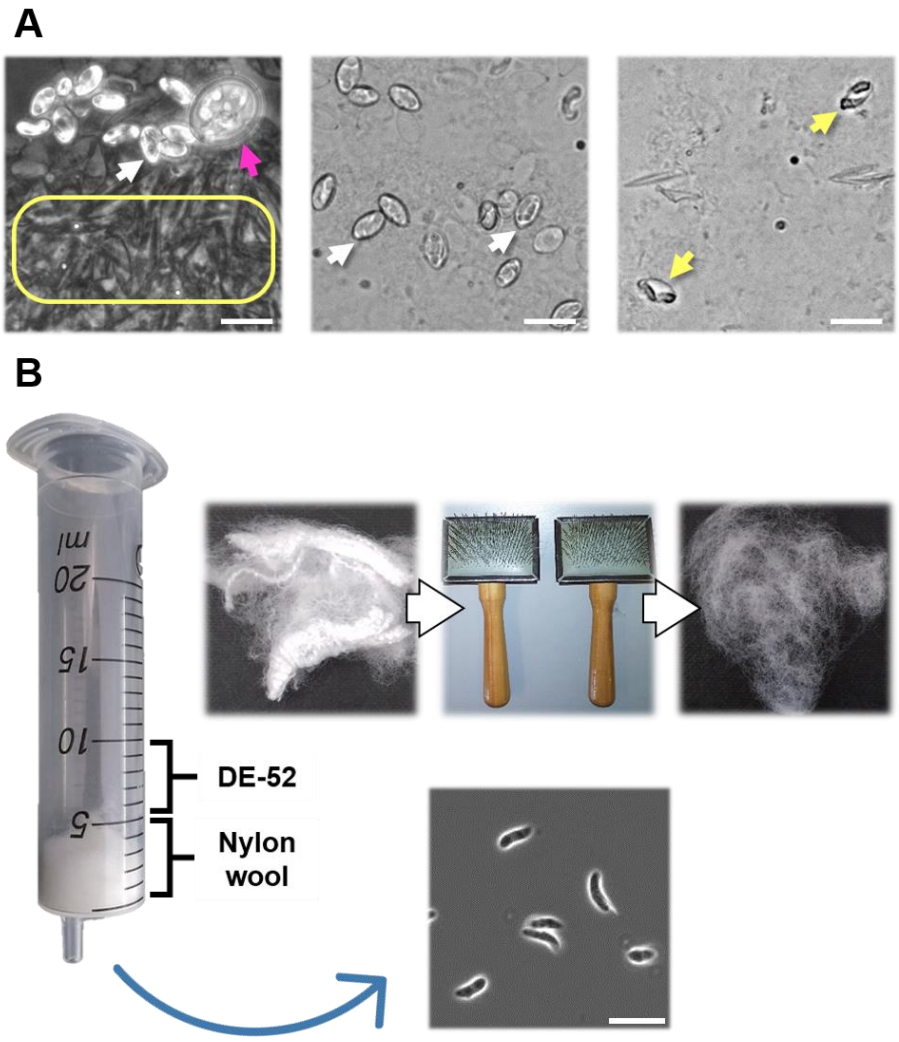

Figure 6. *In vitro* culture of transgenic *Eimeria tenella* Wisconsin parasites expressing the mCitrine reporter in MDBK cells. **A:** intracellular sporozoites after 24 h of infection. Only those successfully transfected show green fluorescence. **B:** late development schizonts showing green fluorescence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

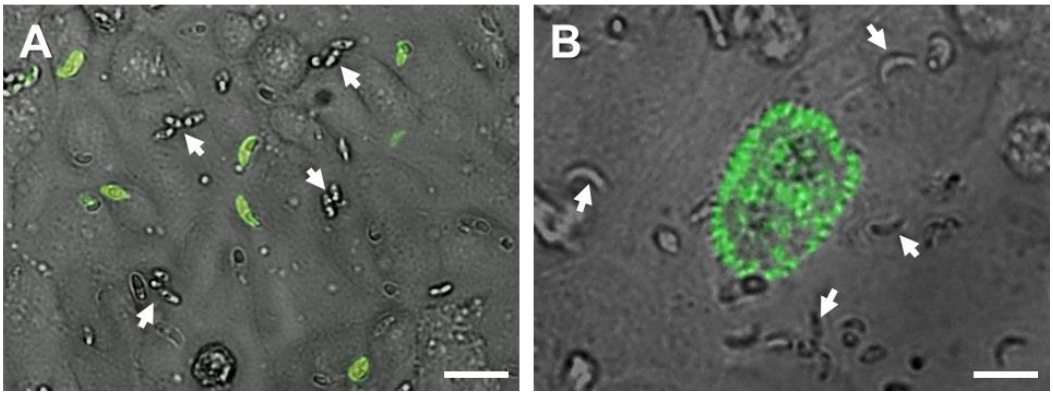

Figure 7. A: Schematic representation of caeca scrapping technique. **B:** Flotation of *E. tenella* oocysts after sodium hypochlorite treatment using saturated salt solution (SSS). Oocysts are present at the interface between the SSS and water phases.

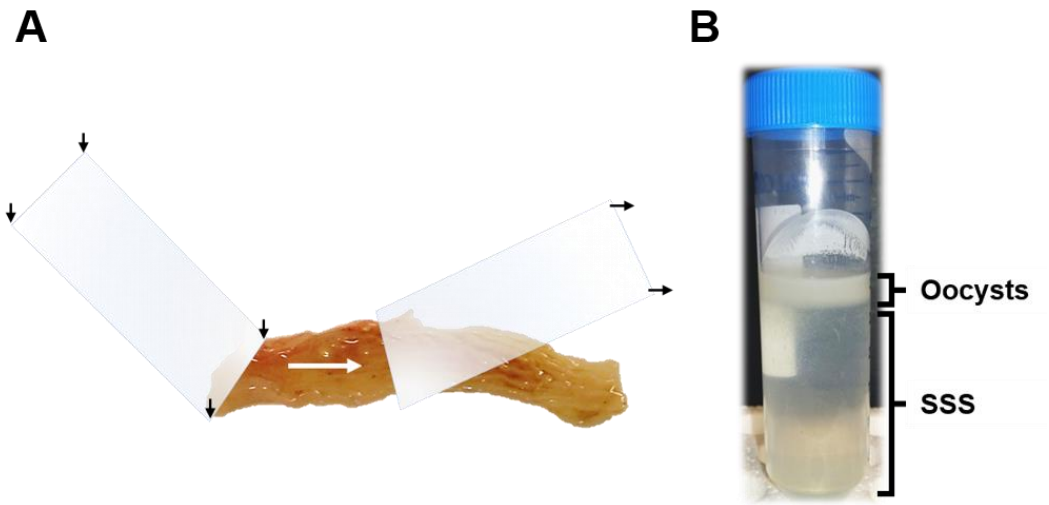

Figure 8. Flow cytometry analysis of transgenic *E. tenella* parasites expressing the mCitrine reporter after first *in vivo* passage. **Top:** exclusion of cell debris by SSC-A/FSC-A gating. **Bottom:** selection of FITC-positive parasites for sorting. Red arrow and box: transgenic parasites expressing mCitrine (note that they represent a minority among the whole population, ~1.4%). SSC-A: side scattered area; FSC-A: forward scattered area; FITC-A: Fluorescein isothiocyanate area.