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TITLE:

Nucleofection and In Vivo Propagation of Chicken *Eimeria* Parasites

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KEYWORDS:

Transfection, merozoites, sporozoites, *Eimeria,* tool, inoculation

SUMMARY:

Here, we provided a method to achieve stable transfection of chicken *Eimeria* parasites by nucleofecting sporozoites or second-generation merozoites. Genetically modified eimerian parasites expressing heterologous antigenic genes could be used as vaccine delivery vehicles.

ABSTRACT:

Transfection is a technical process through which genetic material, such as DNA and double-stranded RNA, are delivered into cells to modify the gene of interest. Currently, transgenic technology is becoming an indispensable tool for the study of *Eimeria*, the causative agents of coccidiosis in poultry and livestock. This protocol provides a detailed description of stable transfection in eimerian parasites: purification and nucleofection of sporozoites or second-generation merozoites, and in vivo propagation of transfected parasites. Using this protocol, we

achieved transfection in several species of *Eimeria.* Taken together, nucleofection is a useful tool to facilitate genetic manipulation in eimerian parasites.

INTRODUCTION:

Eimeria spp. causes coccidiosis, which leads to substantial economic losses in the livestock and poultry industry. Although anticoccidial drugs, and to an extent, attenuated anticoccidial vaccines, have been used widely for the control of coccidiosis, there are still shortcomings regarding their drug resistance, drug residues, and the potential diffusion of vaccine strains that regain virulence[1]. With the development of molecular biology, transfection has become a vital tool for studying gene functions, developing novel vaccines, and screening new drug targets for *Eimeria*.

In the last decades, transfection has been applied successfully for apicomplexan parasites such as *Plasmodium* and *Toxoplasma gondii*[2-6]. A study using β-gal as a reporter for the transfection in *E. tenella* piloted such work in *Eimeria*[7]. The transfection of *E. tenella*[8,9], *E. mitis*[10,] and *E. acervulina* (Zhang et al., unpublished data) was successful in chickens. Recently, we achieved transfection using merozoites of *E. necatrix* through nucleofection[11].

Studies showed that *Eimeria* expressing a heterologous antigen has the potential to be developed as a recombinant vaccine, such as those expressing *Campylobacter jejuni* antigen A (CjaA) or chicken interleukin 2 (chIL-2)[12,13]. Therefore, this protocol describes a nucleofection study of *Eimeria* spp. in chickens. The procedure describes purification of sporozoites or merozoites, nucleofection with plasmid DNA, cloacal inoculation/intravenous injection and in vivo propagation to help researchers starting studies on transgenic *Eimeria* parasites.

PROTOCOL:

Chickens for all animal experiments were housed and maintained according to the China Agricultural University Institutional Animal Care and Use Committee guidelines and followed the International Guiding Principles for Biomedical Research Involving Animals. The experiments were approved by the Beijing Administration Committee of Laboratory Animals.

1. Extraction and purification of sporozoites of *Eimeria* spp. (e.g., *E. tenella*)

1.1 Release of sporocysts

1.1.1 Centrifuge 1 x 10^7 sporulated oocysts in potassium dichromate solution (2.5%, m/v) at 2,300 x *g* for 5 min. Wash them with PBS (phosphate buffer solution) three times.

1.1.2 Resuspend the pellets with 1 mL of PBS and transfer to a 15 mL tube. Add an equal volume of glass beads (1 mm x 1 mm diameter range) and oscillate the oocyst suspension using a vortex mixer to release the sporocysts.

1.1.3 Monitor the release of sporocysts by microscopy every minute. Stop vortexing when more than 90% of oocysts are broken.

NOTE: Most of the oocysts (such as *E. tenella, E. necatrix,* and *E. acervulina*) were broken after 1 min using the vortex mixer.

1.1.4 Transfer the sporocyst suspension to new 1.5 mL tubes and centrifuge at 1,600 x *g* for 5 min.

1.1.5 Resuspend the precipitate with 1 mL of 50% density gradient solution, combine in a 1.5 mL tube, and centrifuge at 10,000 x *g* for 1 min.

NOTE: For the density gradient composition, refer to **Table 1**. The density gradient is a silica-based colloidal medium, consisting of colloidal silica particles of 15–30 nm diameter (23% w/w in water), which have been coated using polyvinylpyrrolidone (PVP).

1.2 Release of sporozoites

1.2.1 Resuspend the precipitate with the excystation buffer (**Table 1**) and incubate in a 42 °C water bath for 40-60 min to release the sporozoites. Stop incubating when more than 90% of sporozoites are released. Then centrifuge at 600 x *g* for 10 min.

NOTE: Shake the tubes once every 5 minutes during excystation.

1.2.2 Resuspend the precipitation with 1 mL of 55% density gradient solution and centrifuge at 10,000 x *g* for 1 min.

1.2.3 Resuspend the precipitation with 1 mL of PBS and count the sporozoites using a hemocytometer.

2. Collection and purification of merozoites of *E. necatrix*

NOTE: Use Arbor Acre (AA) broilers aged 7-14 d in the experiment. Coccidia-free chickens (n=3) were inoculated with 2 x 10^5 oocysts of *E. necatrix.* At 109 h post-infection, the birds were sacrificed by cervical dislocation. The intestine was removed for the collection of the 2nd generation merozoites. For different *Eimeria* species, there was a different time for collection of the 2nd generation merozoites: *E. necatrix* at 109 h, and *E. tenella* at 112 h post-inoculation. For the transfection of *E. necatrix*, merozoites are the optimal choice as the second merozoites are easy to purify.

2.1 Collection of the second-generation merozoites of *E. necatrix*

2.1.1 Cut the chicken intestine longitudinally, from the yolk stalk (the middle of the small intestine) to the ileocecal orifice, and wash it with PBS or HBSS (Hank's Balanced Salt Solution) gently three times in a Petri dish.

2.1.2 Cut the intestine into 0.5 cm x 0.5 cm pieces and place it in a conical flask with a digestion buffer (**Table 1**). Place the flask on a magnetic mixer at 37 °C with a stirring bar for 30-60 min to release merozoites. After 30 min of incubation, monitor the release of merozoites by microscopic examination every 5 min.

2.2 Purify the merozoites by filtration and centrifugation.

2.2.1 Filter the suspension containing digested merozoites using four layers of gauze[14], and centrifuge at 600 x *g* for 10 min.

2.2.2 After centrifugation, discard the supernatant containing intestine debris. Transfer the precipitation with purified merozoites to 1.5 mL tubes.

2.2.3 Resuspend the precipitation with 1 mL of PBS and count the merozoites using a hemocytometer.

3. Nucleofection of merozoites or sporozoites

3.1 Preparation before nucleofection of parasites

3.1.1 Prepare about 10^7 merozoites or sporozoites in one tube. If transfecting merozoites, prepare 3-4 tubes.

3.1.2 Prepare an amount of plasmid DNA or purified PCR fragment that is greater than or equal to 10 µg.

NOTE: The plasmid used in this study contains 2 genes: enhanced yellow fluorescent protein (EYFP) and dihydrofolate reductase thymidylate synthase derived from *Toxoplasma gondii* (TgDHFR-TS)[15].

3.1.3 Prepare 25 U of restriction enzyme. If plasmids are linearized, the restriction enzyme can improve the transfection efficiency. If the plasmids are circular, omit the restriction enzyme.

3.1.4 Prepare 85 µL of nucleofection buffer: mix 20 µL of nucleofection buffer I and 1 mL of nucleofection buffer II, and use a part of the solution. The volume of the total buffer is 100 µL.

3.2 Nucleofection

3.2.1 Centrifuge the sporozoite or merozoite suspension at 600 x *g* for 10 min. Then discard the supernatant.

3.2.2 In the following order, add 85 µL of nuclear transfection buffer, 10 µg of plasmid (PCR fragment), and 25 U of restriction enzyme (usually 5 µL) into the 1.5 mL tube containing sporozoites or merozoites.

3.2.3 Transfer the suspension to a nuclear transfection cup. Put the cup into a nuclear transfer groove.

3.2.4 Turn on the nucleofection device by using the power button and select the transfection procedure **U-033**. If the nucleofection device starts in the **Free Program Choice** mode, exit this mode by pressing the **X** button.

3.2.5 When the program finishes, press the **X** button of the nucleofection device, and the screen should display **OK**, indicating that the nucleofection is successful.

3.2.6 Add 0.5-1 mL of Dulbecco's Modified Eagle's Medium (DMEM)[8] to the nucleofection cup to stop the reaction and transfer the suspension to 1.5 mL tube after mixing gently.

4. Cloacal inoculation or intravenous injection

4.1 Inoculate the nucleofected parasites into 7-day-old chickens. Inoculate the merozoites of *E. necatrix* or the sporozoites of *E. tenella* via the cloacal route, but inoculate *E. acervulina* sporozoites via intravenous injection. Inoculate about 2 x 10^7 million sporozoites into each chicken, and incoluate merozoites 10^7 for each bird.

5. Propagation and FACS sorting

5.1 Collect oocysts from feces 5–9 days post-inoculation with transfected sporozoites. Collect the oocysts on the third day after inoculating with transfected merozoites.

5.2 Use fluorescence-activated cell sorting (FACS) and 150 mg/kg pyrimethamine[8] to successively increase the transgenic population ratio.

NOTE: Use pyrimethamine by adding it directly in the feed. For more convenient use, prepare water-soluble pyrimethamine. Dissolve 1 g of pyrimethamine in 0.2 mL pf H_2SO_4 and 9.8 mL of N-methyl pyrrolidone (NMP), and then add 1.5 mL of this stock solution into 1 L of drinking water for birds.

6. Optional column purification

NOTE: If more pure sporozoites or merozoites are needed, there is an optional method that purifies them through a diethylaminoethyl-52 cellulose (DE-52 cellulose) column.

6.1. Prepare the DE-52 cellulose column at least one day in advance.

6.1.1. Prepare glycine eluent buffer (**Table 1**). Adjust the pH of glycine eluent buffer from 7.6 to 8.0 and prewarmed to 41 °C.

6.1.2. Add 2.5 g of DE-52 cellulose to the column. Add water and soak overnight. Discard the supernatant.

6.1.3. Add water and soak for 1 h. Discard the supernatant.

6.1.4. Add 0.1 M NaOH and soak for at least 2 hours. Repeat this step.

6.1.5. Replace the supernatant with water. After the cellulose completely settles to the bottom (about half an hour), repeat this step.

6.1.6. Discard the supernatant, add 0.1 M HCl, and soak for at least 2 hours. Repeat this step.

6.1.7. Discard the supernatant, and soak the cellulose twice with glycine eluent buffer.

6.1.8. Measure and adjust pH from 7.6 to 8.0 by adding 0.1 M HCl or 0.1 M NaOH.

NOTE: In this part, the liquid has at least 5x more volume than that of DE-52 cellulose.

6.2. Adjust the flow rate between 40-50 r/min.

6.3. When the sedimentation of cellulose is completed, add the sporozoite or merozoite suspension to the chromatographic column. Adjust the flow rate to 30-40 r/min.

NOTE: Resuspend the sporozoite or merozoite precipitation with glycine eluent buffer before adding the chromatographic column.

6.4. Collect with glycine eluent buffer into 50 mL tubes. Stop the collection according to the results of the microscopic examination of sporozoites or merozoites during the elution process.

6.5. Centrifuge the glycine eluent buffer collected at 600 x *g* for 10 min. Transfer the sporozoite or merozoite precipitation to new 1.5 mL tubes.

6.6. Count the sporozoites or merozoites using a hemocytometer.

REPRESENTATIVE RESULTS:

This protocol has been used to transfect eimerian parasites. In this study, the 2nd generation meronts and merozoites of *E. necatrix* were shown in **Figure 2A** and **Figure 2B**, while **Figure 2C** and **Figure 2D** showed the sporocysts and sporozoites of *E. tenella* after using the density gradient solutions. The oocysts of *E. necatrix* (**Figure 3A**) *and E. tenella* (**Figure 3B**) after nucleofecting the merozoites or sporozoites were also shown. The transfection efficiency of first-generation oocysts after nucleofecting the sporozoites is about 3-10%, in general. However, after nucleofecting the merozoites, the transfection efficiency of second-generation oocysts is only a few thousandths (Transfection efficiency of first-generation oocysts could not be calculated).

FIGURE AND FIGURE LEGENDS:

Figure 1. Purification of sporozoites. (**A**) Purify sporozoites through the density-gradient centrifugation with density gradient solutions. (**B**) Purify sporozoites through the DE-52-cellulose column.

Figure 2. The 2nd generation meronts and merozoites of *E. necatrix* along with the sporocysts and sporozoites of *E. tenella.* (**A**) Mature 2nd generation meronts of *E. necatrix.* (**B**) The released 2nd generation merozoites of *E. necatrix*. (**C**) Sporocysts of *E. tenella* after purification. (**D**) Sporozoites *E. tenella* after purification. The scale bar is 10 μm.

Figure 3. The oocysts obtained after infecting with the nucleofected merozoites or sporozoites. (**A**) The oocysts of *E. necatrix* after infecting with the nucleofected merozoites. (**B**) The oocysts of *E. tenella* after infecting with nucleofected sporozoites. The scale bar is 10 μm.

Table 1: Composition of Buffers.

DISCUSSION:

In the 1990s, a transfection system was developed for apicomplexan parasites, and it was used for studies on eimerian parasites. Recently, stable transfection was conducted in *E. tenella*[8,9] and *E. nieschulzi*[15]. We achieved the stable transfection of *E. necatrix* by transfecting second-generation merozoites[11]. Inoculation of transfected sporozoites of *E. acervulina* through the wing vein resolved the the inability of sporozoites of *E. acervulina* to be inoculated via the cloacal route (Zhang et al., unpublished data). Here, we described a detailed transfection procedure to help researchers nucleofect eimerian parasites.

Previous studies showed that it was feasible to inject transfected sporozoites into the intestinal lumen of rabbits in a laparotomy for in vivo stable transfection of *E. magna*[16] and *E. intestinalis*[17]. According to our experience, there was higher efficiency when sorting sporocysts by FACS instead of oocysts. There were also reports about transfection of unsporulated oocysts of *E. maxima* using a gene gun system or successful electroporation of sporulated oocysts with eGFP-Ham-OTU RNA[18,19]. Thus, our future studies explore transfection of oocysts or sporocysts to simplify the transfection procedures in *Eimeria* parasites.

The transfection success in eimerian parasites could enable genetically modified *Eimeria* to be used as vaccine vehicles to carry heterologous antigens, such as CjaA from *C. jejuni*[13]. Although transfection efficiency in *Eimeria* has been significantly improved, gene editing technology continues to have limitations in eimerian parasites. With the development of transfection in *Eimeria*, CRISPR/CAS9 technology in *Eimeria* (Hu et al., unpublished data) could lead to genetic manipulation of *Eimeria*.

In conclusion, this protocol provides a detailed procedure for nucleofection in chicken *Eimeria*. The transfection of sporozoites or merozoites is valuable for the study of gene function in *Eimeria.*

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DISCLOSURES:

None.

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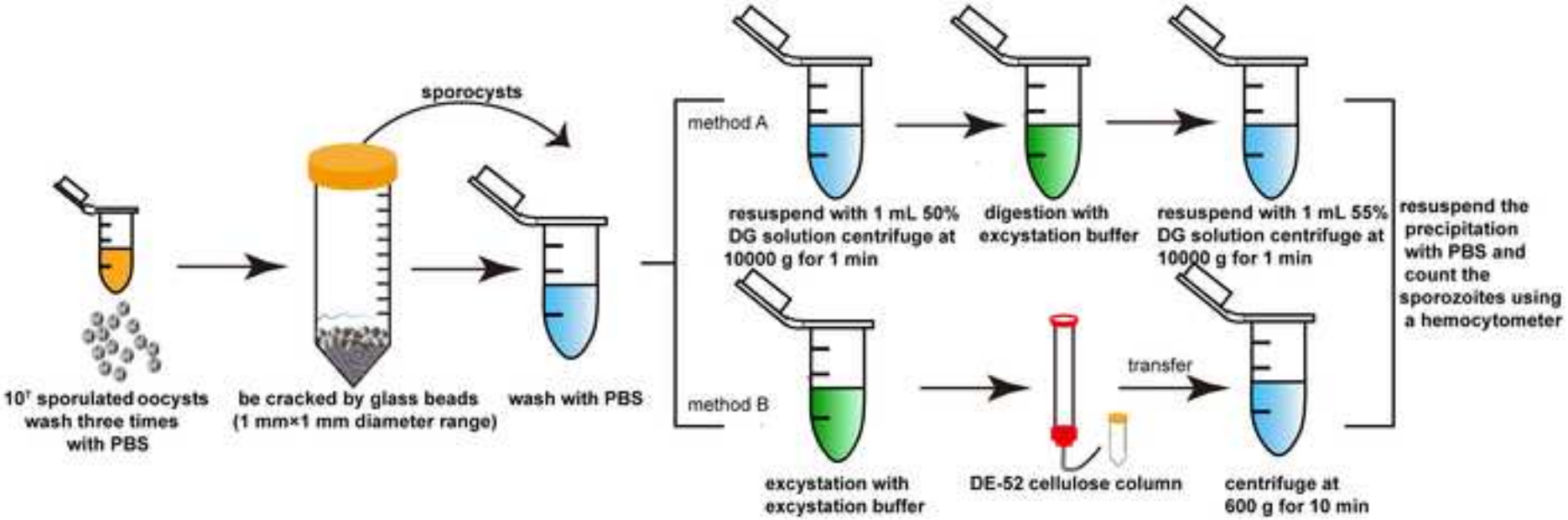
Figure



Figure

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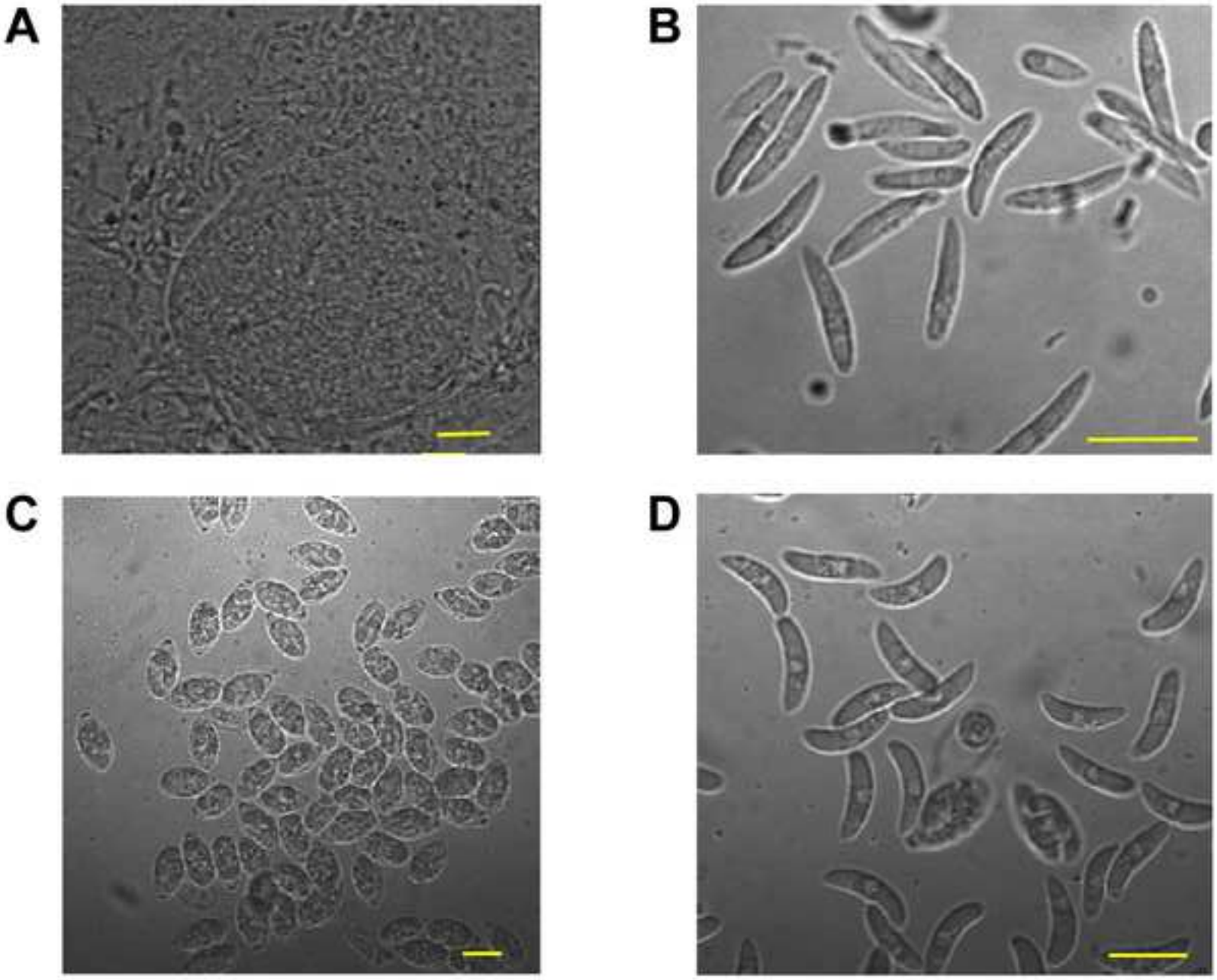

Figure

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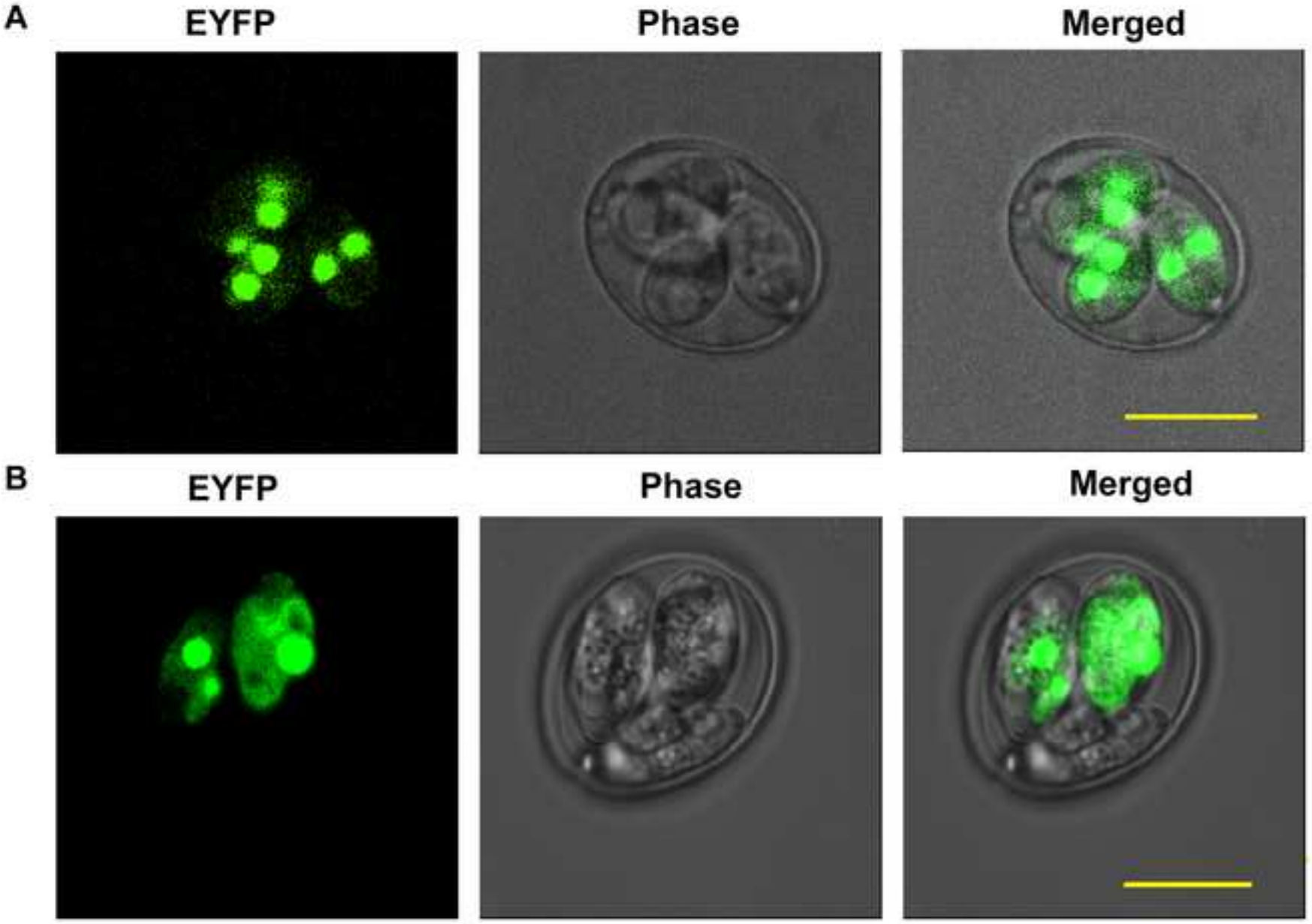

Table 1. Composition of buffers

Buffer
Digestion buffer
0.1 M NaOH
0.1 M HCl
Glycine eluent buffer
Excystation buffer
1 × DG gradient stock solution(Percoll)
Nucleofection buffer I
Nucleofection buffer II

*water：distilled water

Composition
0.25 g trypsin, 0.5 g sodium taurodeoxycholate hydrate, 100 ml PBS or HBSS
2 g NaOH, 500 ml water
4.3 ml concentrated hydrochloric acid, 500 ml water
0.75 g glycine, 7.9 g NaCl, 500 ml water
0.75% trypsin, 10% chicken bile, PBS
90% 1 × DG gradient stock solution(Percoll), 10% PBS (10 X)
ATP-disodium 2 g, $MgCl_2$-$6H_2O$ 1.2 g, 10 ml water
KH_2PO_4 6 g,$NaHCO_3$ 0.6 g, glucose 0.2 g, 500 ml water

Name of Material/Equipment	Company
ATP-disodium	Sigma
Cellulose DE-52	Solarbio
Constant Flow Pump	SHANGHAI JINGKE INDUSTRIAL CO., LTD.
DMEM	MACGENE
Glass beads	Sigma
Glucose	Sigma
Glycine	Biotopped
HBSS	MACGENE
KH_2PO_4	Sigma
Low Speed Centrifuge	BEIJING ERA BEILI CENTRIFUGE CO.,LTD
Magnetic Mixer	SCILOGEX
$MgCl_2$	Sigma
MoFlo cell sorter	BeckMan Coulter, US
$NaHCO_3$	Sigma
Nucleofection device	LONZA/amaxa
PBS	Solarbio
Percoll (DG gradient stock solution)	GE Healthcare
Sodium taurodeoxycholate hydrate	Sigma
Sorvall Legend Micro 17 Microcentrifuge	ThermoFisher Scientific
The composition of DMEM: 4.5 g/L glucose with sodium pyruvate, L-glutamine, and 25 mM HEPES.	
Trypsin	Solarbio
Vortex Mixer	Beijing North TZ-Biotech Develop.co.
Water Bath Thermostat	Grant Instruments(Cambridge) Ltd

Catalog Number	Comments/Description
A26209	
C8350	
HL-2B	
CM15019	
Z250473-1PAK	
No. V900116	
G6200	
CC016	
No. V900041	
DT5-2	
MS-H280-Pro	
449164	
201309995	
144-55-8	
90900012 (Nucleofector Ⅱ)	
P1010	
17-0891-09	
T0875	
75002430	
T8150	
HQ-60-Ⅱ	
GD120,GM0815010	

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Response: We are very grateful to you for pointing out this issue for protocol writing. We followed your suggestions and rewrote the protocol section in the imperative tense.

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11. Figure 1: Please remove "percoll" from the figure and use generic description. Please use a single space between numerical values and their units. Please use mL instead of ml. Please use x g instead of g.

Response: We corrected these errors as required.

12. There is a 2.75 pages limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: We highlighted the protocol steps in yellow for the video preparation.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript is in the scope of Journal of Visualized Experiments. This paper is dealing with the experimental procedure to transfect sporozoites and second-generation merozoites of the avian coccidia, *Eimeria necatrix*. Authors described carefully the protocol to purify each parasite stage and to run transfection. Using drug-selection during in vivo propagation and fluorescent-selection by FACS, authors obtained stably transfected parasites. This is a useful method to produce genetically modified *Eimeria* parasites, which could be used as vaccine vehicles.

Major Concerns:

1. This article is well written and the material and method section is clear (except inoculation route, see below). Although, the originality of the research is poor (stable transfection of *Eimeria* species of sporozoites and merozoites has been regularly published since the very first work: Clark et al., "A toolbox facilitating stable transfection of *Eimeria* species", Molecular & Biochem. Parasitol. 2008, 162:77-86), the main criticism concerns the way parasites are inoculated to chicken. The intravenous injection is cited for *E. necatrix* propagation. This procedure deserves to be fully argued. To our knowledge, except in Long and Rose 1965, Exp. Parasitol. 16:1-7, this procedure has never been used since. Moreover, the cloacal route was used successfully for stable transfection of E. *necatrix* (see Duan *et al.*, "Stable transfection of *Eimeria necatrix* through nucleofection of second-generation merozoites", Molecular & Biochem. Parasitol. 2019, 228:1-5), published by the same lab. This is in contradiction with present results "In the study, inoculation of transfected sporozoites of *E. acervulina* by wing vein solved the problem that sporozoites of *E. necatrix* and *E. acervulina* could not be inoculated via cloacal route" (lines 272-273).

Response: Thanks a lot for pointing out this mistake we made during the preparation of the MS. Now we corrected the description of steps for Cloacal inoculation or intravenous injection.

2. A second major point concerns the result section: Figures 2 and 3 are of poor quality and cannot be published as is.

Response: We replaced the 2 figures with newly prepared ones.

Minor Concerns:

Line 115: please add, "by cervical dislocation" at the end of the sentence "the birds were sacrificed", and delete line 123.

Response: Thank you. We added "by cervical dislocation" at the end of the sentence "the birds were sacrificed", and delete line 123.

Line 139: please add, the reference for the gauze used.

Response: We cited the reference (Eckert, J. *et al.*, 1995) in the revised manuscript.

Line 151: please, replace the volume of restriction enzyme by the unit used.

Response: We have replaced "5 μL" by "25 U".

Line 183: what is the individual dose for each chicken?

Response: We added the description of “The dose of sporozoites for each chicken is about 2 million，and the merozoites were about 10^7”.

Line 190: how was administered pyrimethamine? Was it in food, in the water?
Response: We added a note in our revised manuscript: We use pyrimethamine by adding it directly in the feed. For more convenient use, we prepare water-soluble pyrimethamine (1 g pyrimethamine is dissolved in 0.2 mL H_2SO_4 and 9.8 mL N-methyl pyrrolidone (NMP), then 1.5 mL of this stock solution is added into 1 L drinking water for birds).

Line 191: the drug-selection by pyrimethamine implies that the vector used for transfection contains the DHFR-TS gene. Please indicate this earlier in the manuscript.
Response: Thanks a lot for this suggestion. We added a sentence to descript the selection marker in the plasmid in our revised manuscript (lines 150 and 151).

Lines 229-237: no volume is indicated for any of these steps. Please precise each volume.
Response: We added a note to explain it.

Line 246: at the end of the result section, authors could give the yield of positive transfected parasite obtained at first and the second passage in chicken.
Response: Thank you for this suggestion. We added the transfection efficiency obtained at first and second passages (line 244-247).

Line 260: in Table 1 or Table 2, please add the reference and the composition of DMEM medium.
Response: We added the information in Table 2.

Line 262: in Table 2, please add pyrimethamine reference.
Response: The reference has been cited in the revised manuscript (*Clark* et al., 2008, reference [8]).

Line 270: the first work dealing with stable transfection of *E. tenella* was Clark et al., 2008. Please cite this reference.
Response: We corrected the mistake and cited in the revised manuscript (reference [8]).

Line 290: replace "We will" by "we will".
Response: We did the correction.

Reviewer #2:

Manuscript Summary:

The article describes a method of transfection of *Eimeria* spp. by nucleofecting sporozoites and second-generation merozoites. The method is proposed to facilitate the genetic manipulation of this protozoan. Some minor revisions to clarify the protocol are needed. Additionally, the language of the manuscript should be improved, especially the Discussion.

Major Concerns:

The discussion should be rewritten. A more comprehensive comparison of the protocol described in this study and previous studies should be drawn up. It is not clear when the authors refer to the current study or to previous studies. The language should be reviewed by a native speaker.

Response: We are very grateful to the reviewer for point out the shortage of discussion. In the revised version of MS, we compared the transfection studies recently carried out by several research groups.

Minor Concerns:

-Line 44: replace "merozoites" by "second-generation merozoites".

Response: We did this replace.

-Line 46 and Line 78: clarify if only *E. tenella* was used (as indicated in line 78) or the method was applied to the three Eimeria spp. indicated in line 87

Response: This protocol is suitable for several *Eimeria* spp. Therefore, we have re-stated the sentence in the revised manuscript: Extraction and purification of sporozoites of *Eimeria* spp. (For example *E. tenella*)

-Line 114: both the strain of chickens and age at the time of infection should be indicated.

Response: Thank you very much. We added a sentence “In our experiment, we used Arbor Acre (AA) broilers at the age of 7-14 d to study”.

-Line 117: the time point for the collection of 2nd generation merozoites for each *Eimeria* spp. should be indicated.

Response: We added the time point for the collection of 2nd generation merozoites for *E. necatrix* and *E. tenella*. In this study, the time point for *E. necatrix* at 109 h, and *E. tenella* at 112 h post-inoculation, respectively.

-Line 125: clarify the term "yold stalk" and the different portions of the intestine to be analyzed depending on the species of *Eimeria*.

Response: "Yolk stalk" is in the middle of the small intestine. It refers to a tubular connection between the yolk sac and the embryonic gut in the developing embryo. Such as *E. tenella* parasitized mainly in the cecum. *E. acervulina* parasitized mainly in the duodenum. *E. maxima* and *E. necatrix* parasitized mainly in the small intestine, whereas the gametogony of *E. necatrix* occurs in the cecum due to the migration of the merozoites.

-Line 128: "of small intestine..." Were not the ceca analyzed for *E. tenella*?

Response: Because of the transfection of merozoites mainly used for *E. necatrix*, the parasitic position of 2nd generation merozoites of *E. necatrix* mostly in the middle of the intestine. Thus the purification of second-generation merozoites of *E. necatrix* is described here as an example.

-Line 149: explain why several tubes have to be used for merozoites and not for sporozoites.

Response: Because the transfection efficiency of merozoites is lower than sporozoites, we need more merozoites to ensure transfection successfully. However, 1×10^7 sporozoites are enough for the transfection.

-Line 150-153: indicate the manufacturer of reagents used for transfection.

Response: We have added the manufacturer of reagents used for transfection.

-Line 174: indicate meaning and manufacturer for "DMEM"

Response: We have added the meaning in line 173 and the manufacturer in table 2 for DMEM.

-Line 250: replace "merozoites and schizonts" by "schizonts and merozoites"

Response: We thank the reviewer for pointing out this mistake. We have replaced "merozoites and schizonts" with "meronts and merozoites".

-Line 270: "We attempted to transfer….". Which study do the authors refer to?

Response: The study referred to the article that the reference (Duan et al., 2019). And we have re-stated the sentence in the revised manuscript

-Line 271: "In the study, inoculation….." Do the authors refer to this study or to previous studies?

Response: We thank the reviewer for noting this detail. We revised the sentence and added the reference.

-Line 274: "In addition, we injected…" Do the authors want to say? "In addition, in previous studies we injected….."

Response: This sentence means that it is a feasible method to inject the transfected sporozoites into the intestinal lumen of rabbits in a laparotomy in previous studies. And we have revised the sentence.

Figure 3: the content of each of the three images showed for *E. necatrix* and *E. tenella* should be clarified.

Response: We have added the description of Figure 3.

Figures: the resolution of photographs in both Figures 2 and 3 should be improved.

Response: Thank you. We have replaced the original figures.

Reviewer #3:

Manuscript Summary:

This manuscript provides a detailed method to achieve stable transfection of *Eimeira* parasites of chickens. This method will help to facilitate the application of genetic manipulation in *Eimeria* spp. and explore the *Eimeira* genes' function. So, this manuscript is of great significance and urgency. However, this protocol is lack of continuity and some part is confusing, and need to revise step by step.

Major Concerns:

Lines 80-110: The sporulated oocysts were used. This protocol is lack of continuity and some part is confusing. More detail needed to this section.

This protocol could be (Just an example. Authors need to revise according their experiments):

1. Extraction and purification of sporozoites of *E. tenella*

1.1 Release of sporocysts

1.1.1 Sporulated Oocysts (the amount is about 1×10^7) in potassium dichromate solution (2.5% m/v) was centrifuged at 2300 g for 5 min and the pellet was washed with PBS for 3 times.

1.1.2 The pellets was suspended in ??ml PBS and transfer to ??ml tube. Th equal volume of glass beads (1mm×1mm diameter range) were added. The oocyst suspension was oscillated using Vortex Mixer to release the sporocysts.

1.1.3 Microscopy check was performed every 1 min to monitor the release of sporocysts. Stop vortexing when more than 90 % oocysts was broken.

NOTE: Most of oocysts (E. tenella, E. necatrix and E. acervulina) were broken about 1 min using Vortex Mixer.

1.1.4 Sporocyst suspension was transferred to new 1.5 ml tubes and centrifugate at 1600 g for 5 min. The precipitation was resuspended with 50% Percoll, combined to a 1.5 ml tube, added 50% Percoll to a total volume of 1 ml, and centrifugated at 10000g for 1 min.

1.2 Release of sporozoites

1.2.1 The precipitation was resuspended with the excystation buffer, incubated in water bath of 42 ℃ for 40 min-1 h to release the sporozoites, and then centrifugated at 600 g for 10 min.

NOTE: Shake the tubes once every 5 minutes during excystation.

1.2.2 The precipitation was resuspended with 1 ml 55% Percoll and centrifugated at 10000 g for 1min.

1.2.3 The precipitation was resuspended with 1 ml PBS and the sporozoites concentration was counting using a hemocytometer.

Lines 112-195: Following the above example, revise the protocols of "2. Collection and purification of merozoites of *E. necatrix*", "3. Nucleofection of merozoites or sporozoites","4. Cloacal inoculation/ intravenous injection" and "5. Propagation and FACS sorting".

Response: We are very grateful to the reviewer for rewriting the example procedure. According to your suggestion, we revised it in our new manuscript, and re-stated the protocols of "2. Collection and purification of merozoites of *E. necatrix*", "3. Nucleofection of merozoites or sporozoites"," 4. Cloacal inoculation/ intravenous injection" and "5. Propagation and FACS sorting".

Minor Concerns:

Line 67: should be "spp.".

Response: We changed “spp” into “spp.” in the revised manuscript.

Line 78: specific to *E. tenella*? Or to *Eimeria* spp.?

Response: This protocol is suitable for *Eimeria* spp. Therefore, we re-stated the sentence in the revised manuscript: Extraction and purification of sporozoites of *Eimeria* spp.

Line 149: "if merozoites were used, prepare several tubes"?

Response: We thank the reviewer for noting this detail. We re-stated the sentence in the revised manuscript: If you do the transfection of merozoites, prepare 3-4 tubes in generally.

Line 150: what kind of "plasmid"? Lines 189-150 mentioned FACs and pyrimethamine?

Response: We added a sentence "The plasmid used in this study contains 2 genes, enhanced yellow fluorescent protein (EYFP) and dihydrofolate reductase thymidylate synthase derived from Toxoplasma gondii (TgDHFR-TS)" to explain the "FACs and pyrimethamine" (lines 150- 151).

Line 165: about 10 μl? or 10 μg?

Response: We thank the reviewer for noting this detail. It should be 10 μg, and the volume of the plasmid is usually 10 μl.

Lines 178-180: The two paragraphs have the same meaning.

Response: We deleted this Note from the revised MS.

Lines 183-184: The infected dose? Sporozoites via intravenous injection? Merozoites via cloacal route?

Response: Thank you very much. We added the sentence, "The sporozoites inoculated into each chicken are 2 million, while merozoites for each bird are 10^7." (lines 182 and 183).

Lines 197-198: should be "…… If more purified sporozoites or merozoites needed, there was an ……."

Response: We re-stated the sentence in the revised manuscript: If more purified sporozoites or merozoites needed.

Line 203: should be "……glycine eluent buffer (twice), respectively."

Response: We thank the reviewer for pointing out this mistake. We modified this sentence as "Wash the chromatographic column with water (once) and glycine eluent buffer (twice), respectively".

Lines 209-210: should be" ….column and the flow rate was adjusted to 30- 40 of 100* range."

Response: We thank the reviewer for pointing out this mistake. We changed this sentence as "…adjust the column and the flow rate from to 30- 40 of 100 range".

Lines 219-221: It seems that the pellets needed to be resuspended before counting. What kind of buffer used? The same problem was found in Fig 1.

Response: We agree with this comment. The buffer used to resuspend the pellets is PBS usually. And we re-stated in the revised manuscript.

Line 224: should be "…..DE-52 cellulose was used for the purification….".

Response: We thank the reviewer for pointing out this mistake. We have revised the sentence as

“In general, the purification sporozoites or merozoites need to use for 2.5 g DE-52 cellulose and prepare it at least one day in advance.” according to your suggestion.

Lines 226-227: should be "Adjust the pH of glycine eluent buffer from 7.6 to 8.0 and prewarmed to 41 ℃."
Response: According to your suggestion, we have revised the sentence as “Adjust the pH of glycine eluent buffer from 7.6 to 8.0 and prewarmed to 41 ℃”.

Line 229: should be "Adding water and soaking overnight".
Response: We are grateful to the reviewer for point out this error, and we corrected it in our revised manuscript.

Line 230: should be ". Adding water and soaking for an hour."
Response: We revised it in following your suggestion

Line 231: should be "….discarded. Adding 0.1 M NaOH and soaking for at least 2 hours."
Response: We revised it in our new manuscript.

Lines 224-238: More detail needed in this section.
Response: According to your suggestion, we revised it in our new manuscript.

Line 242: should be "This protocol has been successful……"
Response: According to your suggestion, we revised it in our new manuscript.

Line 248: should be " ….purification of Eimeria (in italic) sporozoites"
Response: According to your suggestion, we revised it in our new manuscript.

Line 252: "were" should be deleted.
Response: According to your suggestion, we revised it in our new manuscript.

Line 253: should be "….D. Sporozoites of *E. tenella* treated with ……"
Response: According to your suggestion, we revised it in our new manuscript.

Lines 255-257: should be "……..after infecting with the transfected merozoites or sporozoites. A. The oocysts of *E. necatrix* (in italic) after infecting with the transfected merozoites. B. The oocysts of *E. tenella* (in italic) after infecting with transfected sporozoites."
Response: We are grateful to the reviewer for the suggestion for improving our manuscript. We revised it in our new manuscript.

Line 260, Table 1 : what kind of "water" was used? 100% percoll=90% percoll + 10% 10*PBS? "Trypsin" should be "trypsin".
Response: The water refers to distilled water. We added the description in table 1. 100% percoll refers to nine-tenths of percoll and one-tenth of PBS (10×) to mix.

Line 271: "In the study"? A reference needed?
Response: We thank the reviewer for noting this detail. We added the reference.

Line 282: should be "heterologous antigens, such as CjaA from Campylobacter jejuni"
Response: We revised it in our manuscript.

Fig 1: should be "10^7 sporulated oocysts". In method 1, "… bile digestive buffer " should be "excystation buffer"? The text explanation of 2^{nd} tube should be lined at the same level with those of 1st and 3rd tubes. In method 2, there is not text explanation to last tube. This figure shows that the last step from both methods is "centrifugation" and "counting". But counting what? Supernatant or pellet?
Response: According to your suggestion, we revised the Figure 1.

The last page, Table of Materials: Uniform format needed. For example, the first letter of some words (Vortex Mixer….) was capital, but not for others (magnetic mixer,….), etc.

Response: We are grateful to the reviewer for point out this problem, and we corrected it in our revised manuscript.

Editorial comments:

The manuscript has been modified and the updated manuscript, **60552_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: We have rechecked the spelling and grammar in our manuscript.

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Response: We removed commercial language from the manuscript and used the common names for them.

3. Please define all abbreviations before use.
Response: Thanks for the suggestion. In the revised manuscript, we redefined all abbreviations before use.

4. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Response: We have revised the text in Protocol and deleted personal pronouns.

5. Please avoid long steps/notes (more than 4 lines).
Response: We have revised the long step and make sure every step less than four lines.

6. Step 1.1.3: Please write this step in the imperative tense.
Response: We have revised this sentence.

7. Step 3.1: Please write this step in the imperative tense.
Response: Thank you. We have revised this sentence.

8. Step 5.1: Please write this step in the imperative tense.
Response: Thank you. We have revised this sentence.

9. Please bold the volume number for all references.
Response: Thank you. We have bold the volume number for all references.

10. Figure 1: Please remove Percoll and registered symbols (®) from the figure.
Response: We have removed commercial language from the figure and used the common names for them.

11. Table 1: Please remove Percoll and registered symbols (®) from the figure.
Response: We removed commercial language from Table 1 and used the common names for them.

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Response: We removed commercial language from the manuscript and used the common names for them.

13. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.
Response: We have removed trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

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