PCR protocols for *Eimeria* spp. Detection and phylogenetic studies

Ecology and Evolution of Molecular Parasite-Host Interactions

Overview

The Polymerase Chain Reaction (PCR) is defined as a simple, sensitive, versatile, robust *in vitro* method for amplifying specific sequences of DNA molecules. Here are described the PCR protocols used in the ecology and evolution of molecular parasite-host interactions group for detection and phylogenetics of *Eimeria* spp. This protocols are based on previously reported primers as well as primers designed by the own group; in order to amplify different genetic regions from the three genomes of this parasite (nuclear, mitochondrial, apicoplast). All of them were optimized for the DreamTaq DNA Polymerase from Thermo Scientific™; however the PCR amplification conditions and concentrations of reagents can be used with kits from different manufacturers.

Protocols

Ap5 tRNA non coding region (Apicoplast genome)

This marker is used for diagnosis of *Eimeria* spp. and the primers were designed and tested in our group (Jost, 2016; Kraft, 2015; Tank, 2015). The region amplified is ~450 bp long.

Reaction setup

In order to reduce the pipetting errors it is necessary to prepare a PCR master mix, which contains all the reagents necessary for the reactions (water, buffer, dNTPs, $MgCl_2$, primers and DNA polymerase) except the DNA which will be added later. The master mix is prepared in a separated tube with the total volume necessary for the desired number of reactions in accordance to Table 1; take into consideration that per PCR round you need four controls (1 positive control, 1 negative control and 2 non template controls [see section “controls” at the end of the protocols]).

Table 1. Reaction setup for PCR amplification with Ap5 primers

Reagent	Initial concentration	Final concentration	Volume per reaction**
Buffer*	10X	1X	2 µL
dNTPs	10 mM	0.5 mM	1 µL
Primer Ap5_F	10 µM	0.25 µM	0.5 µL

Primer Ap5_R	10 µM	0.25 µM	0.5 µL
$MgCl_2$ solution	25 mM	2 mM	1.6 µL
Taq Polymerase	5 U/µL	0.025 U/µL	0.1 µL
DNase free water	-	-	12.3 µL
DNA (Template)	10 - 200 ng/µL	1 - 20 ng/µL	2 µL
FINAL VOLUME OF REACTION			20 µL

* The buffer contains 20 mM of $MgCl_2$ leading to a final concentration of 2 mM, meaning a total concentration of 4 mM $MgCl_2$ in each reaction due to the extra amount added.
** In order to calculate the total volume for the master mix multiply the volume of each reagent by the number of reactions that are necessary (except DNA).

It is necessary that during the whole procedure (master mix preparation, distribution of the master mix into tubes and adding DNA to each tube) the reagents and the DNA be maintained in coolers to avoid degradation of them.

Once that the master mix is prepared, gently mix it and pipette 18 µL of it in each PCR tube (0.2 mL).

Add 2 µL of template and mix again.

Make sure that all liquid is in the bottom of the tube by a quick spin in the centrifuge.

Thermocycling conditions

Place the reaction in the thermal cycler and perform the PCR according to the following conditions:

Step		**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation		95	4 min	1
Amplification	Denaturation	92	30 s	35
	Annealing	56	30 s	
	Extension	72	45 s	
Final extension		72	7 min	1
Hold		4	∞	1

Once the program is finished analyse the products by electrophoresis in a 1% agarose gel (for preparation see the section “agarose gel electrophoresis” at the end of the protocols) or storage the products at 4 °C (separately from DNA or reagents) until posterior use.

COI Cytochrome c oxidase subunit I (Mitochondrial genome)

This marker is widely used for identification of many eukaryotes at species level. The primers Cocci_COI were designed by Mácová (2013) and tested in our group (Jost, 2016; Kraft, 2015). These primers amplify a region ~720 bp long.

Reaction setup

Table 2. Reaction setup for PCR amplification with Cocci_COI primers

Reagent	**Initial concentration**	**Final concentration**	**Volume per reaction**
Buffer*	10X	1X	2 µL
dNTPs	10 mM	0.5 mM	1.0 µL
Primer Cocci_COI_F	10 µM	0.25 µM	0.5 µL
Primer Cocci_COI_R	10 µM	0.25 µM	0.5 µL
$MgCl_2$ solution	25 mM	-	-
Taq Polymerase	5 U/µL	0.025 U/µL	0.1 µL
DNase free water	-	-	13.9 µL
DNA (Template)	10 - 200 ng/µL	1 - 20 ng/µL	2 µL
FINAL VOLUME OF REACTION			20 µL

* The buffer contains 20 mM of $MgCl_2$ leading to a final concentration of 2 mM.

Thermocycling conditions

Place the reaction in the thermal cycler and perform the PCR according to the following conditions:

Step	**Temperature (°C)**	**Time**	**Cycles**

Initial denaturation		95	1 min	1
Amplification	Denaturation	92	30 s	35
	Annealing	55	30 s	
	Extension	72	20 s	
Final extension		72	5 min	1
Hold		4	∞	1

Eukaryotic Small Ribosomal Subunit 18s rRNA (Nuclear genome)

This marker is used for identification at genus level and for phylogenetic purposes of *Eimeria* spp.. The primers used to amplify this marker are Api_18s_kvic (Kvicerova *et al.,* 2008) that amplify a long region ~1500 bp from the position 62-86 to 1568-1586 according to the *E. tenella* U67121 sequence. The primers were tested in our group (Jost, 2016; Kraft, 2015; Tank, 2015).

Reaction setup

Table 3. Reaction setup for PCR amplification with Api_18s_kvic primers

Reagent	**Initial concentration**	**Final concentration**	**Volume per reaction**
Buffer*	10X	1X	2 µL
dNTPs	10 mM	0.5 mM	1.0 µL
Primer Api_18s_kvic_F	10 µM	0.25 µM	0.5 µL
Primer Api_18s_kvic_R	10 µM	0.25 µM	0.5 µL
$MgCl_2$ solution	25 mM	-	-
Taq Polymerase	5 U/µL	0.025 U/µL	0.1 µL
DNase free water	-	-	13.9 µL
DNA (Template)	10 - 200 ng/µL	1 - 20 ng/µL	2 µL
FINAL VOLUME OF REACTION			20 µL

* The buffer contains 20 mM of $MgCl_2$ leading to a final concentration of 2 mM.

Thermocycling conditions

Place the reaction in the thermal cycler and perform the PCR according to the following conditions:

Step		Temperature (°C)	Time	Cycles
Initial denaturation		95	4 min	1
Amplification	Denaturation	92	45 s	35
	Annealing	53	45 s	
	Extension	72	1 min 30 s	
Final extension		72	10 min	1
Hold		4	∞	1

Nested PCR

In case of unspecific products in the PCR (amplicons with a different molecular size than the expected), it is necessary to do a second amplification in order to amplify an inner region in the original PCR products using a different pair of primers that will bind specifically. Primers Eim_18s_1 amplify a region ~918 bp long from the position 542 to 1460 in the *E. tenella* U67121 genome; meanwhile primers Eim_18s_2 amplify a region ~810 bp long from the position 78 to 888 in the same strain genome. For both primer pairs the same reaction setup and thermocycling conditions is used:

Reaction setup

Table 4. Reaction setup for PCR amplification with Eim_18s_1 and Eim_18s_2

Reagent	Initial concentration	Final concentration	Volume per reaction
Buffer*	10X	1X	2 µL
dNTPs	10 mM	0.45 mM	0.9 µL
Primer Eim_18s_1_F or Eim_18s_2_F	10 µM	0.23 µM	0.46 µL

Primer Eim_18s_1_R or Eim_18s_2_R	10 µM	0.23 µM	0.46 µL
$MgCl_2$ solution	25 mM	-	-
Taq Polymerase	5 U/µL	0.045 U/µL	0.18 µL
DNase free water	-	-	13.9 µL
DNA (Template)	10 - 200 ng/µL	1 - 20 ng/µL	2 µL
FINAL VOLUME OF REACTION			20 µL

* The buffer contains 20 mM of $MgCl_2$ leading to a final concentration of 2 mM.

Thermocycling conditions

Place the reaction in the thermal cycler and perform the PCR according to the following conditions:

Step		**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation		95	3 min	1
Amplification	Denaturation	95	30 s	35
	Annealing	54	30 s	
	Extension	72	30 s	
Final extension		72	10 min	1
Hold		4	∞	1

Prokaryotic Small Ribosomal Subunit 16s rRNA (Apicoplast genome)

This marker is used for identification at species level of *Eimeria* spp.. The primers used to amplify this marker were designed by Cai *et al.,* (2003) and they amplify a region ~520 bp from *E. tenella* SSU gene in apicoplast.

Reaction setup

Table 5. Reaction setup for PCR amplification with SSU primers

Reagent	Initial concentration	Final concentration	Volume per reaction
Buffer*	10X	1X	2 µL
dNTPs	10 mM	0.5 mM	1.0 µL
Primer SSU_F	10 µM	0.25 µM	0.5 µL
Primer SSU_R	10 µM	0.25 µM	0.5 µL
$MgCl_2$ solution	25 mM	0.5 mM	0.4 µL
Taq Polymerase	5 U/µL	0.025 U/µL	0.1 µL
DNase free water	-	-	13.5 µL
DNA (Template)	10 - 200 ng/µL	1 - 20 ng/µL	2 µL
FINAL VOLUME OF REACTION			20 µL

* The buffer contains 20 mM of $MgCl_2$ leading to a final concentration of 2 mM, meaning a total concentration of 2.5 mM $MgCl_2$ in each reaction due to the extra amount added.

Thermocycling conditions

Place the reaction in the thermal cycler and perform the PCR according to the following conditions:

Step		Temperature (°C)	Time	Cycles
Initial denaturation		95	6 min	1
Amplification	Denaturation	95	20 s	35
	Annealing	59	30 s	
	Extension	72	1 min	
Final extension		72	10 min	1
Hold		4	∞	1

Prokaryotic Large Ribosomal Subunit 23s rRNA (Apicoplast genome)

This marker is used for identification at species level of *Eimeria* spp.. The primers used to amplify this marker were designed by Cai *et al.,* (2003) and they amplify a region ~450 bp from *E. tenella* LSU gene in apicoplast.

Reaction setup

Table 6. Reaction setup for PCR amplification with LSU primers

Reagent	Initial concentration	Final concentration	Volume per reaction
Buffer*	10X	1X	2 µL
dNTPs	10 mM	0.5 mM	1.0 µL
Primer SSU_F	10 µM	0.25 µM	0.5 µL
Primer SSU_R	10 µM	0.25 µM	0.5 µL
$MgCl_2$ solution	25 mM	0.5 mM	0.4 µL
Taq Polymerase	5 U/µL	0.025 U/µL	0.1 µL
DNase free water	-	-	13.5 µL
DNA (Template)	10 - 200 ng/µL	1 - 20 ng/µL	2 µL
FINAL VOLUME OF REACTION			20 µL

* The buffer contains 20 mM of $MgCl_2$ leading to a final concentration of 2 mM, meaning a total concentration of 2.5 mM $MgCl_2$ in each reaction due to the extra amount added.

Thermocycling conditions

Place the reaction in the thermal cycler and perform the PCR according to the following conditions:

Step		Temperature (°C)	Time	Cycles
Initial denaturation		95	6 min	1
Amplification	Denaturation	95	20 s	35
	Annealing	54	30 s	
	Extension	72	1 min	
Final extension		72	10 min	1
Hold		4	∞	1

Open Read Frame 470 [ORF470] (Apicoplast genome)

This marker is used for identification at species level of *Eimeria* spp. and for phylogenetic purposes; the region amplified is part of a cluster that codifies iron-sulphur biosynthetic enzymes. The primers used to amplify this marker were designed by Zhao and Duszynski *et al.,* (2001) and they amplify a region ~800 bp from the ORF470 in apicomplexans apicoplast.

Reaction setup

Table 6. Reaction setup for PCR amplification with ORF470 primers

Reagent	**Initial concentration**	**Final concentration**	**Volume per reaction**
Buffer*	10X	1X	2 µL
dNTPs	10 mM	0.25 mM	0.5 µL
Primer SSU_F	10 µM	0.25 µM	0.5 µL
Primer SSU_R	10 µM	0.25 µM	0.5 µL
$MgCl_2$ solution	25 mM	0.5 mM	0.4 µL
Taq Polymerase	5 U/µL	0.025 U/µL	0.1 µL
DNase free water	-	-	14 µL
DNA (Template)	10 - 200 ng/µL	1 - 20 ng/µL	2 µL
FINAL VOLUME OF REACTION			20 µL

* The buffer contains 20 mM of $MgCl_2$ leading to a final concentration of 2 mM, meaning a total concentration of 2.5 mM $MgCl_2$ in each reaction due to the extra amount added.

Thermocycling conditions

Place the reaction in the thermal cycler and perform the PCR according to the following conditions:

Step		**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation		95	4 min	1
Amplification	Denaturation	92	45 s	35
	Annealing	50	45 s	

	Extension	72	1 min 30 s	
Final extension		72	7 min	1
Hold		4	∞	1

Controls

The usage of controls is crucial for the proper interpretation of your PCR results. In every run of PCR three type of controls are required:

Positive control

This is a previously amplified sample and it is known that it contains the target sequence. For our PCRs, the DNA extracted from colon content of **infected** mice with *E. falciformis* is used as positive control.

Negative control

This is DNA from the same type of sample but this does not contain the target sequence. This kind of controls are used to detect potential unspecific amplifications in the sample. In our case, DNA extracted from colon content of **non-infected** mice is used.

Non template controls (NTC)

Substances that do not contain DNA and therefore they always have to be negative. This controls are used to detect contaminations. Water and elution buffer are used as NTC.

Agarose gel electrophoresis

Agarose gel electrophoresis is the most used method for separating nucleic acid fragments, based on the capacity to move negatively charged nucleic acid molecules through an agarose matrix by an electric field. Adjusting the agarose concentration modify the size of the gel pores controlling the separation of a wide range of different-size nucleic acid molecules, 1 -1.5% agarose gels have good resolution (~0.4 - 6 kb). The migration of nucleic acids in agarose gels is also affected by the choice of running buffer (TAE or TBE) and the applied voltage (~ 90 - 100 mV). Shorter fragments run faster and further than longer fragments. For the visualization of the PCR products, different fluorescent dyes that intercalate with dsDNA can be used.

Preparation of the gel

Weight the correct amount of agarose in accordance to the size and concentration of the agarose (Table 7) and put it into an dry and clean Erlenmeyer flask.

	Small gel (half of the chamber/one row of wells)		Big gel (compleat chamber/two row of wells)	
Component	1%	1.5%	1%	1.5%
Agarose	0.5 g	0.75 g	1 g	1.5 g
Buffer (TAE 1X)*	50 mL		100 mL	
Heat in a microwave, at 30 s intervals remove the flask and swirl the contents to mix well and repeat until the agarose is completely dissolved.				
Wait until the agarose cool down (around 60 - 70°C) and add the fluorescent dye in the following proportions:				
Roti-Safe	2.5 µL		5 µL	
Mix the agarose and pour it into the gel casting tray blocked in the open edges (can be in the chamber or alternatively with tape in order to create a mold) with a comb to create the wells.				
Let it solidify at room temperature and then remove the comb.				
Place the gel in the electrophoresis chamber and fill it with buffer TAE 1X				

* 40 mM Tris-acetate, 1 mM EDTA (Alternatively TBE [45 mM Tris-borate, 1 mM EDTA])

Set up of the samples for the gel

Mix 3 µL of gel loading buffer 6X with 7 µL of PCR product and pippett all the mixture in one well.

Use the proper electrophoresis program according to the agarose concentration; for 1% gels use 100 mV/130 mA/60 min, and with 1.5% gels use 90 mV/130 mA/80 min.

The DNA will move **from the negative (black) electrode to the positive (red) electrode**.

Recommendations

Before starting

1. Do all the calculations for the PCR reactions.

2. Label all the tubes that you will need, using a clear structure and write it down in your lab book.
3. Clean with ethanol 70 and RNase AWAY solution the area dedicated to work PCR and the pipettes.
4. Unfreeze all the components for the reaction ($MgCl_2$, PCR buffer, dNTPs Mix, primers, nuclease free water) and homogenize them gently.
5. Unfreeze, separately, the DNA of your samples and your controls in the 4 °C fridge, with this you will avoid the degradation of your templates.
6. Make sure that the tips and tubes that you will use are clean.
7. Because we do not have our own cycler is necessary to book one in other laboratories, for that just put your name and the time when you are going to use it.

During the preparation

1. Be concentrated in all the moments in order to avoid skip or add a double amount of one reagent of the master mix.
2. One suggested way to prepare the reactions and avoid cross contaminations is :
    a. Prepare the master mix, distribute it into the tubes and storage again the reagents at -20°C.
    b. Prepare your non-template controls.
    c. Take out the samples from the fridge and add the DNA to the tubes.
    d. Finally, add the negative and positive controls, in this order.
3. Always homogenize all the components gently.
4. Set up the PCR reaction in a cooling block.
5. Use your own set of reagents.
6. Prepare master mix for n+1 samples.

After the preparation

1. Clean the working space and pipettes with ethanol 70 and RNase AWAY solution.
2. Close the waste bag and throw it into the contaminated waste container.
3. Set the pipettes in the top volume in order to extend their calibration.

COI Cytochrome c oxidase subunit I (Mitochondrial genome)

This marker is widely used for identification of many eukaryotes at species level. The primers Eim_COI_M were designed by Heitlingler (2013) and tested in our group (Jarquin et al., In prep). This primer pair complement results for samples negative using Cocci_COI primers. These primers amplify a region ~680 bp long.

Reaction setup

Table 1. Reaction setup for PCR amplification with Eim_COI_M primers

Reagent	**Initial concentration**	**Final concentration**	**Volume per reaction**
Buffer*	10X	1X	2.5 µL
dNTPs	10 mM	0.5 mM	1.25 µL
Primer Eim_COI_M_F	10 µM	0.25 µM	0.625 µL
Primer Eim_COI_M_R	10 µM	0.25 µM	0.625 µL
$MgCl_2$ solution	25 mM	0.5 µM	0.5 µL
Taq Polymerase	5 U/µL	0.025 U/µL	0.125 µL
DNase free water	-	-	16.875 µL
DNA (Template)	10 - 200 ng/µL	1 - 20 ng/µL	2.5 µL
FINAL VOLUME OF REACTION			25 µL

* The buffer contains 20 mM of $MgCl_2$ leading to a final concentration of 2 mM.

Thermocycling conditions

Place the reaction in the thermal cycler and perform the PCR according to the following conditions:

Step		**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation		95	3 min	1
Amplification	Denaturation	92	30 s	35
	Annealing	52	30 s	
	Extension	72	30 s	
Final extension		72	5 min	1
Hold		4	∞	1

Eimeria detection in tissue by qPCR

The intensity of *Eimeria* spp. is assessed using qPCR by relative quantification from intracellular stages of the parasite compared to host DNA (house mouse nuclear genome) as internal reference. Cecum and ileum tissue of mice are screened using primers targeting a short mt COI region in order to detect DNA from intracellular stages of *Eimeria* (Eim_COI_qX-F, TGTCTATTCACTTGGGCTATTGT; Eim_COI_qX-R GGATCACCGTTAAATGAGGCA), while *Mus musculus* primers targeted the *CDC42* nuclear gene (Ms_gDNA_CDC42_F CTCTCCTCCCCTCTGTCTTG; Ms_gDNA_CDC42_R TCCTTTTGGGTTGAGTTTCC).

Reaction setup

Table 1. Reaction setup for qPCR amplification with host and parasite primers

Reagent	**Initial concentration**	**Final concentration**	**Volume per reaction**
iTaqTM Universal SYBR® Green Supermix*	2X	1X	10µL
Primer F**	1000 nM	400 nM	0.8 µL
Primer R**	1000 nM	400 nM	0.8 µL
DNase free water	-	-	7.4 µL
DNA (Template)	50 ng/µL	2.5 ng/µL	1 µL
FINAL VOLUME OF REACTION* **			20 µL

* The Supermix contains antibody mediated hot-start iTaq DNA Polymerase, dNTPs, MgCl2, SYBR® Green I Dye, enhancers, stabilizers, and a blend of passive reference dyes (including ROX and fluorescein).

** Prepare individual reactions for each primer pair per sample.
*** Include a NTC with DNase free water.

Thermocycling conditions

The conditions are adjusted to the real time platform Eppendorf Mastercycler ep *realplex*:

Step		**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation		95	2 min	1
Amplification	Denaturation	95	15 s	40
	Annealing	55	15 s	
	Extension	68	20 s	
Melting curve analysis		65 to 95	3 s/step	-

Intensity estimation

To assess the intensity it is necessary to calculate the ΔCt between mouse and Eimeria (CtMouse- Ct*Eimeria*). The threshold for detection was estimated at ΔCt = -5 and results above this value were considered as an estimate of parasite tissue load.