



Deliverable D-JRP19-WP4.8
Report on the development
of Standard Operating
Procedures (SOPs) for the
post DNA extraction capture
of *Cryptosporidium* spp.
DNA

Workpackage 4 of
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Responsible Partners: P41-SVA, P30-RIVM



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D-JRP-PARADISE-WP4.8

REPORT ON THE DEVELOPMENT OF STANDARD OPERATING PROCEDURE (SOP) FOR THE POST DNA EXTRACTION CAPTURE OF *CRYPTOSPORIDIUM* SPP. DNA

This is a public deliverable of One Health EJP Joint Research Project: JRP19-ET1.1-PARADISE – PARASite Detection, ISolation and Evaluation

(<https://onehealthejp.eu/jrp-paradise/>)

Work Package:

JRP- PARADISE– WP4 Parasite enrichment strategies;

Task:

JRP-PARADISE-PARADISE-WP4-T2 Development of post-DNA extraction enrichment strategies.

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1. Background

Cryptosporidium parvum is the most important zoonotic species in the genus *Cryptosporidium*. It causes gastrointestinal disease in humans and other animals, particularly young ruminants, and has a global distribution. Infection is transmitted by direct faecal-oral contact or by ingestion of water and food contaminated with parasite oocysts.

Detection of parasites on food and in water is complicated by low contamination levels, often resulting in under-detection. Besides the need for more sensitive approaches for detection of parasite DNA on food, a more sensitive method will also be useful for detection of low numbers of *C. parvum* oocysts in manure. This is required to estimate the prevalence of this parasite, as, otherwise, asymptomatic ruminant carriers could remain undetected.

PARADISE WP4 aims to improve the performance of current approaches to detect parasite contamination in food through the development of parasite enrichment strategies, which should improve the sensitivity of detection and deliver cost effective methods applicable to food matrices.

Objectives of PARADISE WP4 are:

- ✓ To develop two pre-DNA extraction protocols based on two alternative affinity reagents, nanobodies (single-chain variable fragment antibodies) and aptamers (oligonucleotides that bind to specific target molecules);
- ✓ To assess nanobodies and aptamers application for magnetic capture of *C. parvum* and *G. duodenalis* (oo)cysts in different matrices;
- ✓ To develop a protocol for post-DNA extraction to concentrate parasite DNA based on hybridization of target-specific biotinylated probes.
- ✓ To evaluate enrichment strategies by inter-laboratory comparison

This deliverable reports on the third objective of PARADISE WP4, i.e., the development of Standard Operating Procedures (SOP) for the post DNA extraction capture of *Cryptosporidium* spp. by use of hybridization biotinylated, single-stranded DNA probes.

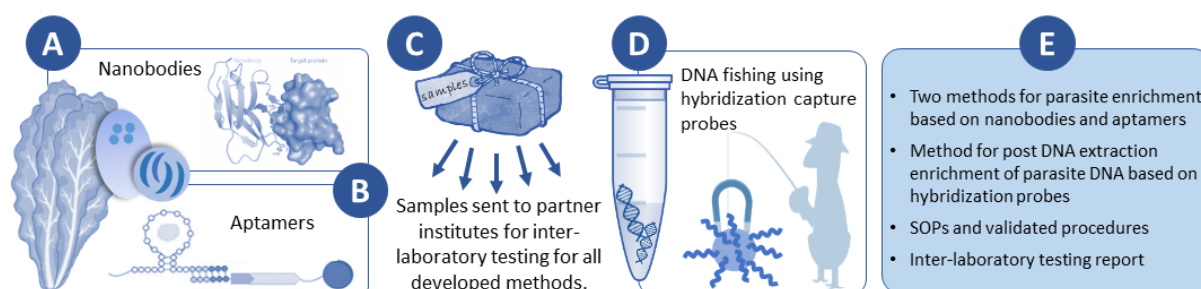


Figure 1. Illustration of WP4. D) is task WP4-T2, post-DNA extraction enrichment of specific markers. Figure is adopted from the PARADISE project proposal.

2. Procedure of development of Standard operating procedure for the post DNA extraction capture of *Cryptosporidium* spp. DNA

Description of procedure

We developed a protocol to capture DNA by using hybridization probes for specific targets, namely the glycoprotein 60 (*gp60*) gene and the 18S rRNA gene. These targets were chosen to allow for detection (both), species determination (18S rRNA gene), and subtyping (*gp60* gene).

The optimization was performed by SVA using the 18S rRNA gene as target, and by RIVM using the *gp60* gene as target. Optimization included probe selection, lysis, capture and qPCR.

After optimization, protocols were compared and integrated to allow dual detection using probe sets for both target genes in one experiment. To validate the protocol, *C. parvum* positive manure, counted



oocysts and probes were shared between SVA and RIVM and tested in parallel (Table 1). Experiments were performed to determine the detection limit of naturally infected faeces and artificially spiked manure. Results yielded comparable outcomes, and therefore an applicable SOP.

Table 1. Samples used for protocol validation

Sample	Lab Number of Replicates	Number of oocysts/sample	Dilution
Clean oocysts isolated with FACS	SVA (4) / RIVM (4)	100	
	SVA (4) / RIVM (4)	50	
	SVA (4) / RIVM (4)	10	
	SVA (4) / RIVM (4)	5	
Faecal sample spiked with clean oocysts	SVA (4) / RIVM (4)	100	undiluted
	SVA (4) / RIVM (4)	50	undiluted
	SVA (4) / RIVM (4)	10	undiluted
	SVA (4) / RIVM (4)	5	undiluted
Faecal sample 1, high number of oocysts	SVA (2) / RIVM (2)	~66 000 000	undiluted
	SVA (2) / RIVM (2)	~6 600 000	1:10
	SVA (2) / RIVM (2)	~660 000	1:100
	SVA (2) / RIVM (2)	~66 000	1:1 000
	SVA (2) / RIVM (2)	~6 600	1:10 000
	SVA (2) / RIVM (2)	~660	1:100 000
	SVA (2) / RIVM (2)	~66	1:1 000 000
Faecal sample 2, high number of oocysts	SVA (2)	~13 400 000	undiluted
	SVA (2)	~1 340 000	1:10
	SVA (2)	~134 000	1:100
	SVA (2)	~13 400	1:1 000
	SVA (2)	~1340	1:10 000
	SVA (2)	~134	1:100 000
	SVA (2)	~13	1:1 000 000
Faecal sample, moderate number of oocysts	SVA (2) / RIVM (2)		undiluted
Faecal sample, low number of oocysts	SVA (2) / RIVM (2)		undiluted
Negative controls	SVA (14) / RIVM (12)	0	undiluted

3. Standard operating procedure for the post DNA extraction capture of *Cryptosporidium* spp. DNA

Chemicals

The chemicals below are required for different steps in the protocol.

Chemical	Concentration	(Suggested) supplier
Demineralized water	-	-
Molecular grade water / PCR grade water (DNase/RNase free)	-	Sigma
Tris-HCl pH8.0	1 M	Sigma
EDTA	0.5 M	Sigma
NaCl	5 M	Sigma
SDS	20 %	Sigma
0.5 mm zirconium oxide beads	-	BioSpec Products
2 mm zirconia beads	-	BioSpec Products
streptavidine Sepharose	-	GE Healthcare
PBS	1x	Sigma
Dynabeads M270 streptavidine	-	Invitrogen



Buffers, capture oligonucleotides and helper probes

Lysis buffer (note: first add demineralized water, and SDS last)

	Stock concentration	Buffer concentration	Volume in buffer (ml)
Tris-HCl pH8.0	1 M	100 mM	0.8
EDTA	0.5 M	5 mM	0.08
NaCl	5 M	200 mM	0.32
SDS	20%	0.4 %	0.16
Demineralized water	-	-	6.64
Total volume			8

B&W 1x working solution

	Stock concentration	Buffer concentration	Volume in buffer (ml)
Tris-HCl pH8.0	1 M	5 mM	0.8
EDTA	0.5 M	0.5 mM	0.16
NaCl	5 M	1 M	32
MilliQ water	-	-	127.04
Total volume			160

Capture oligonucleotides and helper probes

Oligo name	Oligo Function	Sequence and tags	Target
CDH5	5' helper probe	GCTACCACATCTAAGGAAGGC	18S rRNA gene
CUH5	5' helper probe	GGGAATTAGGGTTCGATTCC	18S rRNA gene
CNC5	5' capture probe	BIO-TEG-GAGAGGGAGCCTGAGAAACG	18S rRNA gene
CUC3	3' helper probe	GCATAGTTTATGGTTAAGACTACGA	18S rRNA gene
CDC3	3' helper probe	CCTAACTTTCGTTCTTGATTAATGAAAA	18S rRNA gene
CNC3	3' capture probe	BIO-TEG-GGTATCTGATCGTCTTCGATCC	18S rRNA gene
CparGP60FqPCR_Cap	5' capture probe	BIO-TEG-CCACTACTCCAGCTCAAAGTGA	gp60 gene
CparGP60RqPCR_Cap	3' capture probe	BIO-TEG-CGGGATCTGTTTGGTCTTTTAT	gp60 gene

Capture oligonucleotides [stock 100 μ M] are diluted 1:100 to 1 μ M (=user solution)

Helper probes [stock 100 μ M] are diluted 1:10 to 10 μ M (= user solution)

To each sample we then add:

2.5 μ L capture probes (1 μ M), so **2.5 pmol**

10 μ L helpers (10 μ M), so **100 pmol**

Protocol

1. Add 400 μ l or 800 mg of 0.5 mm zirconium oxide beads, 200 μ l or 600 mg of 2 mm zirconia beads (BioSpec Products Inc., Bartlesville OK, USA) in a 15 ml tube
2. Weigh 2g faeces in a 15ml tube filled with beads from step 1.



3. Add 8 ml lysis buffer.
4. Beadbeating 2x 6.5 m/s for 1 min (MP FastPrep 24).
5. Incubate at 98°C for 30 minutes.
6. Repeat step 4 and 5.
7. Centrifuge the sample for 15 min at 3500 * g (at RT, max deceleration).

Note: The *Cryptosporidium* oocysts are lysed and the DNA is in the supernatant.

8. Bring all supernatant (approximately 8 ml) in clean 15 ml tube for continuation in the protocol.

Samples can be stored at -20 °C before continuation with step 9. Thaw sample at room temperature before step 9.

9. Washing streptavidine Sepharose.
 - a. Add n (=number of samples) * 90 µl streptavidine Sepharose to a clean 2 ml tube
 - b. Mark the tube at the level of the liquid.
 - c. Wash 3 times by adding 500 µl-1000 µl 1xPBS, centrifuging (20s short spin) and removing the supernatant.
 - d. Suspend the washed streptavidine Sepharose in 1x PBS to the original volume (to the mark of step 9b).
10. Add 90 µl washed streptavidine Sepharose to each sample.
11. Incubate 45 min, tumbling at 10 rpm (using a blood tube rotator or similar), at room temperature.
12. Centrifuge the sample for 15 min at 3500*g.

Note: The *Cryptosporidium* DNA is in the supernatant. The streptavidine removed the free biotin in the solution.

13. Bring all supernatant in clean 15 ml Greiner tube.

Samples can be stored at -20 °C for a maximum of 1 week before continuation with step 14. Thaw sample at RT before step 14.

14. Add 2.5 µl [1 µM] of each (F and R) capture oligo to each sample (for both *gp60* and 18S rRNA) and 10 µl [10 µM] of each helper probe (for 18S rRNA).
15. Add 2ml 5M NaCl.
16. Incubate 15 min at 98 °C.
17. Incubate 45 min at 56 °C. Rotate the samples, or invert tubes at least 2x during incubation.
18. Cool the samples by tumbling at 10 rpm (using a blood tube rotator or similar), for 10 min (to room temperature).
19. Vortex and brief spin to remove liquid from the lids.

Note: The target *Cryptosporidium* DNA is bound to the probes (DNA-probe complex) and is in the supernatant.

20. Washing dynabeads M270.
 - a. Resuspend the dynabeads by gently mixing (by inverting and swirling, do not vortex).
 - b. Take the required volume (n * 90 µl) in a 1,5 ml tube.
 - c. Place the tube in a magnet set for 2 minutes.
 - d. Remove the supernatant.
 - e. Remove the tube from the magnet and resuspend in n * 90 µl in 1x B&W buffer.
 - f. Repeat step 20c and 20d.



- g. Resuspend the dynabeads in $n \times 90 \mu\text{l}$ in 1x B&W buffer.
21. Add $90 \mu\text{l}$ washed dynabeads to the 10 ml sample.
 22. Incubate the samples for 60 min, rotate 10 rpm, at room temperature.

Note: The *Cryptosporidium* DNA-probe complex is bound to the magnetic beads.

23. Place the 15ml tubes in a magnet and lay on a shaking plate for 10 min (shake gently).
24. While the tube is in the magnet, discard the supernatant.
25. Suspend the beads (do not vortex) in 1 ml 1x B&W buffer and pipette it in a 1.5 ml tube.
26. Place the 1.5ml tube in a magnet.
27. While the tube is in the magnet, discard the supernatant.
28. Repeat step 26 to 28 twice.
29. Suspend the beads in $100 \mu\text{l}$ sterile PCR grade water in a new 1,5 ml tube.
30. Place the 1.5 tube for exactly 10 min at 100°C (make sure you put the tubes in at a time interval in which you also take them out. Each tube should incubate for exactly 10 min).

Note: The target *Cryptosporidium* DNA is released from the magnetic beads by heating and the target DNA is in the supernatant.

31. Place the 1.5 ml tube in a magnet.
32. Pipette the supernatant after 5 sec in a clean tube (quick pipetting as the pipette tip may melt). The use of LoBind tubes is suggested.
33. Proceed with PCR or store the DNA at -20°C .



PCR protocol

Chemicals

SensiFast Probe Lo-Rox Kit (Bioine/Meridian)

Molecular Grade Water (RNAse/DNAse free water)

Probes and primers

Oligo name	Oligo sequence (5' → 3')	Label 5'	Label 3'	Function
CparGP60F	CCACTACTCCAGCTCAAAGTGA	VIC replaceme nt	BHQ1	qPCR primer F gp60
CparGP60R	CGGGATCTGTTTGGTCTTTTAT			qPCR primer R gp60
CparGP60Tp	GGTTCGGAGAAGGTACCCCAGCT			qPCR probe gp60
Caf-probe	AGCGTACCAACAAGTAATTCTGTATCGATG			CIAC (internal control) qPCR probe
StrCr.Fw	GGTTGTATTTATTAGATAAAGAAC	Atto 647N	BHQ3	qPCR primer F 18S rRNA
StrCr.Rw	TAGGCCAATACCCTACCGTC			qPCR primer R 18S rRNA
StroupPro3.M	CATATCATTCAAGTTTCTGACCTATC	FAM	MGB	qPCR probe 18S rRNA

Note: probes should be HPLC purified by the manufacturer

Equipment

Roche LightCycler 480

Protocol

Note: Perform each reaction in triplicate. The Competitive Internal Amplification Control (CIAC) is a synthetic linear DNA added to control for PCR inhibition. The CIAC uses the same primers as the *gp60* target, with a different probe and is ready to use.

1. Prepare the Primer/probe (PP) mix and master mix according to the following table.
For each target prepare a separate PP mix and master mix.

Table 2. Primer probe/CIAC mix gp60 gene

stock conc.	Primer/probe or DNA name	μl
100 pmol/μl	CparGP60Tp	5
100 pmol/μl	CparGP60F	10
100 pmol/μl	CparGP60R	10
100 pmol/μl	CIAC probe	5
	CIAC DNA	200
	TE buffer	270
	Total	500



Table 3. Primer probe mix 18S rRNA gene

stock conc.	Primer/probe or DNA name	μL
100 pmol/μl	StroupPro3.M	5
100 pmol/μl	StrCr.Fw	10
100 pmol/μl	StrCr.Rw	10
	TE buffer	475
	Total	500

Table 4. Master mix

mix	μl
Sensifast	1000
PP/CIAC Mix <i>gp60</i> OR PP mix 18S rRNA	500
Total	1500

2. Add 15 μl master mix and 5 μl target DNA in wells of a 96-well qPCR plate
3. Cover the qPCR plate with a transparent cover
4. Spin down briefly to make sure all liquid is at the bottom of the well
5. Run the qPCR program, see table below. (plate can be stored before running at 4 °C for 6 hours, wrapped in aluminum foil)

Table 5. PCR program used in LC480

Step	Temperature	time	Number of cycles
1	95 °C	3'	1 x
2	95 °C	5"	45 x
3*	60 °C	30"	

*Acquisition at the end of step 3 using the following filters: 440-488 nm, 465-510 nm, 498-580 nm, 533-610 nm, 618-660 nm.

Analyze data using LC480 software using the 2nd derivative at high confidence.