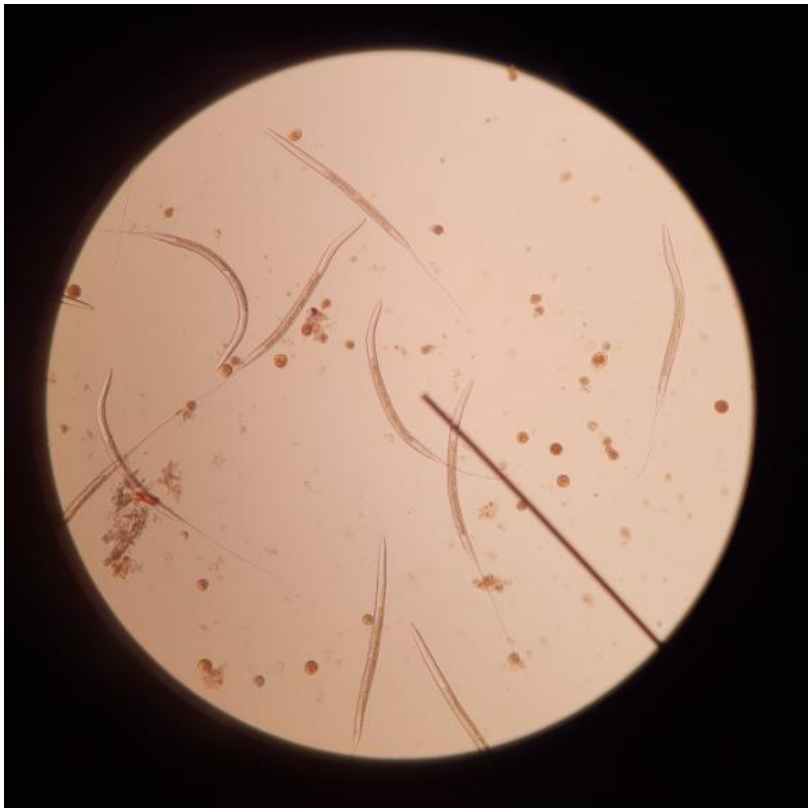
Characterization of the 5'-end of glc-3 in *Cylicostephanus goldi* nematodes by a genome walking method using a rolling circle amplification of genomic templates for inverse PCR (RCA-GIP)



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ABSTRACT

Background: Gastrointestinal nematodes of the cyathostomin species pose a threat to equine health worldwide. Even more so, since cyathostomin species are developing resistance against all groups of veterinary anthelmintics. Macrocyclic lactones, such as ivermectin and moxidectin, are still the most effective anthelmintic drugs registered for use in horses. However, recent research shows a reduced egg reappearance period and a reduced efficacy, suggesting a developing resistance. Glutamate gated chloride channels are the receptor targets for macrocyclic lactones. It was discovered that the glutamate gated chloride channel-α4 is a ivermectin sensitive glutamate gated channel and so the gene encoding for this channel (glc-3) is strongly suspected to play a role in developing resistance. Especially the 5'-end of this gene, which has proven to comprise several variations on mRNA level. So far, however, the genomic sequence of the 5'-end of glc-3 has not been mapped. This research focusses on the characterisation of the 5'-end of glc-3 and the possible relation with developing resistance.
Objectives: To characterize the 5'-end of the glc-3 gene by a genome walking method using DNA from the nematode species *Cylicostephanus goldi*.
Methods: Different lysis methods have been researched such as worm lysis buffer in combination with proteinase K and an alkaline lysis buffer from the REPLI-g Mini kit or REPLI-g Single Cell kit. PCR was used to visualise efficacy of lysis methods on different life stages of cyathostomins. Polymerase chain reaction was also used to examine successfulness of the multiple displacement amplification. A genome walking method was performed using rolling circle amplification of the products of the multiple displacement amplification with primers turned outwards. This way large quantities of linear templates are generated suitable for inverse PCR. This allows for sequencing and identification of unknown genomic sequences flanking known regions.
Results: Lysis of eggs, L1s and L3s with worm lysis buffer and proteinase K was successful most of the time. Lysis with the alkaline lysis buffer showed very inconsistent results, with only a small success rate. DreamTaq PCR with primer pair CY1-CY18 has proven to be a sufficient method of visualising the efficacy of the lysis methods as well as visualising downstream obtained products. Multiple displacement amplification was successful for two lysed eggs and genome walking was performed on the product. Genome walking was partially completed for both eggs and resulted in products ready for sequencing. Sequencing of the genome walking product revealed the template to be glc-3.
Conclusion: cyathostomin eggs have proven to be promising substitutes for adult cyathostomins in performing downstream molecular work such as polymerase chain reaction, multiple displacement amplification and genome walking. Genome walking on lysed eggs has led to amplification of glc-3, which is a promising step towards the characterization of the 5'-end of this gene.

1. Introduction

Gastrointestinal nematodes belonging to the cyathostomin species are the most prevalent and potentially pathogenic parasites in equid populations worldwide (Collobert-Laugier, 2002; Corning, 2009). Over 50 species of cyathostomins are recognized, however little is known about species specific pathogenicity nor the factors that determine species distribution within the host (Lichtenfels, 2008; Stancampiano, 2010).

As the different stages of cyathostomins reside in the large intestines of horses, they cause damage to the intestinal wall. Damage to the intestinal mucosa can cause weight loss, lethargy, ventral edema and sometimes diarrhoea (Corning, 2009; Nielsen, 2018). The encysted L4 stage of these nematodes pose the biggest thread to equine health. Mass migration of these L4's into the intestinal lumen causes devastating damage to the large intestinal wall and thereby a huge reduction in nutritional metabolism. This clinically recognized syndrome, called larval cyathostominosis, especially seen in younger horses during late winter has a mortality rate of up to 40-70% (Abbott, 1998; Love, 1992; Lyons, 1994)

Management of equine cyathostomin infection has proved to be challenging and subject to many different factors such as population dynamics, larval pasture burdens, weather and seasonal changes (Nielsen M. B.-T.-D., 2018). The repeated non selective use of anthelmintic drugs in the past, aimed to decrease the prevalence of *Strongylus vulgaris* (Nielsen, 2012) has resulted in development of resistance against all three groups of anthelmintics; benzimidazoles (BZ), tetrahydropyrimidines (THP) and macrocyclic lactones (ML) (Kaplan, 2004; Geurden, 2014; McArthur, 2015; Nielsen, 2018). It has long been known that there is a widespread resistance against BZ and THP such as pyrantel (Kaplan, 2004). Due to heavy reliance on the use of MLs, a developing resistance against ivermectin (IVM) and moxidectin (MOX) has been noticed since over a decade (Geurden, 2014; Peregrine, 2014). Lyons *et al.* (2011) and Geurden *et al.* (2014) reported a reduced egg reappearance period (ERP) and a reduced efficacy measured over a prolonged period of time. This strongly suggest a developing resistance against these types of anthelmintics (Lyons, 2011; Molento, 2008).

Glutamate-gated chloride channels (GluCls) are the receptor targets for the ML group of anthelmintics (Wolstenholme, 2011). Glutamate is the physiologic ligand of this receptor and is potentiated by IVM. When IVM irreversibly binds to the receptor, the channel remains open resulting in a constant flow of Cl^- ions, which leads to continuous hyperpolarisation of the cell. This in turn causes permanent paralysis of the cyathostomin, ultimately leading to its death (Wolstenholme, 2005). In the nematode *Caenorhabditis elegans*, Cully *et al.* (1994) was able to identify two subunits within the GluCl. Only the –α- subunit seems to be IVM-sensitive, while the β-subunit seems IVM-insensitive (Cully, 1994; Tandon, 2006). The genes encoding for subunits α and β were respectively termed glutamate-gated chloride channel 1 (glc-1) and glutamate-gated chloride channel 2 (glc-2). Further research identified multiple genes encoding for multiple α-subunits, amongst which is glc-3 encoding for GluClα4 (Wolstenholme, 2005). The GluClα4 is a IVM sensitive glutamate gated channel and variation in glc-3 is strongly suspected to play a role in the developing IVM resistance (Wolstenholme, 2011).

In vitro research suggests there is a variation in the 5'-end of glc-3 on mRNA level between IVM resistant and sensitive cyathostomin larvae (unpublished data). This variation translates into four different mRNA sequences, from now on termed as band A, B, D and E. Only variant A and B are believed to encode for functional proteins and can both be found within the same individual larvae. However, variant A can be found in pools with IVM sensitive and resistant L3 larvae, while variant B was only present in resistant larvae (unpublished data). When viewing both bands on mRNA level, band A comprises a low complexity part and a conserved coding part (CCP) and band B comprises a

signal peptide and a CCP. However, so far no definite conclusion can be drawn to what the structure of the 5'-end of glc-3 should look like.

Recent (unpublished) research within the department focussed on finding the origin of band A and B, using DNA from adult cyathostomins to examine glc-3 variation. This research continues this search focussing on characterization of the 5'-end of glc-3 in *Cylicostephanus goldi* nematodes by a genome walking (GW) method using rolling circle amplification.

2. Materials and methods

2.1 Parasite source

On three different occasions adult cyathostomins were collected at an equine abattoir. Samples were also taken from one horse presented for necropsy at the Veterinary Pathology Diagnostic Centre, Faculty of Veterinary Medicine, Utrecht University. From each horse six samples were taken in total; collecting two plastic bags with approximately 500 g of faeces from each anatomic region of the large intestines (dorsal - and ventral colon and caecum). Processing of the faeces was done by an agar gel method (van Wyk, 1980) developed for collection of gastro-intestinal nematodes, see appendix 1. The abundant isolated L4s were divided over multiple Falcon tubes and mainly stored in PBS at -20 ^{o}C. Some of the L4s, specially prepared for an experiment regarding fixation techniques, were stored under different circumstances i.e. in 70% or 100% ethanol (EtOH) at -20 ^{o}C and at room temperature. The 16 collected adult cyathostomins were stored in PBS at -20 ^{o}C. Prior to storage they were decapitated for morphological identification. The heads were cleared in chloro-lactophenol and the species identified according to Lichtenfels *et al.* (2008).

2.2 Faecal sample collection

Faecal samples of clinically healthy horses were collected from the department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University. The samples were fresh (< 2 hours after defaecation) as they were collected from recently cleaned stables. The history of anthelmintic treatment was unknown. All samples were examined for strongylus type eggs with the McMaster method.

2.2.1 Larval culture

Multiple samples with an EPG ≥ 250 were used to culture larvae. In total 2.5 g of sawdust was added to 25 g of each faecal sample in a glass jar. The substance was mixed, moistened with tap water placed in a 23-25°C incubator for 7 days. After 7 days the L3s were collected and stored per jar at 4 ^{o}C in tap water. Two larval cultures were left for ±9 days to optimise circumstances for development of *Strongylus vulgaris* larvae.

2.2.2 Faecal sample processing

Samples with high EPG (≥ 500) were used for collection of strongylus type eggs. A total of 50 g faeces was placed on a sieve, mesh size 0.106 µm and rinsed with tap water. The filtrate was collected in a bucket and pored over a mesh size 0.038 µm sieve, which was small enough to collect the majority of the eggs. Absence of eggs in the filtrate was confirmed by microscopic evaluation.

The residue was complemented with distilled water (dH_2O) up till 50 ml in Falcontubes. The tubes were centrifuged for 1 min, at 1000 x g. The supernatant was disposed and the pellet complemented with 20 ml dH_2O. Next, 12.5 ml of sucrose solution (100 ml dH_2O + 70 g sucrose; SD=1.18) was added on the bottom of the tube. The tubes were placed in the centrifuge for 15 min at 2000 x g, with no breaks. The interphase was obtained and suspended in 50 ml dH_2O. Another centrifugation cycle (2 min, 2000 x g) provided a clear sample with concentration of purified eggs at the bottom.

Under a microscope individual eggs where collected and a known number of eggs were brought into separate microcentrifuge tubes. Multiple tubes were left overnight at room temperature to produce L1's for further use.

A total of 1 ml of the eggs suspension was supplemented with 15 ml of 70% EtOH to fixate overnight at room temperature. The following day, the eggs were rinsed and centrifuged 3 times to remove the EtOH from the eggs. The tubes were centrifuged for 1 min, at 1000 g. The supernatant was disposed and the pellet complemented with 20 ml dH_2O. Next, 12.5 ml of a sucrose solution (100 ml dH_2O + 70 g sucrose; SD=1.18) was added on the bottom of the tube. The tubes were placed in the centrifuge for 15 min at 2000 x g, with no breaks. The interphase, was obtained and suspended in 50 ml dH_2O. Another centrifugation cycle (2 min, 2000 x g) provided a clear sample with concentration of purified EtOH fixated eggs at the bottom. The same procedure was done for the eggs recovered from the undifferentiated adult female cyathostomins.

2.3 Lysis method

To prepare templates for PCR, first the eggs, larvae or adults had to be lysed overnight. Therefore, Worm Lysis Buffer (WLB) and Proteinase K (20 mg/ml, Invitrogen, ThermoFisher Scientific, The Netherlands) were mixed and added to the eggs/larvae/adults. The ratio WLB: Proteinase K used in this research was 100 µl to 0.5 µl (1xWLB). In general 100 µl 1xWLB was added when working with larvae or adults (like in earlier experiments) and 25 µl 1xWLB when working with eggs. Sometimes a two-fold concentration of WLB and Proteinase K were used (2xWLB). See Appendix 2 for composition of WLB. Samples in WLB were kept at – 80 ^{o}C for 15 min after which they were incubated overnight at ± 56 ^{o}C. The following morning Proteinase K was inactivated at 95 ^{o}C for 15 min (Eppendorf Thermomixer® C) and the lysed samples were stored at -20 °C.

In each experiment multiple eggs, L1s or L3s were often lysed in similar ways to produce duplicate samples. As eggs, L1 or L3s could not be separated prior to lysis, these were no true duplicates and will be termed replicates from now on. As replicates often show the same outcome they will be mentioned but the data not always shown.

2.4 Polymerase Chain Reaction(PCR)

The PCR reaction was facilitated by the C1000™ Thermal Cycler (Bio-Rad laboratories B.V. The Netherlands) with the DreamTaq PCR mix (Appendix 3). The main primer pair used in the DreamTaq PCR were CY1-CY18 which are encoding for ribosomal RNA (rRNA) genes in cyathostomin spp. CY1 (5'GGTCAAGGTGTTGTATCCAGTAGAG3') as the forward primer and CY18 (5'CTTAGACATGCATGGCTTAATC3') as the reverse primer. The following primer pairs have also been used; F532-R823 (specific for GluCl-α4 = glc-3), F350-R370 (CCP of glc-3), FA250-RA200 (Variant A of glc-3), FB140-RB30 (Variant B of glc-3). The primer pair used for GW was F532-R370 (GluCl-α4/CCP). Appendix 4 contains all primers including function and sequence. Appropriate positive and negative control sample were included.

Amplification was carried out as follows: denaturation at 95 ^{o}C for 3 min, then amplification for 35 cycles at 95 ^{o}C for 30 sec, 50-60 ^{o}C for 30 sec, and 72 ^{o}C for 60 sec with a final elongation step of 72 ^{o}C for 7 min. During the cycle the lid temperature is kept at 105 ^{o}C. Afterwards the sample is kept at 4 ^{o}C.

2.5 PCR product evaluation

Evaluation of PCR obtained product was done by gel electrophoresis. A ±1% agarose gel in Tris, acetate, EDTA (TAE) buffer supplemented with Midori Green (Nippon Genetics Europe) was used to visualise the PCR products. A DNA ladder (2 µl of 1 kb (Promega, Madison, VS) was added to compare the PCR products. In general, the protocol consisted of a run of 30 min by 75 V facilitated by the PowerPac™ Basic (Bio-Rad laboratories B.V. The Netherlands). In some cases another 20 min at 75 V

was added, to further enhance product separation on the gel. Imaging of the agar-gel was done by the Molecular Imager® Gel Doc™ XR+ combined with Image Lab™ Software on a PC.

2.6 The GW method

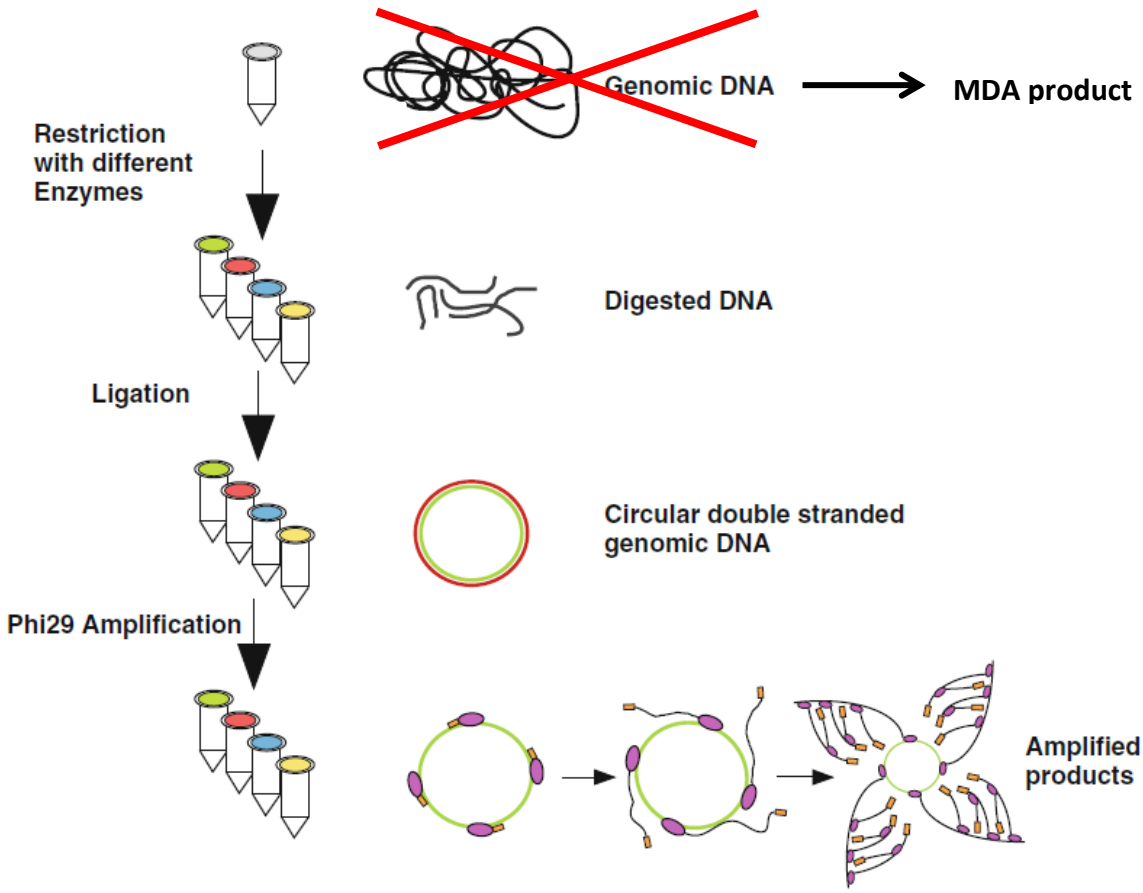

Figure 1: a schematic overview of the GW method described by Tsaftaris et al. (2010). The method used in this research is based on this GW method, with an altered first step. As described in more detail below, the GW was performed on MDA products. The MDA products are digested with 4 different restriction enzymes (separate tubes) and ligated to produce circular double stranded DNA. Amplification is done by *φ29* DNA polymerase (violet oval) and random primers (orange). An aliquot of each *φ29* reaction serves as a template in an inverse PCR to obtain both the 5'- and 3'- ends at the same time (Tsaftaris, 2010).

2.6.1 Multiple Displacement Amplification

In order to amplify genomic DNA from cyathostomin adults, larvae or eggs the REPLI-g Mini Kit or REPLI-g Single cell kit (Qiagen, The Netherlands) was used. See respectively Appendix 5 and 6 for the protocols. A total of 2.5 µl of a sample was mixed with an alkaline denaturation buffer (from one of both kits) and PBS or Tris-EDTA (TE), incubated on ice for 10 or 30 min, after which a stop solution was added to neutralise the sample. A master mix was prepared and mixed with the lysed sample after which it was incubated for 8 h at 30 ^{o}C. Inactivation of the REPLI-g Single cell DNA polymerase was done by heating the sample at 65 ^{o}C for 3 minutes. Obtained MDA product was stored at 4 ^{o}C for short –term and -20 ^{o}C for long term storage. Appendix 7. displays the further steps of GW.

Quantification of the MDA product was done with the Qubit 3.0 Fluorometer (Invitrogen, ThermoFisher Scientific, the Netherlands). A total of 1 µl of a 1:100 MDA product dilution was used. According to manufacturer's protocol a Qubit Working Solution was prepared for each standard (S1

and S2) and for each sample to be measured. All tubes were vortexed briefly and incubated at room temperature for 2 minutes before reading in the Qubit Fluorometer. The quantification was displayed as tube concentration (ng/ml) and as sample concentration (ng/µl).

2.6.1.1 *Hydrolysis with S1 Nuclease*
Prior to the digestion step (see 2.6.2 digestion) an (extra) alternative set of samples containing MDA product were incubated with S1 nuclease (ThermoFisher Scientific, The Netherlands). This is a single-strand-specific endonuclease, hydrolysing DNA or RNA into 5' end mononucleotides, such as in loops and gaps. The reaction mixtures were already prepared separately to enhance the next step in line, the digestion with restriction enzymes. The S1 nuclease reaction mixture consisted of 0.1 µl S1 nuclease, 0.9 µl 5X reaction buffer for S1 nuclease (ThermoFisher Scientific, The Netherlands) and 2.5 µl undiluted MDA product. The mixtures were incubated at room temperature for 30 minutes. Next, inactivation was done by adding 2 µl 0.5 M ethylenediaminetetraacetic acid (EDTA) and heating at 70 ^{o}C for 10 minutes.

2.6.2 Digestion
Four restriction mixtures were prepared separately for each restriction enzyme; Xbal, BamH1, EcoR1 and Xhol (ThermoFisher Scientific, The Netherlands). The mixture consisted of 19 µl nuclease free water, 2.5 µl 10x fast digest buffer (ThermoFisher Scientific, The Netherlands), 2.5 µl MDA product (or 2.5 µl S1 hydrolysed MDA product) and 1 µl of the restriction enzymes. The mixtures were incubated according to their specific requirements i.e. Xbal, BamH1 and Xhol at 37 ^{o}C for 10 minutes and EcoR1 at 37 ^{o}C for 20 minutes. Next, inactivation was done by heating the samples to 65 ^{o}C for 20 minutes (Xbal) or to 80 ^{o}C for 5 minutes (BamH1, Xhol, EcoR1).

2.6.3 Ligation
The ligation mixture was prepared separately for each restriction digestion (Xbal, BamH1, EcoR1 and Xhol). The ligation mixture consists of 425 µl nuclease free water, 50 µl 10X T4DNA ligase buffer (ThermoFisher Scientific, The Netherlands), 5 µl T4 ligase (ThermoFisher Scientific, The Netherlands) and 20 µl of the restriction digestion. High dilution is needed to minimalize ligation other than self-ligation. The samples were incubated at room temperature for 2 h after which the ligase is inactivated at 70 ^{o}C for 5 minutes.

2.6.4 Precipitation
The precipitation mixture consisted out of 500 µl 100% EtOH, 50 µl 3 M sodium acetate (pH 5.3) and 500 µl of the ligation mixture. The precipitation mixtures are incubated at -80 ^{o}C for 45 minutes. The precipitation mixtures are centrifuged at 20,000 x g for 15 minutes, followed by elimination of the supernatant. The pellet is dissolved in 200 µl chilled 70% EtOH and centrifuged at 20,000 x g for another 15 minutes. The majority of the supernatant is discard. The remaining supernatant is allowed to vaporise in the open air and the pellet dissolved in 20 µl TE buffer.

2.6.5 PCR with GW primers
The precipitation products for the different restriction enzymes were amplified by DreamTaq PCR with the primer pair coding for the CCP of glc-3. F350 (5'CATATGATCGAYAAGCCRAACG3') as the forward primer and R370 (5'GCARATGCATYGGACATGAAAG3') as the reverse primer. The direction of these primers is outwards. Also the primer pair F532-R823 was used, coding for the glc-3. Since there were two reverse primers available, one general primer for glc-3 and one specific for *C. Nassatus*, both primers were used in the PCR mix in equal amounts. Another primer pair (F532-R370) was used, which is the forward primer (5'CAGTACTATCCTATGGATGTGCAAA3') for GluCl-α and the reverse primer for the CCP of glc-3. The PCR reaction was facilitated by the C1000™ Thermal Cycler (Bio-Rad laboratories B.V. The Netherlands).

2.7 Gel Band Purification
Purification of PCR product from agarose gels was done with GFX PCR DNA and Gel Band Purification Kit, according to the protocol in Appendix 8. The section of the agarose gel containing the band of interest was excised under exposure of long wavelength (365 nm) ultraviolet light and placed in a microcentrifuge tube with buffer. The mixture was heated to 60 ^{o}C for 15-30 minutes to dissolve the agarose. A sample binding and washing step, on a MicroSpin column, was alternated with spinning the tube at 16 000 x g for 30-60 seconds. Eventually the DNA was eluted in a TE elution buffer and ready to use for downstream application.

2.8 Sanger sequencing
Sequencing of PCR products was performed by BaseClear B.V., Leiden, The Netherlands. Preparation of samples consisted of treating 5 µl PCR products directly with 2 µl ExoSAP-IT reagent (Affymetrix, ThermoFisher Scientific, The Netherlands). The mixture incubated at 37 ^{o}C for 15 minutes to degrade remaining nucleotides and primers. Next, the sample was incubated at 80 ^{o}C for 15 minutes to inactivate the ExoSAP-IT reagent. Incubation steps were facilitated by the C1000™ Thermal Cycler (Bio-Rad laboratories B.V. The Netherlands). Following incubation, samples were brought into tubes ready for transport, each containing 3 µl of the PCR reaction product, 2.5 µl forward or reverse primer and 14.5 µl milliQ. The sample was send to BaseClear B.V.

3. Results

3.1 Comparison of different cyathostomin life stages as PCR template

3.1.1 Testing cyathostomin eggs as PCR template
To investigate whether cyathostomin eggs would be a good alternative for cyathostomin adults, a test was performed with multiple life stages. The test was run with fresh eggs, L1's or L3's in dH_2O, eggs fixated overnight in 70% EtOH and eggs collected from an adult cyathostomin fixated in EtOH. All samples were lysed in 25 µl 1xWLB/Prot K. To investigate whether a higher concentration of WLB and Proteinase K made a difference in PCR product, some samples were lysed with 2xWLB/Prot K. All conditions were tested with 2 individual eggs, L1's or L3s. Only one set of all conditions is shown as the outcome was completely similar to the other. The results are shown in Figure 2.
The results show a clear product for fresh and EtOH fixated eggs, L1 and L3. However, no product could be seen with eggs retrieved from the EtOH fixated adult cyathostomin. This is in accordance with earlier research on EtOH fixated adult cyathostomins done at this research department. The question is whether the lack of product is attributable to inhibiting factors present in the adult cyathostomin or that it might be caused by problems within the DNA itself, for instance denaturation by long term fixation in EtOH or incomplete uncoiling because of a lack of rehydration.

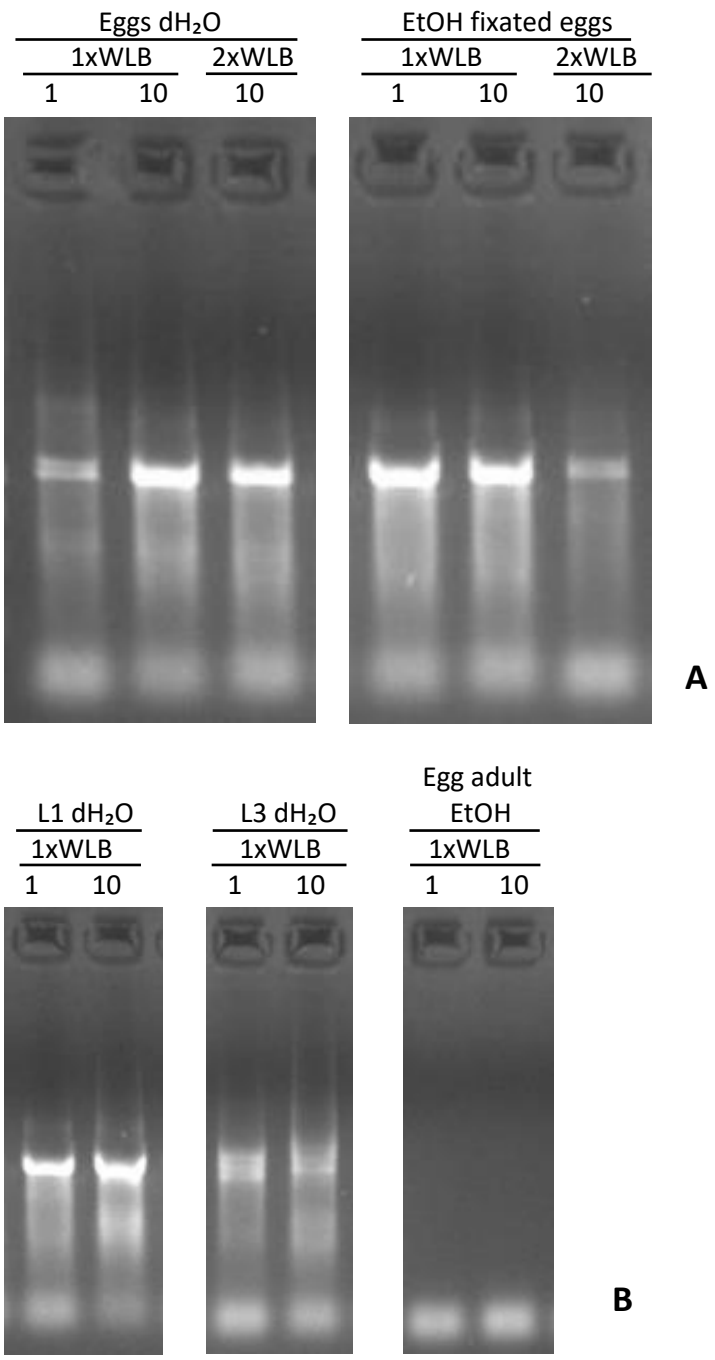

Figure 2: CY1-CY18 DreamTaq PCR fragments obtained from different templates and separated on agarose gel. A) templates: 1 or 10 eggs, fresh or EtOH fixated, suspended in dH2O and lysed in 1xWLB or 2x WLB. B) templates:1 or 10 L1s or L3s suspended in dH_2O, or 1 or 10 eggs isolated from EtOH fixed adults were lysed with 1xWLB.

3.1.2 Testing on inhibiting factors

To further investigate the lack of amplification eggs retrieved from EtOH fixed adults another PCR was performed. The template for this PCR, a single lysed fresh egg was serial diluted with the lysed single egg retrieved from the EtOH fixated adult cyathostomin. Hypothesized was that in case the eggs retrieved from EtOH fixed adults contained inhibiting factors, the fresh egg sample should lose its signal with increasing concentrations of the lysed egg from EtOH fixed adults,

Another serial dilution was performed with 10 eggs retrieved from the EtOH fixated adult cyathostomin lysed together in 25 ul 1xWLB. These lysed eggs were diluted in dH_2O. With each

diluting step, possible present inhibiting factors should have less influence, which might result in a visible PCR product after all. The results are shown in Figure 3 a and 3b. Replicates showed similar outcome (not shown). All dilutions of the lysed fresh egg, with the lysed eggs from the EtOH fixated adult cyathostomin gave a PCR product, whilst the lysed egg retrieved from the EtOH fixated adult did not give any signal after several dilution with dH_2O. From this the provisional conclusion can be drawn that inhibiting factors are not the problem.

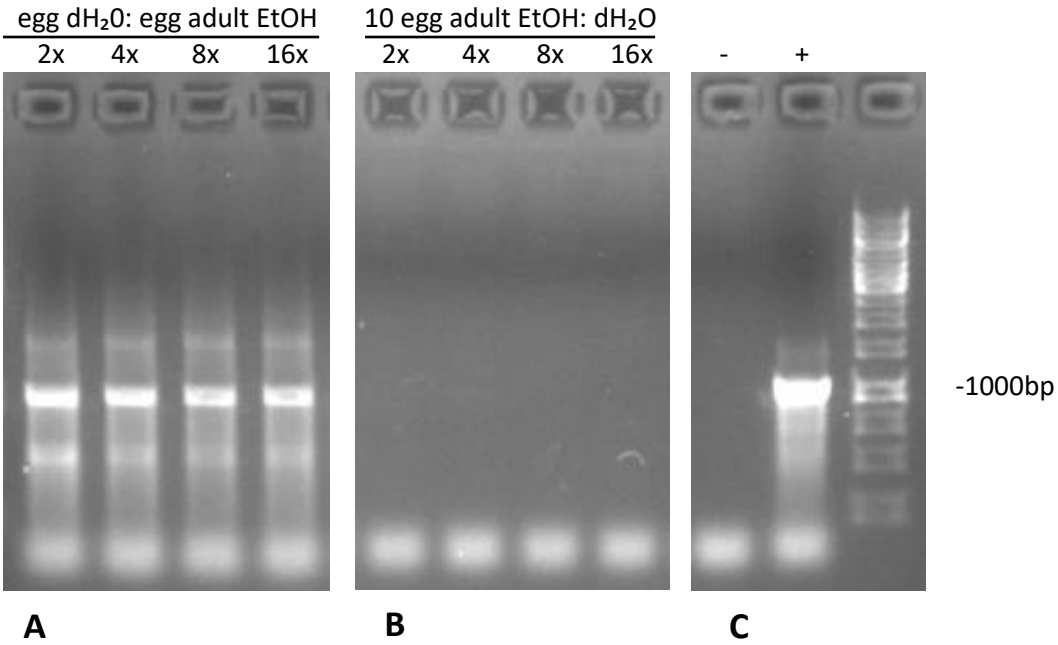

Figure 3: CY1-CY18 DreamTaq PCR fragments obtained from different templates and separated on agarose gel. A) egg in dH_2O diluted with sample containing eggs from EtOH fixated adult (2x, 4x, 8x, 16x dilution). B) 10 eggs from EtOH fixated adult diluted with dH_2O (2x, 4x, 8x, 16x). C) Negative control: dH_2O. Positive control: single fresh eggs in dH_2O in 25 ul 1xWLB lysis buffer. DNA ladder 1 kb.

3.2 Comparison of lysis methods for GW

In order to examine the possible use of fresh cyathostomin eggs in the GW method, the first step was verifying if the mild alkaline lysis buffer required for MDA (REPLI-g Mini or REPLI-g sc kit) was strong enough to lyse eggs and L1's.

To visualise if the alkaline lysis buffer had worked, CY1-CY18 PCR was performed on the lysate of eggs and L1's in PBS. Replicates were included in the test. Eggs were lysed for either 10 or 30 minutes. As a positive control, a fresh egg lysed with 1xWLB and diluted several times in dH_2O was included, see Figure 4.

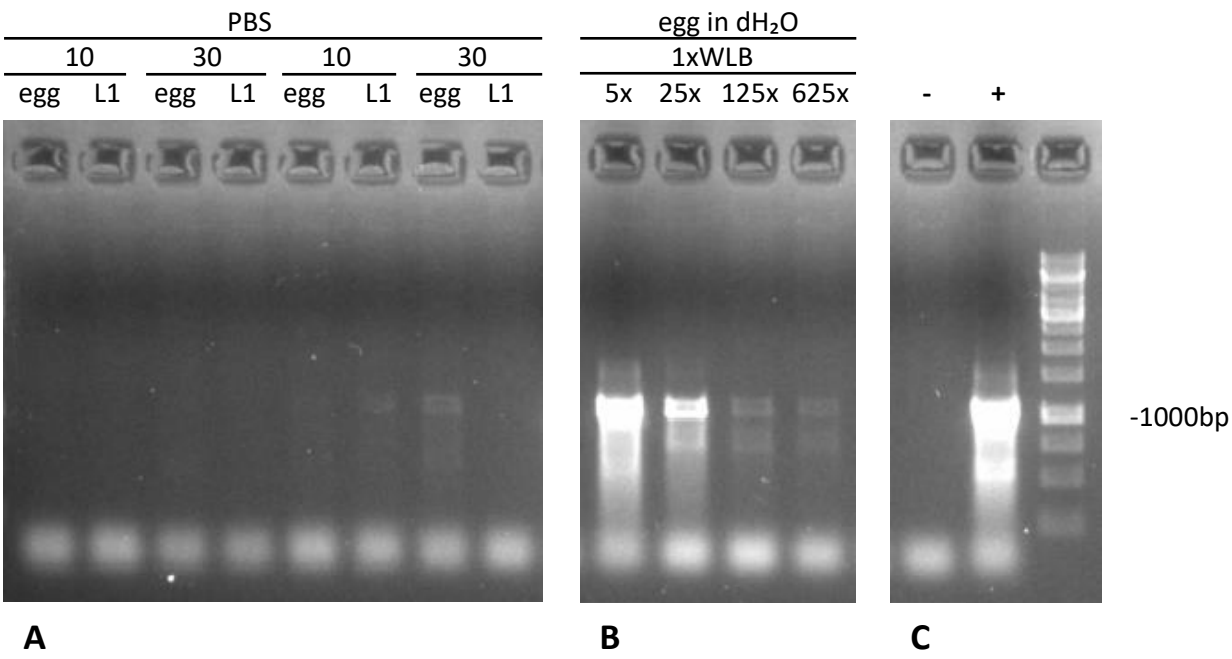

Figure 4: CY1-CY18 DreamTaq PCR fragments obtained from different templates and separated on agarose gel. A) 1 egg or L1 in PBS and lysed with alkaline lysis buffer. Incubation on ice for 10 or 30 minutes. B) A single egg diluted in dH_2O (5x, 25x, 125x, 625x) and lysed with 1xWLB as extra positive control and DNA ladder. C) Negative control: dH_2O. Positive control: single fresh eggs in dH_2O in ul 1xWLB lysis buffer. DNA ladder 1kb.

There seems no difference between 10 or 30 min lysis on ice before the stop solution is added. Also no difference was noticed between eggs or L1's. Some of the eggs/L1s give a vague product on the gel. Others do not seem to give any product, even though they are replicates of the ones that do. The results from this test raised some new questions with respect to the compatibility of the alkaline lysis buffer and the DreamTaq PCR or the efficacy of the alkaline lysis. To investigate these options, two sets of dilutions were made from a fresh egg and a L1 and another DreamTaq PCR with CY1-CY18 performed.

3.2.1 Compatibility of DreamTaq master mix with alkaline lysis buffer

One fresh egg and one fresh L1, both in PBS, were lysed separately with alkaline lysis buffer. After that, both lysates were serial diluted with dH_2O, with 625x being the highest dilution. The same dilution series (5x, 25x, 125x, 625x) with a fresh egg, but lysed with 1xWLB was used for comparison. Replicates were included. The results, shown in Figure 5, show no PCR product for the alkaline buffer lysed egg or L1. The control dilution series show a clear product for all 4 dilutions, with a declining visibility as was expected for increasing dilutions. This indicates that it is a matter of DNA absence rather than incompatibility of the DreamTaq master mix and the alkaline lysis buffer.

To be able to clarify these results further a combination of both lysis buffers was tested. Tubes containing 10 eggs each were either lysed with only alkaline lysis buffer or lysed with 1xWLB/Prot K after the lysis with the alkaline lysis buffer. It was hypothesized that when the alkaline buffer is not strong enough to lyse the eggs, the WLB and Proteinase K would make the DNA available after all. The results are shown in Figure 6. The control sample of 2xWLB/Prot K contained 10 eggs in dH_2O.

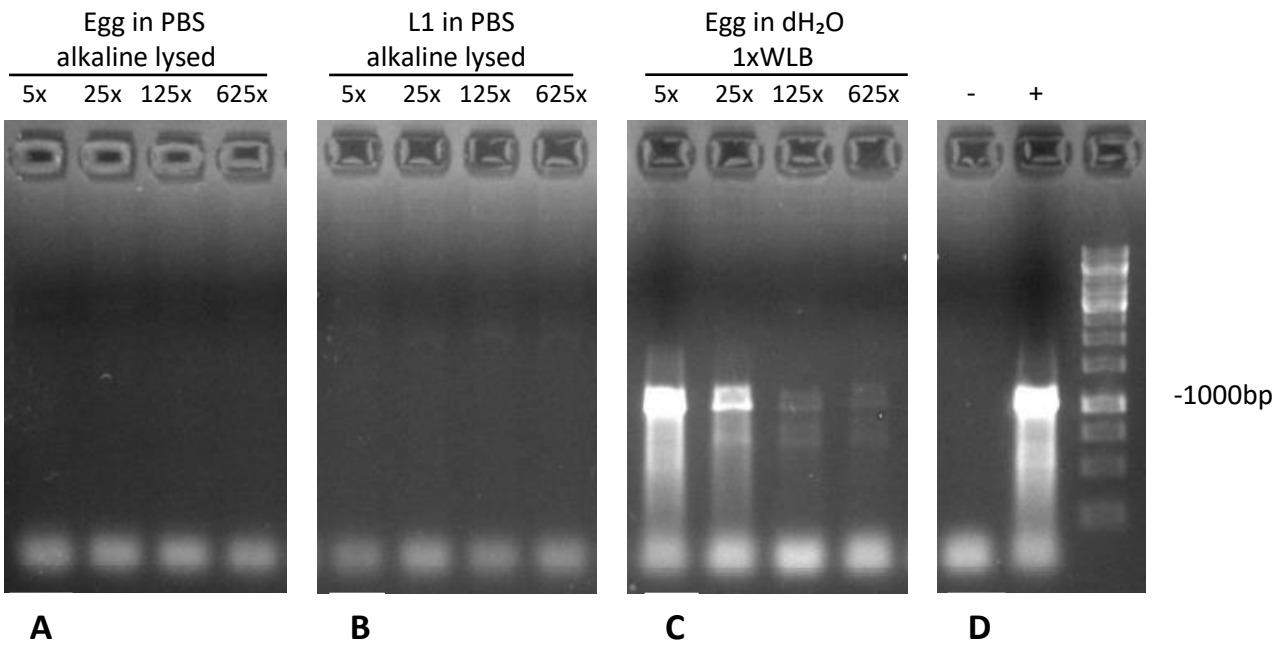

Figure 5: CY1-CY18 DreamTaq PCR fragments obtained from different templates and separated on agarose gel. A) a single fresh egg in PBS and lysed with alkaline lysis buffer and diluted (5x, 25x, 125, 625x) with dH_2O. B) a single fresh L1 in PBS and lysed with alkaline lysis buffer and diluted (5x, 25x, 125x, 625x) with dH_2O. C)A serial dilution of 1 fresh egg lysed with 1xWLB in dH_2O as extra positive control and DNA ladder. D) Negative control: dH_2O. Positive control: single fresh eggs in dH_2O in ul 1xWLB lysis buffer. DNA ladder 1kb.

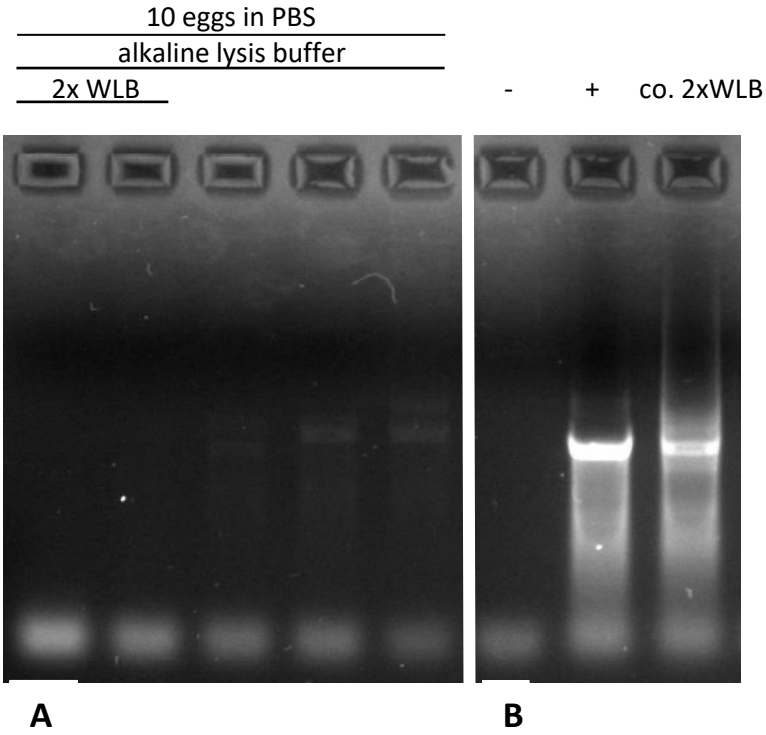

Figure 6: CY1-CY18 DreamTaq PCR fragments obtained from 10 eggs derived templates and separated on agarose gel. A) 10 fresh eggs in PBS lysed with (1) alkaline lysis buffer or lysed with (2) alkaline lysis buffer and 2xWLB/prot K. B) Negative control: dH_2O. Positive control: single fresh eggs in dH_2O in 25 ul 1xWLB lysis buffer. Co. 2xWLB on 10 eggs in dH_2O.

The results from this experiment match the earlier findings that PCR product yield varies between samples, even when they are duplicates. These are true duplicates, as the 5 lanes are from the same 10 eggs, separated after the first lysis step with alkaline lysis buffer. The lanes show a very slight product only for lysis with the alkaline lysis buffer. Addition of 2xWLB does not result in a (visible) product on the gel. The lysis with 2xWLB was correctly executed as this lane shows a clear PCR product.

3.2.2 Varying incubation temperature and length for alkaline lysis

As some of the eggs lysed with alkaline lysis buffer show a faint PCR product (Figure 4) and others did not, it does seem possible to yield DNA from eggs with this lysis buffer. As the two REPLI-g kits use varying incubation times and temperatures an experiment was set up to test these different conditions. Therefore, the protocol was extended beyond the incubation of 10 minutes to an incubation of 30 and 60 minutes, and also the temperature was varied between incubation on ice, - 20 ^{o}C and - 80°C. All possible combinations between length and temperature were tested and replicates included, see Figure 7.

The results from this experiment are somewhat odd as both 30 and 60 min, as well as incubation of ice, - 20 ^{o}C and - 80°C show a clear product on gel, whilst the duplicates of all these samples do not show any product. Again this test was done with single eggs, which might explain the contradicting outcome of the replicates caused by different species, developmental stages or other unknown factors. On the other hand, this experiment clearly shows that in some cases alkaline lysis is sufficient in yielding sufficient template.

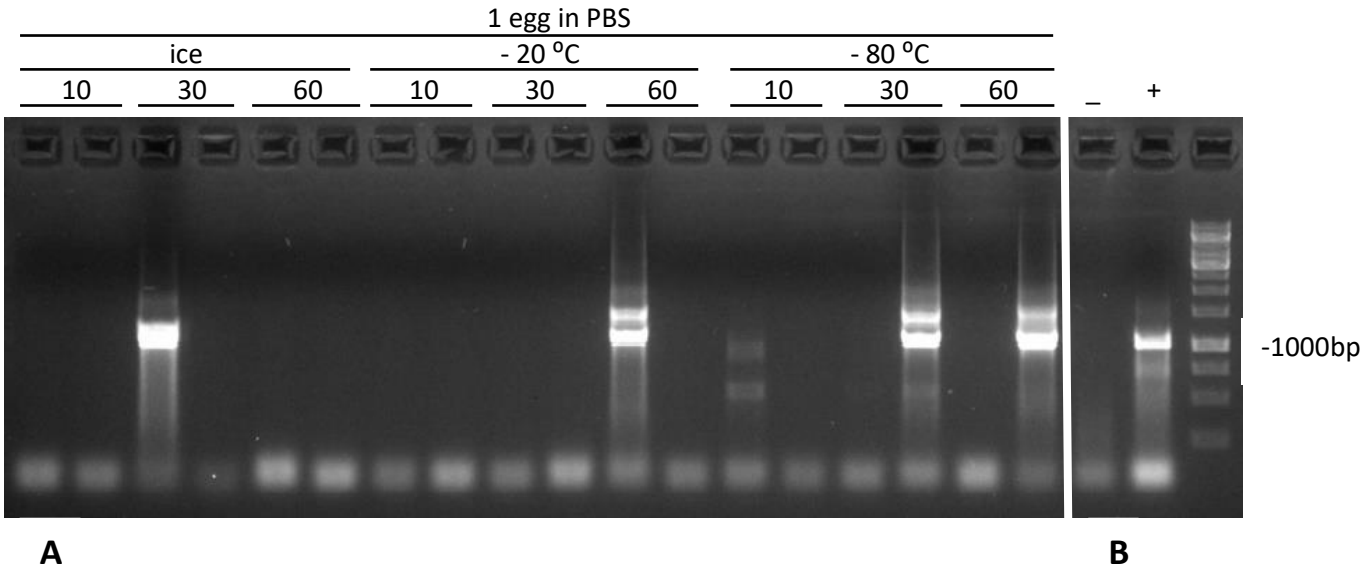

Figure 7: CY1-CY18 DreamTaq PCR fragments obtained from different templates and separated on agarose gel. A) Single eggs in PBS lysed with alkaline lysis buffer and incubated at different temperatures (ice, - 20 ^{o}C, - 80 ^{o}C) and incubation lengths (10, 30 or 60 min). All with replicates. B) Negative control: dH_2O. Positive control: single fresh eggs in dH_2O in 25 ul 1xWLB lysis buffer. DNA ladder 1 kb.

3.2.3 Alkaline lysis buffer combined with PBS vs. TE

The protocol for the REPLI-g Mini Kit and REPLI-g Single cell kit do not differ much. However, one of the differences is the starting solution in which the eggs/L1s are suspended, PBS and TE respectively. A test was run with 10 eggs in PBS and 10 eggs in TE to examine whether there would be a difference in DNA yield. The results are shown in Figure 8. Again, only in one sample a faint product can be seen on the gel, with the duplicate showing no product. These were no true duplicates.

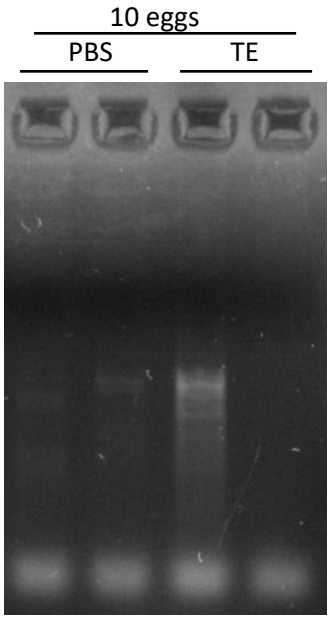

Figure 8: CY1-CY18 DreamTaq PCR fragments obtained from 10 eggs in PBS or TE and separated on agarose gel. Incubated for 30 minutes and ysed with alkaline lysis buffer. Replicates included.

3.3 Repeated PCR evaluation of EtOH fixated eggs

Within the ongoing research, there has been a constant struggle with the use of EtOH fixated adult cyathostomins for the different molecular techniques described in this thesis. To evaluate whether the same struggles are encountered when using EtOH fixated eggs, multiple test were run on EtOH fixated eggs throughout this research. EtOH fixated eggs were lysed with 1xWLB or 2xWLB and Proteinase K and amplified by DreamTaq PCR with primer pair CY1-CY18. Eggs fixated for 10, 20 and 60 days in 70% EtOH at room temperature including replicates are shown in Figure 9. The results show equal outcomes for the different evaluation moments. For all single lysed eggs the lanes show a clear PCR product, with some showing a very clear band and others showing multiple bands in one lane. Striking is the absence of a product in two of the lanes with a template of 10 eggs. It was hypothesized that in case one egg shows a clear PCR product, the chance of more eggs showing a PCR product should increase with every extra egg added to the tube. The contrary appears to be true as the lanes show diminished or no bands at all. The reasons for this might be the inconsistency in the quantity of WLB and Proteinase K used, as the samples with 1 egg were lysed with 1xWLB and the samples with 10 eggs with 2xWLB.

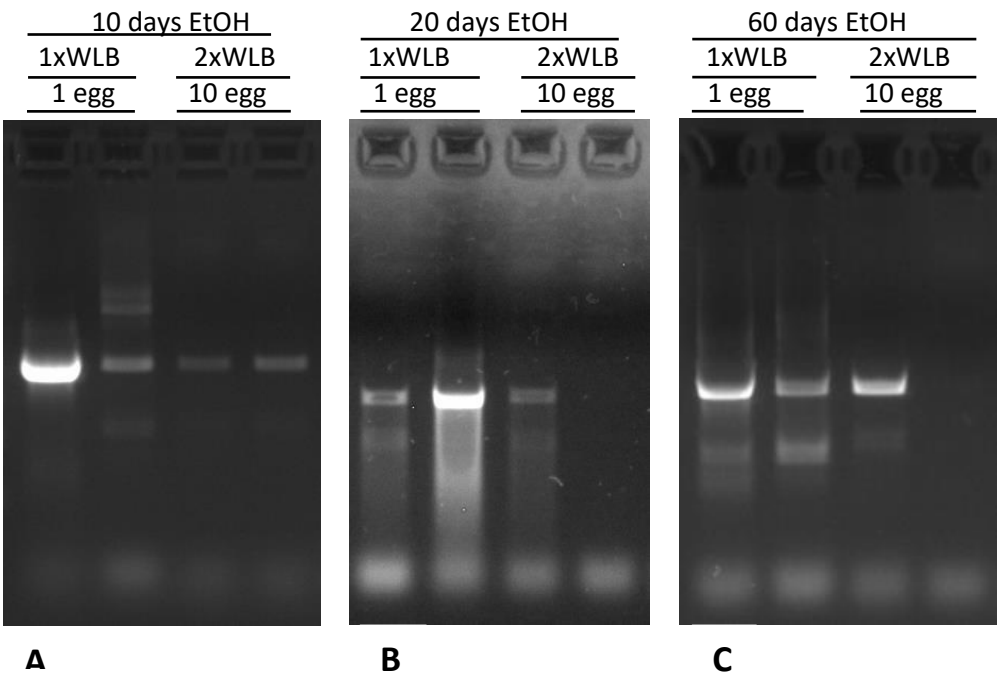

Figure 9: CY1-CY18 DreamTaq PCR fragments obtained from different templates and separated on agarose gel. Replicates included. A) 1 or 10 eggs fixated in 70% EtOH at room temperature for 10 days lysed with 1xWLB or 2xWLB. B)) 1 or 10 eggs fixated in 70% EtOH at room temperature for 20 days lysed with 1xWLB or 2xWLB. C)) 1 or 10 eggs fixated in 70% EtOH at room temperature for 60 days lysed with 1xWLB or 2xWLB.

4. Fixation techniques for use in larvae and adult cyathostomins

In order to evaluate the possible difference between fixation in 70% or 100% EtOH, as well as storage at ± 21°C (room temperature) or -20 °C, an experiment with L4s was set up. The L4 stadium was chosen, as we wanted to cautiously handle the very few fresh available adult cyathostomins. The L4s of unknown species were obtained from one horse at the equine abattoir and fixated over ± 60 h in 70% and 100% EtOH at ± 21 °C and -20 °C. Two 70% EtOH fixated undifferentiated adults (from an earlier obtained badge) were included as extra negative control since these are known to lack PCR product when amplified with DreamTaq PCR. The 1xWLB was tested on 10 eggs in dH_2O, so far known to always show a PCR product. The L4s and two undifferentiated adults were lysed with 1xWLB/Prot K and amplified by DreamTaq PCR, with primer pair CY1-CY18. The test was run in duplicate, See Figure 10. These were no true duplicates as all tubes contained a different L4 or undifferentiated adult. Only the L4s in 70% EtOH show a vague product on the gel, with no visible difference between the two different temperatures. As was expected, adults fixated in 70% EtOH at room temperature do not give a product. Also no product can be seen for L4s fixated in 100% EtOH. In addition also L4s diluted in PBS (- 20°C) and in SAF (± 21°C) were lysed with 1xWLB/Prot K (not shown). However, not one of the L4s showed a visible PCR product.

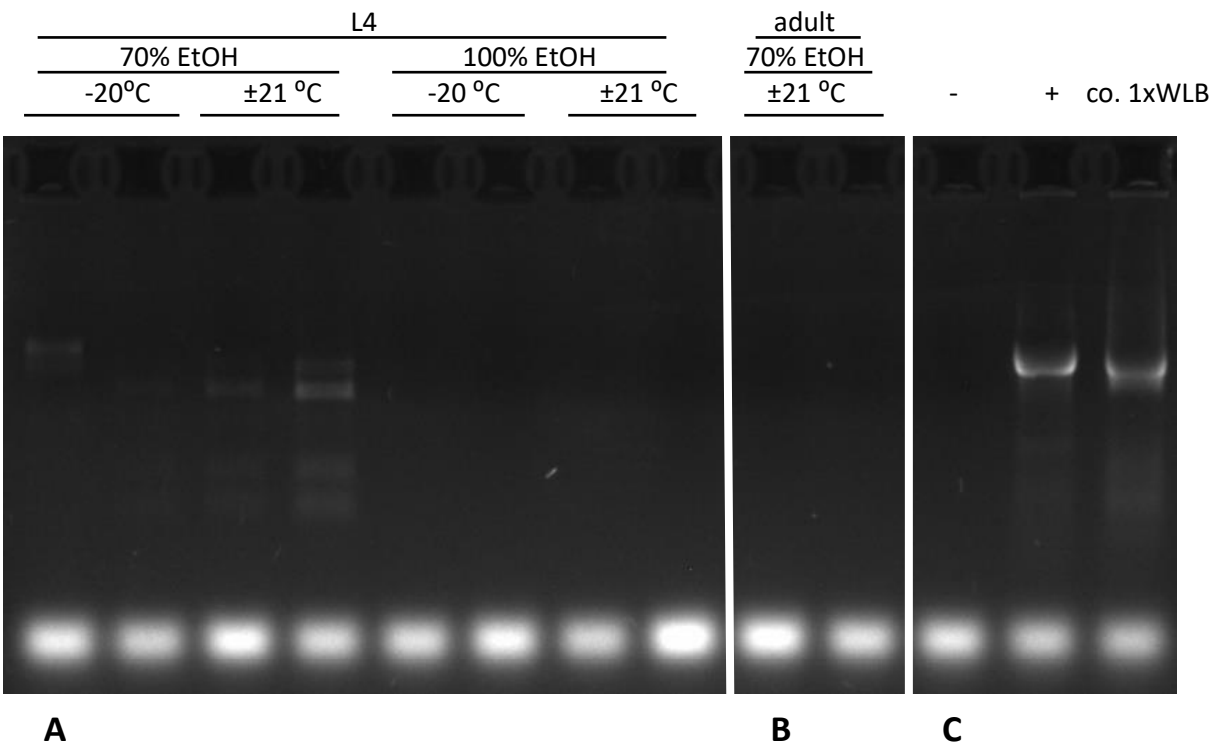

Figure 10: CY1-CY18 DreamTaq PCR fragments obtained from different templates and separated on agarose gel. All lysed with 1xWLB. Replicates included. A) L4s in 70% or 100% EtOH and stored at ±21 °C or -20 °C. B) two undifferentiated adults in 70% EtOH and stored at ±21 °C. C) Negative control: dH_2O. Positive control: single fresh eggs in dH_2O in 25 ul 1xWLB lysis buffer. Control 1xWLB on 10 fresh eggs in dH_2O.

5. *Identification of Strongylus vulgaris in larval cultures*

To elucidate the absence of PCR product in many lysed eggs samples, as well as the inconsistency between duplicates, two larval cultures of the original faecal samples were performed. It is known that different species, i.e. *Strongylus spp.* might not be compatible with the primers used in the current PCR protocol (personal communication F. Kooyman). A total of 100 L3 larvae were microscopically differentiated in regard to the number of intestinal cells they contained. *Strongylus vulgaris* has 28-32 intestinal cells and cyathostomins only have 8 (Lichtenfels, 2008). When microscopically differentiating 100 L3 larvae, all had 8 intestinal cells and hence classified as cyathostomin species.

6. *WLB lysis method versus alkaline lysis method*

Earlier experiments were performed with only small amounts of eggs or L1s, with varying results. Therefore an experiment with multiple replicates was set up. Figure 11 shows the results of an experiment in which multiple samples with 1 egg, 1L1 or 5 eggs are lysed with 1xWLB/Prot K or with alkaline lysis buffer. As can be seen in the figure, independent of the stage (eggs or L1s) lysis with alkaline lysis buffer hardly gives any PCR product, lysis with 1xWLB- or 2xWLB/Prot K in turn, shows highly consistent results. However, since the alkaline lysis is part of GW, we choose to continue with the three eggs that show a product on the gel (Figure 11, red rectangle).

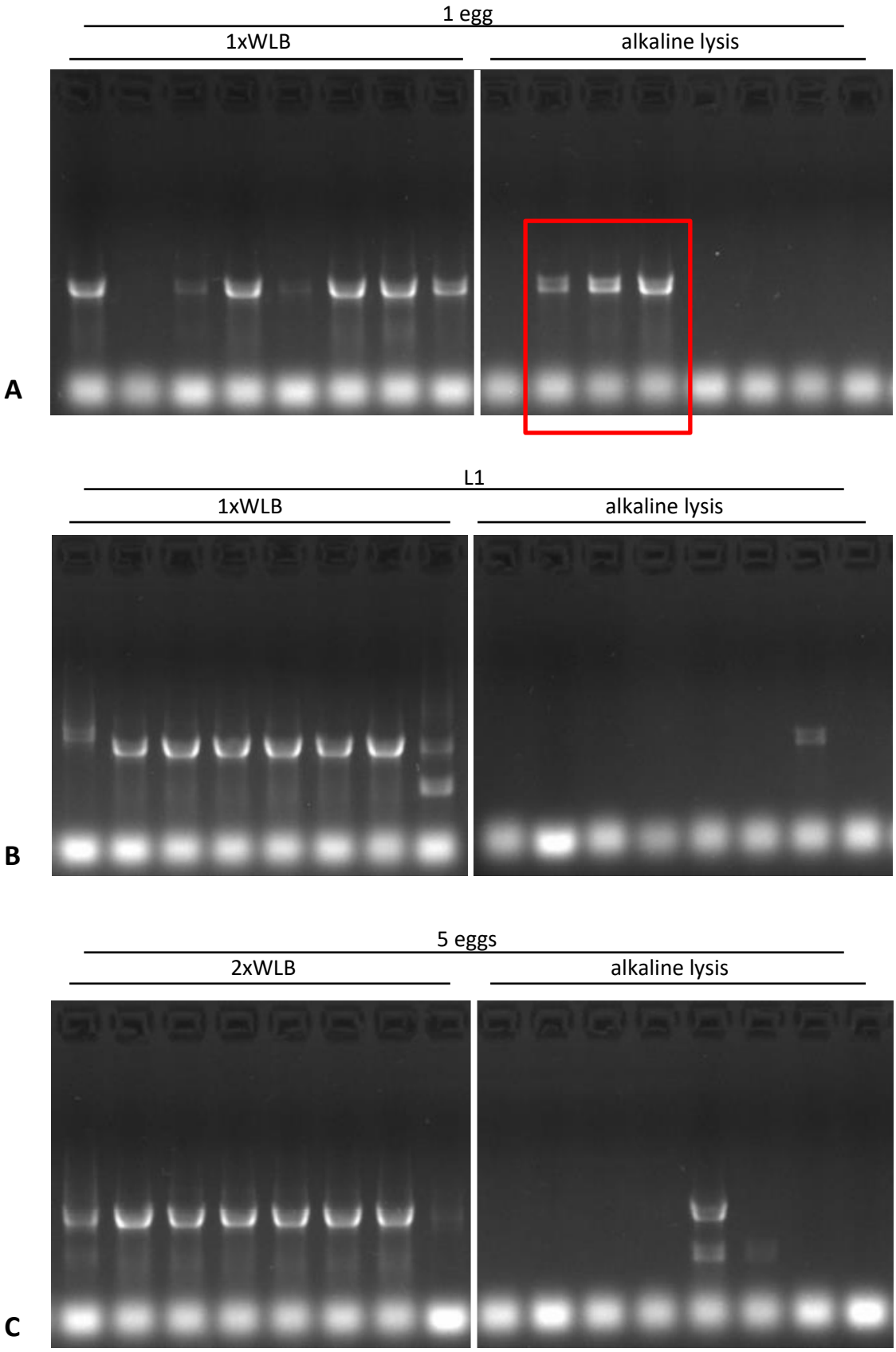

Figure 11: CY1-CY18 DreamTaq PCR fragments obtained from different templates and separated on agarose gel. A) multiple fresh single eggs lysed with 1xWLB or alkaline lysis buffer. The red rectangle shows the CY1- CY18 fragments of the only three single eggs successfully lysed with alkaline lysis buffer. B) multiple fresh single L1s lysed with 1xWLB or alkaline lysis buffer. C) multiple samples with 5 eggs lysed with 2xWLB or alkaline lysis buffer.

As three of the single eggs (red rectangle in Figure 11) showed a good PCR product we decided to carry on with these three single eggs executing the full REPLI-g protocol for MDA. However, the REPLI-g Single Cell kit demands the egg to be suspended in TE. Therefore, 6 more single eggs in TE were lysed with alkaline lysis buffer to be able to compare results between the eggs in PBS and TE. As expected from earlier obtained results, only 1 out of 6 eggs in TE showed a faint PCR product on the gel, See Figure 12.

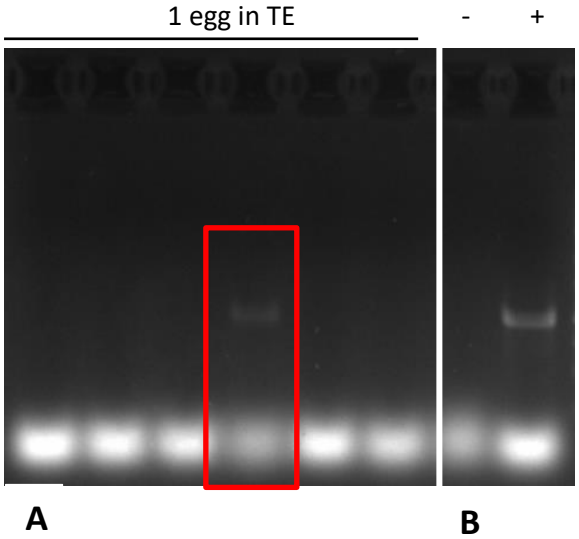

Figure 12: CY1-CY18 DreamTaq PCR fragments obtained from single eggs in TE and separated on agarose gel. A) Multiple single eggs in TE lysed with alkaline lysis buffer. Red rectangle shows faint CY1-CY18 fragments of one egg. B) Negative control: dH_2O. Positive control: single fresh eggs in dH_2O in 25 ul 1xWLB lysis buffer.

7. MDA product dilutions

As no optimal lysis method was found, it was decided to carry on with the 4 alkaline lysed eggs that resulted in a PCR product; 3 in PBS (red rectangle, Figure 11) and 1 in TE (red rectangle, Figure 12). These 4 lysed eggs were used as a template for the MDA executed with the REPLI-g Single Cell kit. After the full REPLI-g Single Cell kit protocol was run with the 4 lysed eggs, a serial dilutions (10x, 100x, 1000x and 10.000x) of the MDA product was made. The same set of dilutions was made for only the lysed eggs (prior to MDA), to compare with.

To see whether the MDA method had been successful, the MDA product dilutions and the lysed eggs dilutions were amplified in CY1-CY18 PCR. The products were brought on gel, see Figure 13. Only egg 3 shows a faint product for the first two dilutions (10x, 100x). This emphasizes the minimal amount of DNA present in the lysed egg samples just after lysis with the alkaline lysis buffer. For egg 2 and 3 MDA was successful as all 4 dilutions show a very clear PCR product. The single egg 4 in TE lysed with alkaline lysis buffer showed no product on the gel, and so this egg had not been used any further (figure not shown).

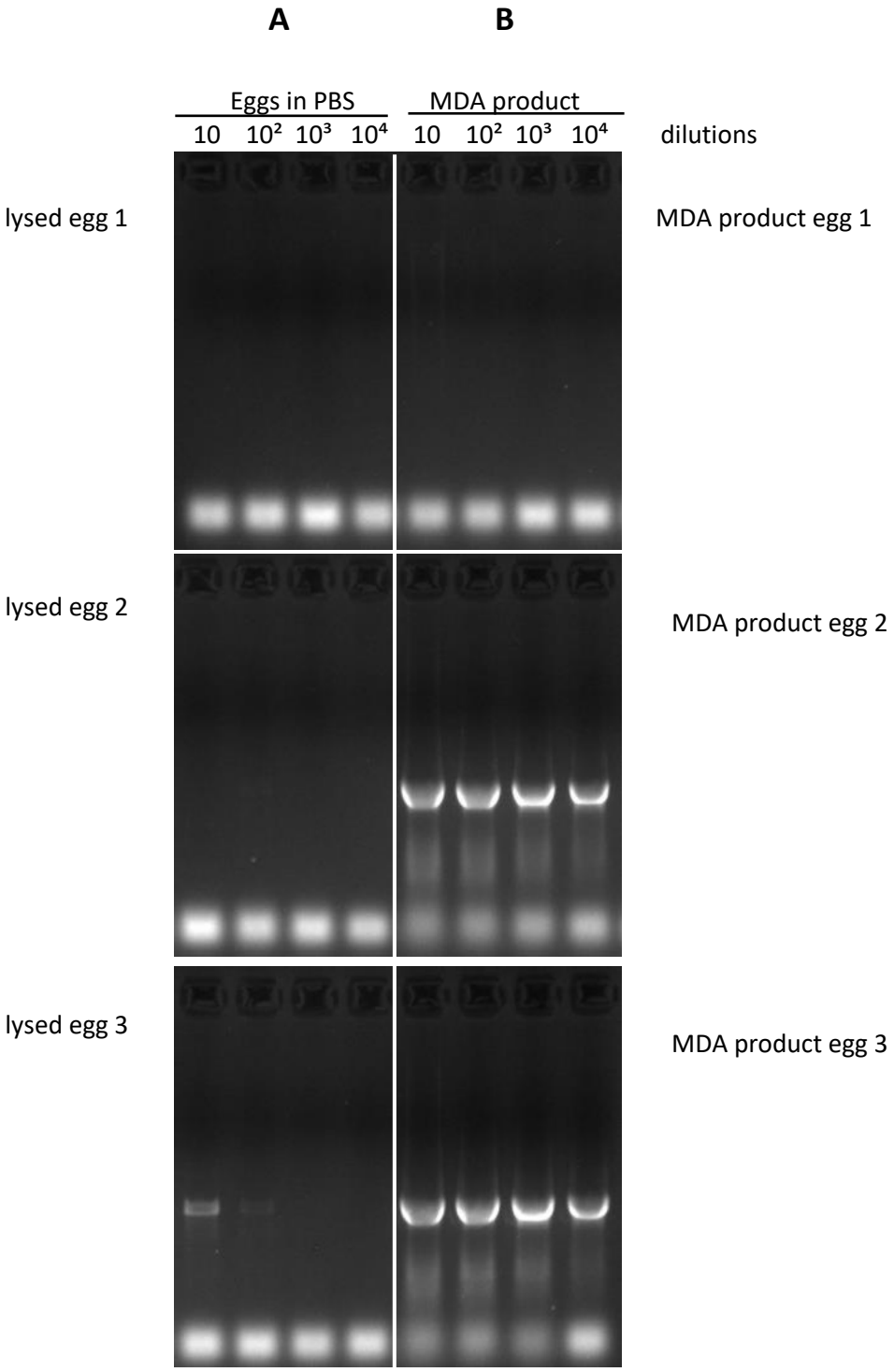

Figure 13: CY1-CY18 DreamTaq PCR fragments obtained from different templates and separated on agarose gel. A) templates: serial dilutions of three single eggs in PBS lysed with alkaline lysis buffer and B) serial dilutions of their amplified (MDA) products .

The amount of DNA was measured with the Qubit 3.0 Fluorometer according to manufacturer's protocol. For this quantification 1 µl of the 1:100 MDA product dilution was used. The quantity of DNA was according to expectation. In order to estimate the amount of DNA needed for downstream applications, further dilutions were made up to 10.000.000 times, see Figure 14.

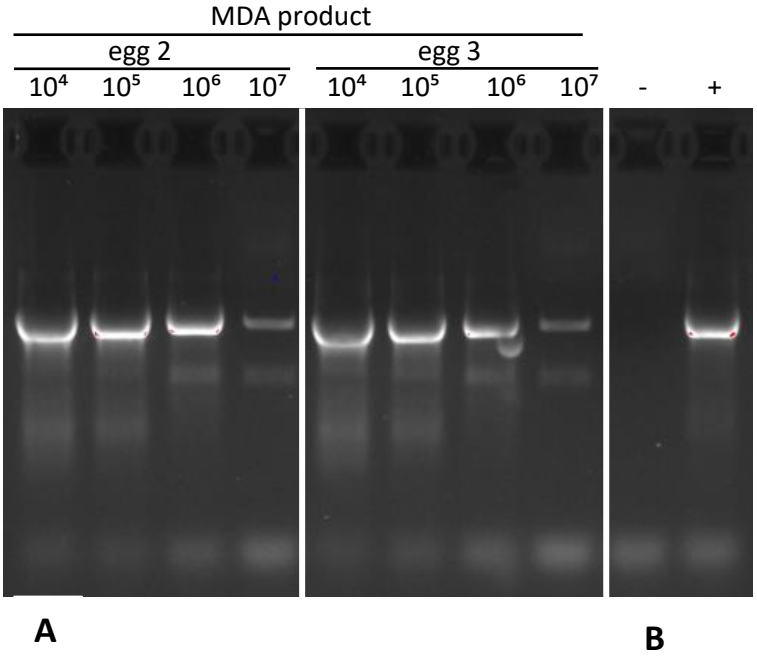

Figure 14: : CY1-CY18 DreamTaq PCR fragments obtained from MDA product as template and separated on agarose gel. A) templates: dilutions of the MDA products of egg 2 and 3 in dH2O (10.000x, 100.000x, 1.000.000x and 10.000.000x). B) Negative control: dH_2O. Positive control: single fresh eggs in dH_2O in 25 ul 1xWLB lysis buffer.

Tabel 1: Qubit 3.0 fluorometer DNA quantification of MDA product of egg 2 and 3. The table shows both sample concentration (1 µl) and the total amount of dsDNA in 25 µl MDA product)

egg	Sample concentration	Total amount of dsDNA in 25 µl
2	3.18 µg dsDNA/µl	79.5 µg dsDNA
3	4.13 µg ds DNA/µl	103.25 µg dsDNA

8. Sequencing of MDA product

The CY1-CY18 fragments of the MDA product of egg 2 and 3 (Figure 13) were sequenced, analysed, aligned and phylogenetically tree was constructed, see Figure 15. Egg 3 was undoubtedly an egg of the species *Cylicostephanus longibursatus.* Egg 2 is very likely from the same species. However, this cannot be stated with the same certainty.

Even though the two eggs were not of the preferred species i.e. *Cylicostephanus goldi,* it was decided to carry on with the available MDA product and start the next step in GW.

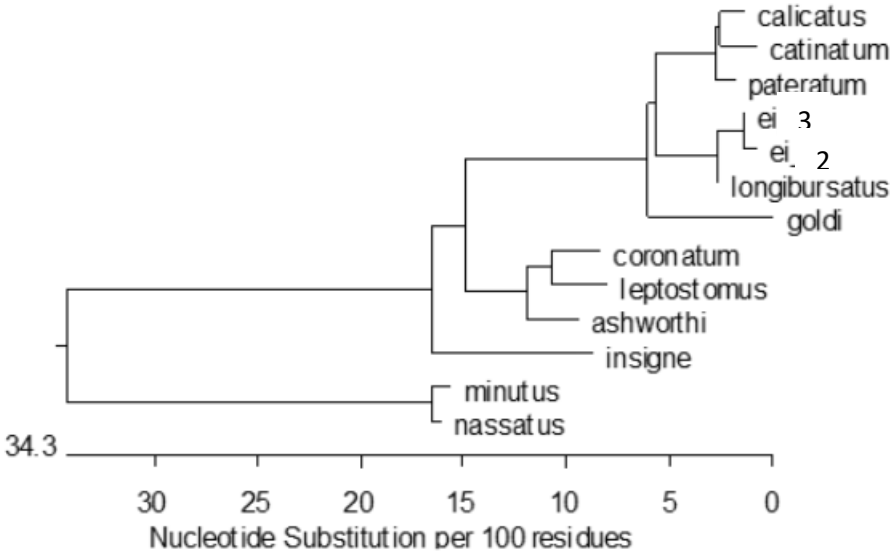

Figure15: BaseClear sequencing results from egg 2 and 3 demonstrating the two samples to be highly related to *Cylicostephanus longibursatus.* The phylogenetic tree consist of multiple cyathostomin species (from top to bottom): *Cylicostephanus calicatus, Cyathostomum catinatum, Cyathostomum pateratum, Cylicostephanus longibursatus, Cylicostephanus goldi, Coronocyclus coronatus, Cylicocyclus leptostomus, Cylicocyclus ashworthi, Cylicocyclus insigne, Cylicostephanus minutes, Cylicocyclus nassatus* (Stancampiano, 2010)

9. Genome walking

The continuation of the GW protocol requires digestion, ligation and precipitation of the undiluted MDA product of egg 2 and 3. To do so, the same version of the GW method used in earlier research at this department was adopted. Another extended version, with addition of a step in which S1 nuclease is added to the reaction mixture, was used as well. See Appendix 7 for the full GW protocol. The results of the GW procedure are visualised with DreamTaq PCR with primer pair F350 – R370 for the CCP of glc-3. The direction of primers is outwards. The melting point of both primers is 50 ^{o}C and the annealing temperature adjusted accordingly. The results of GW on the MDA templates for egg 2 and 3, incubated with – or without S1, are shown in Figure 16. No clear bands can be distinguished, however, large smears are slightly visible. Three options for the lack of product might be (1) the absence of DNA, (2) the incomplete digestion with the different restriction enzymes, making it impossible for the primer pair to bind to their sequence or (3) the primer pair used was insufficient.

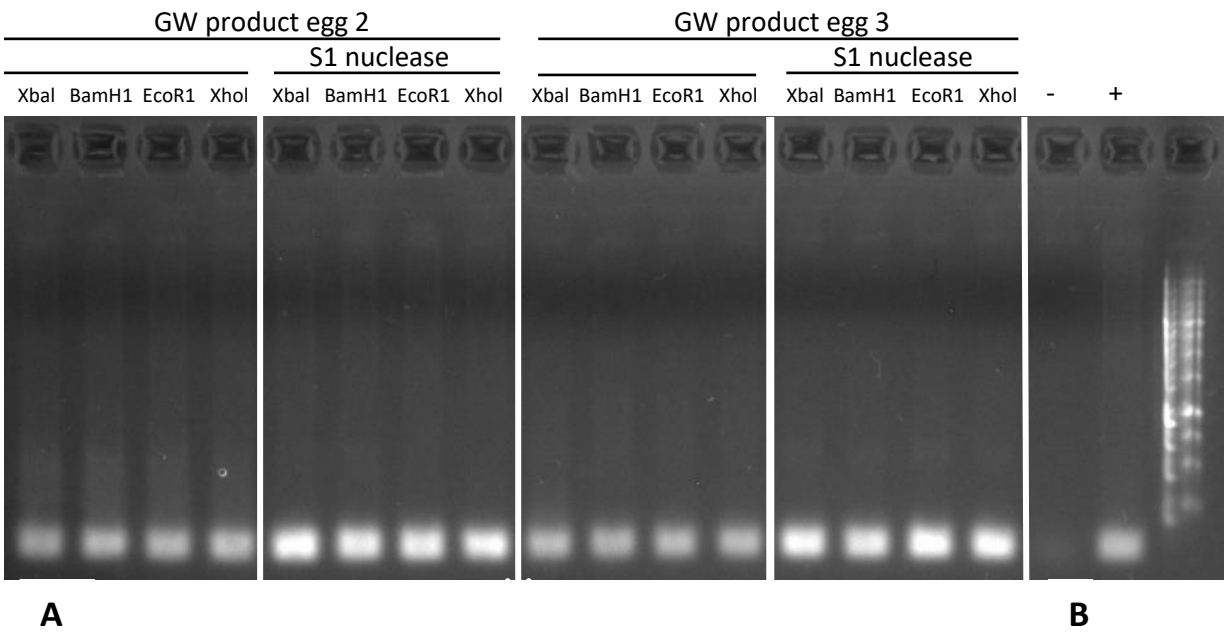

Figure 16: F350-R370 DreamTaq PCR fragments obtained from GW produced templates and separated on agarose gel. A) the MDA product of egg 2 and 3, incubated with and without S1 nuclease, and mixed with the four different restriction enzymes used; XbaI, BamH1, EcoR1 and XhoI. B) Negative control: dH_2O. Positive control: single fresh eggs in dH_2O in 25 ul 1xWLB lysis buffer. DNA ladder 1 kb.

In order to detect whether DNA is still present after digestion and precipitation the F532-R832 PCR was performed, the GW product of egg 2, see Figure 17. For all but one restriction enzyme, the gel clearly shows strong bands, indicating that there was DNA present after digestion, ligation and precipitation. For the lane of EcoR1 a mistake was made when loading the product on gel, therefore no statement can be made about this restriction enzyme.

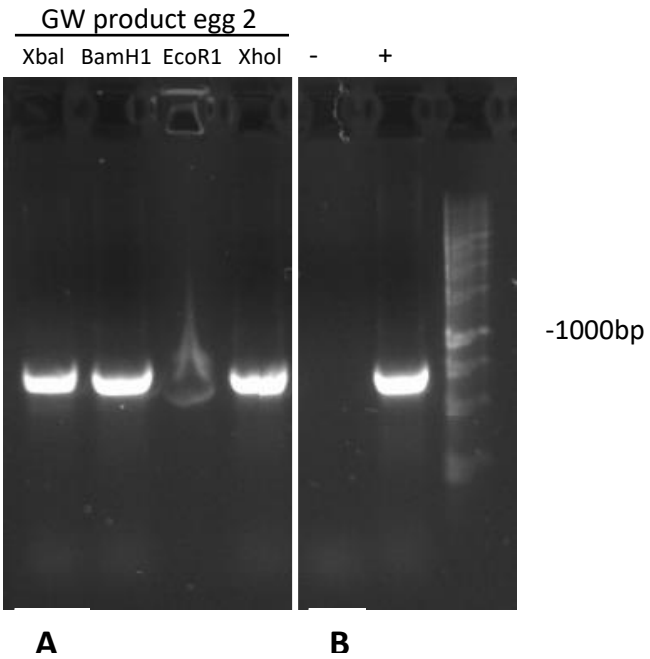

Figure 17: F532-R823 DreamTaq PCR fragments obtained from the GW product of egg 2 as template and separated on agarose gel. A) templates: GW product of egg 2 and the four different restriction enzymes used; XbaI, BamH1, EcoR1 and XhoI for digestion. The lane of EcoR1 is incorrect due to a loading mistake. B) Negative control: dH_2O. Positive control: single fresh eggs in dH_2O in 25 ul 1xWLB lysis buffer. DNA ladder 1 kb.

To verify the digestion step, the reactions (5 µl from egg 2 and 3) were put on a ± 1% agarose gel with the same amount of the non-digested MDA product. All digestion mixtures were brought on gel after addition of 2 µl 6X Loading Dye (Thermofisher Scientific, the Netherlands). Figure 18 shows a difference between the undigested and the digested MDA product for all restriction enzymes. The 10 kb band is not present anymore in the restriction digestions. Also, a difference can be noticed between the different restriction enzymes. All together this indicates that the restriction enzymes have actually worked properly and that plenty of DNA is present in the sample, at least prior to ligation and precipitation.

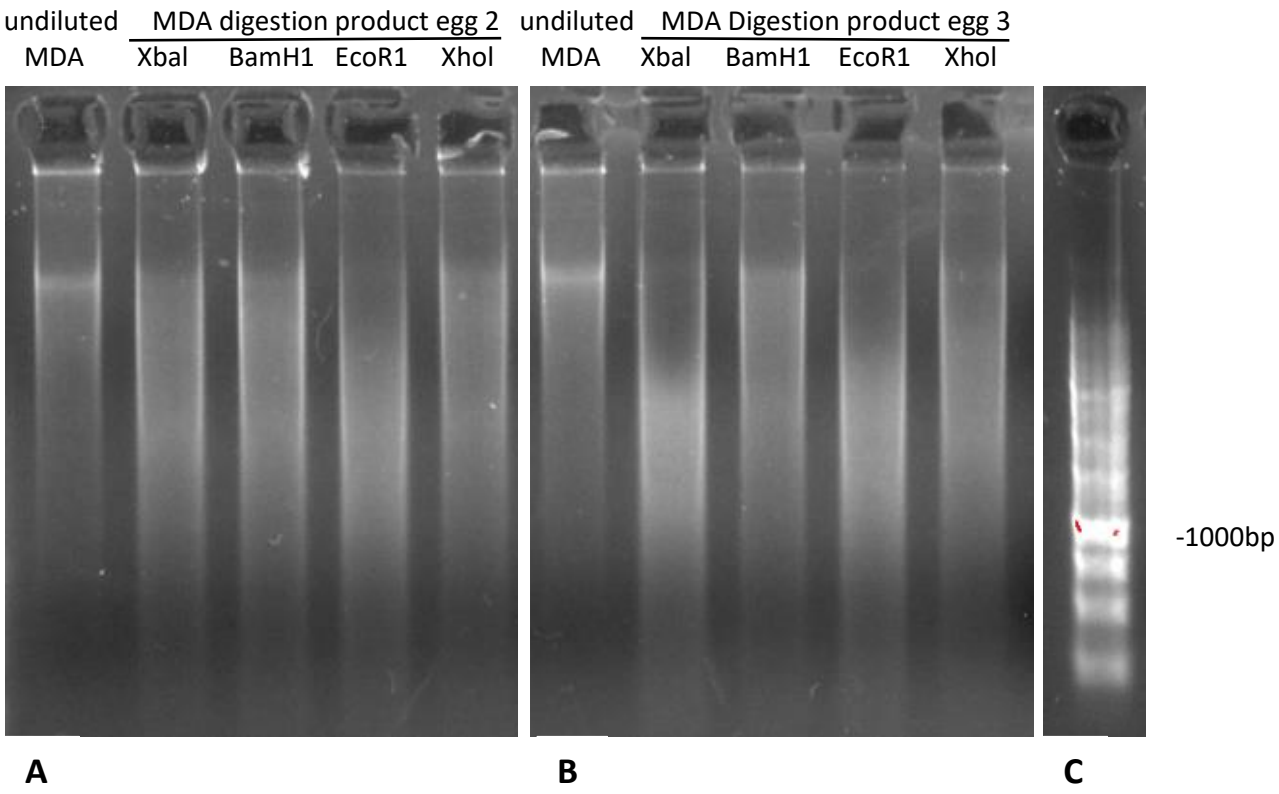

Figure 18: products of the MDA product digestion step of egg 2 and 3, separated on agarose gel. All samples supplemented with 6X Loading Dye prior to application on the gel. A) MDA product of egg 2 for restriction enzymes XbaI, BamH1, EcoR1 and XhoI, compared with the undiluted MDA of egg 2. B) MDA product of egg 3 for restriction enzymes XbaI, BamH1, EcoR1 and XhoI, compared with the undiluted MDA of egg 3. C) DNA ladder 1 kb.

As there seemed to be plenty of DNA present after digestion and considering the restriction enzymes had worked properly, the mistake was to be found in the primer pair used. Therefore, the DreamTaq PCR was repeated with another combination of primers pairs i.e. F532 – R370. This is the forward primer for glc-3 in combination with the reverse outward primer for the CCP for glc-3. The PCR was repeated for GW product incubated with – and without S1 nuclease. See Figure 19a and b.

The GW product for the different restriction enzymes of egg 2 and 3 show multiple faint bands on gel. This indicates that the GW procedure has worked for the different restriction enzymes. There seems a slight difference in density of the bands when comparing lanes with – and without S1 nuclease. The samples in which S1-nuclease (Figure 19a) is added are more clear and distinct with a greater number of different sizes.

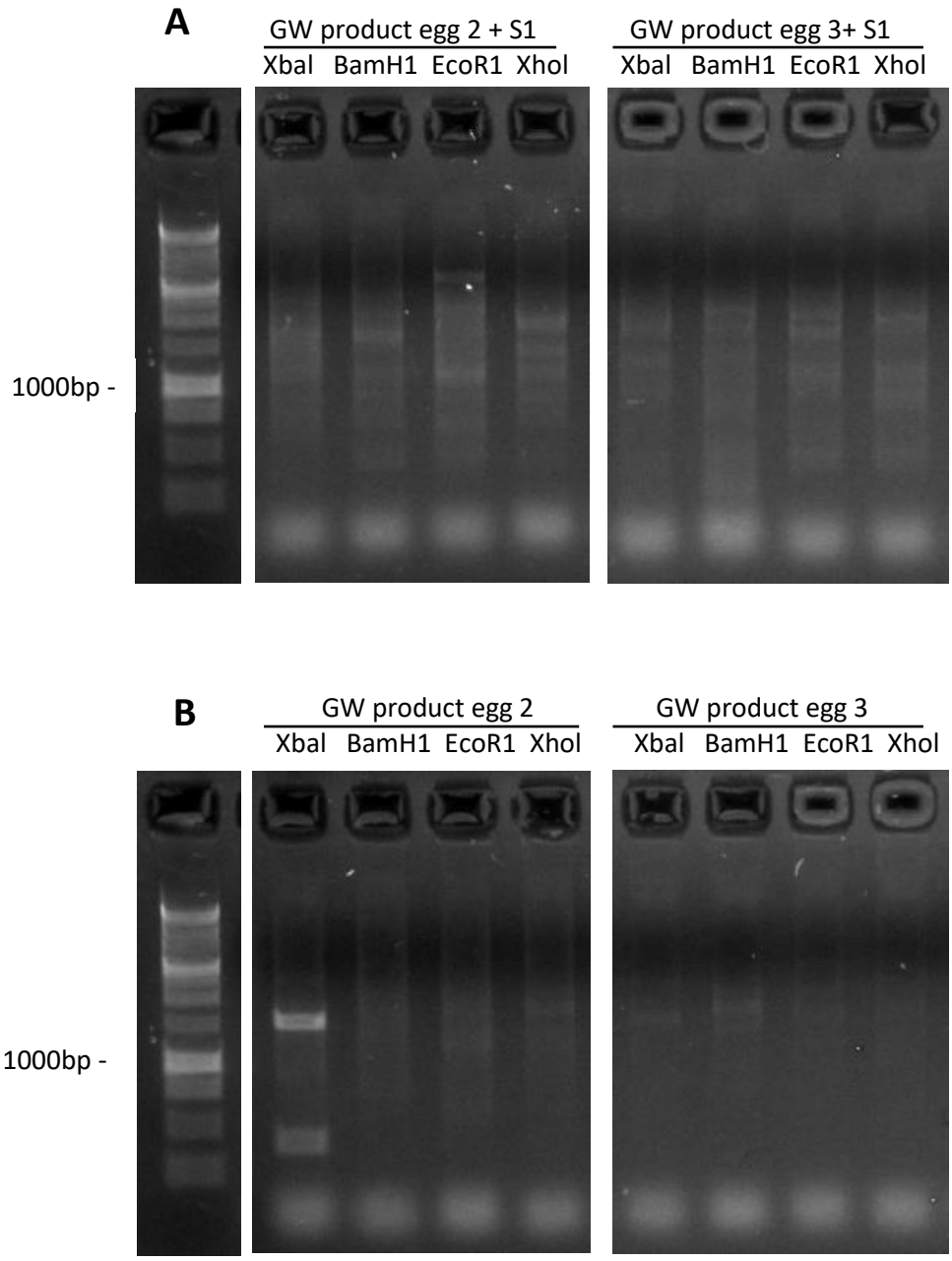

Figure 19. F532-R370 DreamTaq PCR fragments obtained from the GW product of egg 2 and 3 as template and separated on agarose gel. A) the products of the GW method, incubated with S1 nuclease and the four different restriction enzymes used; XbaI, BamH1, EcoR1 and XhoI. B) the products of the GW method, without S1 nuclease, for egg 2 and 3 and the four different restriction enzymes used; XbaI, BamH1, EcoR1 and XhoI.

Only the lane of XbaI for egg 2 shows two distinct bands on the gel. The stored GW product of this restriction enzyme was run again on gel and the two different bands isolated for sequencing.
Both bands visible in the lane of XbaI in Figure 19b were excised from the gel and processed with the Gel Band Purification kit, according to Appendix 8. Eventually the DNA was eluted in TE and prepared for sanger sequencing by BaseClear. The results obtained revealed the DNA to be gc-3.

4. Discussion

The aim of this study was to characterize the 5'- end of glc-3 in one species of cyathostomin, i.e. *C. goldi*. The main reason for choosing this specific species is due to *C. goldi* being the only cyathostomin species on which whole genome sequencing data is available.

Several difficulties were faced in the process of characterizing the 5'- end of this gene, starting with availability of adult *C. goldi*. The only adult *C. goldi* available were fixated in 70% EtOH at room temperature. As mentioned before, EtOH fixated adults do not give satisfying results in the downstream protocols used. A tremendous amount of work (earlier research) was put into optimising these protocols for 70% EtOH fixated adults, but yet without any success. It remains unclear why this fixation step in EtOH does not provide sufficient or useful DNA for further use, as many other researchers use a variety of fixation techniques without reporting any difficulties. Storage of adult worms in liquid nitrogen is an often encountered fixation technique (Cwiklinski, 2012; Cwiklinksi, 2013; Hodgekinson, 2001). Also storage in 100% EtOH at different temperatures (-20 ^{o}C or - 80°C) is frequently used without any remarks made of insufficient fixation (Lake, 2009; Peachey, 2017). EtOH has been known to be a perfect fixative for many different types of tissue and that with a simple rehydration step the DNA uncoils back into its original shape (Srinivasan, 2002). At first we hypothesised that fixation in 70% EtOH at room temperature in combination with the next step in line, obtaining DNA, was the stumbling stone. This, however, seemed rather doubtful as other research groups used a variety of methods and kits to obtain DNA, all with satisfactory results to a certain extent. Lake *et al.* (2009) for instance, describes three different DNA extraction methods from adult cyathostomins fixated in 100% EtOH, amongst which are also QIAmp DNA Mini Kit and lysis with WLB. This is similar to the techniques used in this research, but the results do not correspond. As in general 70% EtOH at room temperature was used to fixate adult cyathostomins in this research, the solution to the problem was hoped to be found in varying the storage temperature and percentage of EtOH in which the adults cyathostomins are fixated. L4s were used to test these different circumstances, but failed to show a PCR product on the gel. There seems no preference between 70% and 100% ethanol, nor the different temperatures, as no difference in circumstances leads to a better result. Perhaps de developmental stage of the larvae plays a role as to not being compatible with the lysis methods or the Dreamtaq PCR. For now, no real conclusions can be drawn to whether or not adult cyathostomins would react the same to different fixation methods.

To overcome the problem with EtOH fixated adults fresh adult *C. goldi* were searched for in slaughterhouse material, which was challenging and did not succeed. The three large intestinal sites were sampled, but no adult *C. goldi* were harvested. The predominant species found within the samples was *Coronocyclus labratus*. Stancampiano *et al.* (2010), sampled 50 Italian horses, describes a prevalence of only 4% for C. goldi, which suggest that not finding adult goldi's is not that strange. On the contrary, the same article describes a 4% prevalence for C. labratus as well. Collobert-Laugier *et al.* (2002) sampled 42 horses in France to clarify distribution and prevalence of cyathostomin populations for equids in France. They identified 20 species of cyathostomins with *C. goldi* being the 5th most prevalent species. Geographic location, management strategies and population factors might possibly make a big difference into species prevalence and abundance. A while ago, Ogbourne (1976) mentioned *Cylicocyclus insigne*, *C. goldi* and *C. longibursatus* as the three most common species. A few years later *Eysker et al*. (1985) mentioned a list of 11 common species, out of a total 20 from the subfamily Cyathostominea, found within that research. This list also includes *C. insigne*, *C. goldi* and *C. longibursatus.* Earlier research within this department focussing on the prevalence of larvae in the mucosa, predominantly found *C. insigne* (≥50%) and actually mentioned the absence of *Cylicostephanus minutus* and *C. goldi.* Without any real knowledge of prevalence and distribution of Cyathostomin species (within horses) in The Netherlands, it became clear that it is very hard and even unwise to rely on a certain species of cyathostominae for research.

Not being able to proceed with EtOH fixated nor fresh adult cyathostomins, a set of experiments with strongyle type eggs was set up. The most striking about these experiments is the inconsistency found within the results. Rather as a rule than an exception, replicates show different outcomes. An important factor might be the difference between eggs in developmental stage, but more probable the species of origin. With the McMaster method the only identification done is whether the egg is a strongyle type egg. The eggs recovered from the faeces can thus be from large - (Strongylinae) or small strongyles (Cyathostominae). The CY1-CY18 PCR will not work on *strongylus spp*. (personal communication, F. Kooyman). To determine the presence of *Strongylus spp.* larval cultures were performed. In the larval culture within this research, all differentiated L3 larvae were cyathostominae. No indication for the presence of *Strongylus vulgaris* was found. Figure 20 shows an L3 cyathostomin under the microscope.

Figure 20: microscopic close up (100x) of a cyathostomin L3 stage in which 8 intestinal cells are clearly visible

Another problem facing the use of eggs is the method for isolating sufficient amounts of DNA. In several experiments it became clear that PCR outcome is highly unpredictable, no matter the content of the sample i.e. one egg or multiple eggs, or the method of lysis. Harmon *et al.* (2006) tested multiple egg disruption procedures for isolating DNA from *Ostertagia osteragi* eggs, which share many morphological similarities with equine strongyle type eggs. Several procedures, amongst which is also digestion with proteinase K, showed good results. However, 100% disruption was only accomplished with a bead beater system using zirconia/silica or ceramic beads for several minutes (Harmon, 2006). Studies in human medicines demonstrate DNA isolation from hookworm eggs directly from faeces, without the need for prior egg isolation (Gruijter, 2005; Verweij, 2001). However, this technique requires only a small amount DNA as it is used for PCR diagnosis. Much larger quantities of DNA are needed for downstream molecular research. The Qiagen QIAmp DNA stool kit is described as a useful commercial kit for rapid reproducible DNA extraction, however when high sensitivity is desired the DNeasy plant kit can be used (Harmon, 2006). It should be emphasized that even though steps were taken and results obtained, that reproducibility of results are of major importance for further research. Therefore, more DNA extraction methods should be examined to find a 100% repeatable protocol.

The method of GW has been used for long and proven an efficient method for isolation of the 5'- and 3'- sequences from a known gene (Tanksley, 1995). The original idea required a gene-specific primer and attachment of anchor sequences, which could be utilised as a universal primer binding templates. As the universal primer corresponds to all anchor sequences present in the cDNAs, nonspecific products are formed, causing a lot of background noise (Polidoros, 2006). To overcome this difficulty DNA templates were circularized, allowing for gene-specific primers to be used in inverse PCR (Polidoros, 2006; Sato, 2004; Tsaftaris, 2010). At the same time both 5'- and 3'-end of the cDNA can be isolated simultaneously. The only drawback is to find restriction enzymes cutting the right size to enable circularization of the genomic template. To bypass this limitation four restriction enzymes were used in this research, which resulted in an equal amount of circularized genomic libraries as for all restriction enzymes clear products could be seen on the gel. Sequencing of the latest obtained product revealed the amplification of glc-3. Further research with newly adapted primers and sequencing of the generated PCR templates might lead to the actual characterization of the 5'-end of glc-3.

New whole genome sequencing techniques, such as nanopore technology, could be exploited too. In this way longer reads (in excess of 100 kb (Jain, 2016)) can be obtained and sequencing performed more rapidly. However, this technique requires a lot library to work with (Bowden, 2019). Because of the variation between adult cyathostomins, pooling them to require the right amount of library is not an option. This would bring us back to use of nanopore technique in a PCR-based protocol on eggs or L3s.

5. Conclusion

Regardless of the life stage, 70% or 100% EtOH fixation remains a nonviable fixation method for downstream molecular work with cyathostomins. Varying temperature or incubation time does not alter the outcome. On the other hand, fresh cyathostomin eggs have proven to be a fairly promising substitute for adult cyathostomins in performing downstream molecular work such as PCR, MDA and GW. However, results have been very inconsistent regarding individual lysis and amplification of eggs with DreamTaq PCR. No clear explanation was found to clarify this. In the future research should lead to a better understanding of these unknown obstructive factors and optimisation of protocols used. Due to successful MDA and GW we were able to amplify glc-3. Sequencing showed this gene originating from *C. longibursatus*. Continuing research should now focus on developing GW primers which make it possible to map the 5'-end using the same genome walking method. When characterisation of the 5'-end has been successful, possibly the same method can be repeated on eggs obtained from a fresh *Cylicostephanus goldi* female when on hand.

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Appendix 1.

Collection of gastro-intestinal nematodes from horses by the slab gel method
Source: van Wyk, J.A., Gerber, H.M. and Groeneveld H.T. (1980) Onderstepoort J. Vet. Res. **47**, 147-158

Materials: incubator at 39 ^{o}C
Bucket
Dinner tray(s) (30 x 40 cm)
Gauze cloth
Cylinders
Plastic beakers
Spatula
Water bath set at 55 ^{o}C
Straightened paperclip (s) or 1 ml plastic pipets

Solutions: 0.9% sodium chloride: dissolve 90 gram of salts to 10 litre warm tap water in a bucket

2% agar in 0.9% sodium chloride (NaCl): dissolve 2 gram agar and 0.9 gram NaCl in 100 ml MilliQ. Boil this solution in the microwave until all the material is dissolved. Cool in water bath of 55 ^{o}C

Protocol

1. Collect the contents of the large intestines and dilute this twice with warm 0.9% NaCl
2. Leave the contents on the table for 10 minutes
3. Remove the upper part as far as possible, repeat sedimentation in smaller containers if the volume is large, 100 ml is the maximum volume for one gel
4. Put a part of wet gauze cloth on a dinner tray, one tray for every 100 ml suspension
5. Add 100 ml of the agar-solution (of 55 ^{o}C) to 100 ml of content and mix immediately with the spatula
6. Pour the mixture on the tray with gauze cloth, leave about 5 cm blanc (for hanging) and leave the gel on the table to set for at least 15 minutes
7. Take the gauze cloth with the gel surface inside and fold this zigzag, stick the straightened paperclip or 1 ml plastic pipette through the curtain and hang it in a cylinder with warm physiologic salt. Put the cylinder at 39 ^{o}C for 60 minutes. The worms will migrate out of the agar, the dirt will remain in the agar.
8. Optional: when not all worms have migrated out of the agar, hang the curtain in a new cylinder with physiologic salt and leave for another hour in the incubator.

Appendix 2.

Worm Lysis Buffer (WLB) preparation protocol (10 mL)

50 mM KCl	500 µl 1M KCl
10 mM Tris, pH 8.0	100 µl 1M Tris
2.5 mM MgCl2	1000 µl 25 mM MgCl2 (Fermentas)
0.45% NP-40	45 µl 10% NP-40
0.45% Tween-20	45 µl 10% Tween-20
0.01% gelatine	100 µl 1% gelatine
	1790 µl
Milli Q	8210 µl
Total	10000 µl

Appendix 3.

DreamTaq Master Mix

DreamTaq Master Mix - For 2.5 µl template

Forward primer 10 µM	1
Reversed primer 10 µM	1
10x DreamTaq Green Buffer	2.5
dNTP 10 mM	0.5
DreamTaq Polymerase (5 U/ul)	0.25
H_2O	17.25
Total	22.5 (+ 2.5 µl template)

PCR cycle DreamTaq PCR

3 min 95 °C

30 sec 95 °C
30 sec 50-60 °C 35 x
60 sec 72 °C

7 min 72 °C

Appendix 4.

Primer pair	Primer	Sequence	Melting temperature (T_m)	Target
CY1-CY18	**CY1 Forward**	GGT CAA GGT GTT GTA TCC AGT AGA G	**60** °C	**rRNA**
	CY18 Reverse	CTT AGA CAT GCA TGG CTT AAT C	**60** °C	
F532 – R823	**F532**	CAG TAC TAT CCT ATG GAT GTG CAA AC	**55** °C	**GluCl-α (glc-3)**
	R823-nas	CAA AAC GAY ACC CAT GAT ACT ATG AC	**55** °C	
	R823-cat	CAG AAC GAC ACC CAY GAY ACT ATG AC	**55** °C	
F350-R370	**F350**	CAT ATG ATC GAY AAG CCR AAC G	**50** °C	**CCP of glc-3**
	R370	GCA RAT GCA TYG GAC ATG AAA G	**50** °C	
F532-R370 (genome walking)	**F532**	CAG TAC TAT CCT ATG GAT GTG CAA AC	**50** °C	**GluCl-α (glc-3)**
	R370	GCA RAT GCA TYG GAC ATG AAA G	**50** °C	**CCP of glc-3**
FA250-RA200	**FA250**	TGC KGT TTT ATT AGG WTT TCA GC	**54** °C	**Variation A of glc-3**
	RA200	CGD GAA TGC TGT CTT GCW CC	**54** °C	
FB140-RB30	**FB140**	ACT GCT CCT CCR YAT TCC C	**50** °C	**Variation B of glc-3**
	RB30	GTT YAG CAG TAC RGC CCC	**50** °C	

Appendix 5.

Protocol: Amplification of Genomic DNA from Blood or Cells using the REPLI-g Mini kit
Source: REPLI-g Mini/Midi Handbook

Things to do before starting

- Prepare Buffer DLB by adding 500 µl nuclease-free water to the tube. Mix thoroughly and centrifuge briefly. Below referred to as reconstituted buffer.

Procedure

1. Prepare sufficient Buffer D2 (denaturation buffer) for the desired number of reactions.

Component	Volume*
DTT, 1 M	5 µl
Reconstituted Buffer DLB	55 µl
Total volume	**60 µl**

*Volume for up to 15 reactions

2. Place 2.5 µl PBS into a microcentrifuge tube.
3. Add 0.5 µl cell material (> 600 cells/ µl) to the PBS.
4. Add 3.5 µl Buffer D2. Mix by vortexing and centrifuge briefly.
5. Incubate for 10 min on ice.
6. Add 3.5 µl Stop Solution. Mix by vortexing and centrifuge briefly.
7. Thaw REPLI-g Mini DNA Polymerase on ice. Thaw all other components at room temperature, vortex, then centrifuge briefly.
8. Prepare a master mix. Mix and centrifuge briefly.

Component	Volume/reaction
Nuclease-free water	10 µl
REPLI-g Mini Reaction Buffer	29 µl
REPLI-g Mini DNA Polymerase	1 µl
Total volume	**40 µl**

9. Add 40 µl of master mix to 10 µl denatured DNA (step 6).
10. Incubate at 30 ^{o}C for 10-16 h.
11. Inactivate REPLI-g Mini DNA Polymerase at 65 ^{o}C for 3 min.
12. If performing PCR analysis, dilute the amplified DNA 1:20 and use 3 µl of diluted DNA for each PCR
13. Store amplified DNA at 4 ^{o}C for short-term storage or -20 ^{o}C for long-term storage.

Appendix 6.

Protocol: Whole genome amplification from biopsies using the REPLI-g Single Cell kit
Source: REPLI-g Mini/Midi Handbook

Things to do before starting

- Prepare Buffer DLB by adding 500 µl H_2O sc to the tube. Mix thoroughly and centrifuge briefly. Below referred to as reconstituted buffer.

Procedure

1. Place the biopsy tissue (approximately 2mm³) in a microcentrifuge containing 10 µl TE buffer. Incubate at room temperature (20-25 °C) for 10 min, vortexing occasionally.
2. Prepare sufficient Buffer D2 (denaturation buffer) for the total number of whole genome amplification reactions

Component	**Volume***
DTT, 1 M	5 µl
Reconstituted Buffer DLB	55 µl
Total volume	**60 µl**

*Volume for up to 6 reactions

3. Add 10 µl Buffer D2 to each microcentrifuge tube containing biopsy tissue. Mix by vortexing briefly and place on ice for 30 min.
4. Add 10 µl Stop Solution to each microcentrifuge tube containing lysed tissue and mix briefly by vortexing. Spin down the tissue debris by pulse centrifugation
5. Thaw REPLI-g sc DNA Polymerase on ice. Thaw all other components at room temperature, vortex, then centrifuge briefly.
6. Prepare a master mix. Mix and centrifuge briefly.

Component	**Volume/reaction**
H_2O sc	9 µl
REPLI-g sc Reaction Buffer	29 µl
REPLI-g sc DNA Polymerase	2 µl
Total volume	**40 µl**

7. Add 40 µl of master mix to 10 µl lysed and neutralized tissue cells (step 4.) Mix well by vortexing for 10 s and centrifuge briefly.
8. Incubate at 30 °C for 8 h.
9. Inactivate REPLI-g sc DNA polymerase by heating the sample at 65 °C for 3 min.
10. Store amplified DNA at 4 °C for short-term storage or -20 °C for long-term storage.

Appendix 7.

- Protocol: genome walking including S1 nuclease.

  When performing the protocol without S1 nuclease, start from step 1. digestion.

- ***S1 nuclease***

  1. *Prepare the following reaction mixture:*

Component	***Volume***
S1 Nuclease	*0.1 µl**
5X Reaction Buffer for S1 Nuclease	*0.9 µl**
Undiluted MDA product	*2.5 µl*
Total volume	***µl***

**use 1 µl of a dilution of 1:10 µl (S1 nuclease : S1 nuclease dilution buffer) in total*

  2. *Incubate the mixture at room temperature for 30 min.*
  3. *Stop the reaction by adding 2 µL of 0.5 M EDTA and heating at 70 °C for 10 min.*

- **Digestion**

  - Thaw restriction enzymes (XBal, EcoR1, BamH1 and Xhol) on ice.
  - Tubes with MDA-product should only be opened in the electrophorese laboratory.

  1. Prepare the restriction mixture for each restriction enzyme separately.

Component	**Volume**
Nuclease free water	19 µl
10x Fast Digest buffer	2.5 µl
Undiluted MDA product	2.5 µl
Restriction enzyme	1 µl
Total volume	**25 µl**

  2. Mix by vortexing and centrifuge briefly
  3. Incubate the samples according to the following table:

Restriction enzyme	**Time**	**Temperature**
Xbal	10 min	37 ^{o}C
BamH1	10 min	37 ^{o}C
Xhol	10 min	37 ^{o}C
EcoR1	20 min	37 ^{o}C

4. Inactivate the samples according to the following table:

Restriction enzyme	Time	Temperature
Xbal	20 min	65 ^{o}C
BamH1	5 min	80 ^{o}C
Xhol	5 min	80 ^{o}C
EcoR1	5 min	80 ^{o}C

❖ **Ligation**

5. Prepare the ligation mixture for each restriction enzyme separately.

Component	Volume
Restriction mixture	20 µl
10x T4DNA ligase buffer	50 µl
T4 ligase	5 µl
Nuclease free water	425 µl
Total volume	**500 µl**

6. Mix by vortexing and centrifuge briefly. Incubate for 2 h at room temperature.
7. Inactivate ligase at 70 ^{o}C for 5 min.

❖ **Precipitation**

8. Prepare the precipitation mixture for each restriction enzyme separately.

Component	Volume
Ligation mixture	500 µl
3M sodiumacetate (pH 5,3)	50 µl
100% EtOH	500 µl
Total volume	**1050 µl**

9. Incubate at - 80 ^{o}C for 45 min
10. Spin down for 15 min at 20 000 x g.
11. Discard the supernatant.
12. Wash the pellet with 200 µl chilled 70% EtOH.
13. Spin down for 15 min at 20 000 x g again and let the supernatant vaporize in the open air.
14. Dissolve the pellet in 20 µl TE buffer.

Appendix 8.

Protocol for purification of DNA from TAE and TBE agarose gels
Source: GE Healthcare Product Booklet: illustra GFX PCR DNA and Gel Band Purification Kit

1. **Sample Capture**
    a. Weigh a DNase-free 1.5 ml microcentrifuge tube and record the weight.
    b. Using a clean scalpel, long wavelength (365 nm) ultraviolet light and maximal exposure time, cut out an agarose band containing the sample of interest. Place agarose gel band into a DNase-free 1.5 ml microcentrifuge tube.
    c. Weight the microcentrifuge tube plus agarose band and calculate the weight of the agarose slice.
    d. Add 10 µl Capture buffer type 3 for each 10 mg of gel slice. NOTE: if the gel slice weighs less than 300 mg, add 300 µl Capture buffer type 3. DO NOT add less than 300 µl Capture buffer type 3 per sample.
    e. Mix by inversion and incubate at 60 ^{o}C for 15-30 minutes until the agarose is completely dissolved. Mix by inversion every 3 minutes.
    f. Once the agarose has completely dissolved check that the Capture buffer type 3-sample mix is yellow or pale orange in colour.
    g. For each purification that is to be performed, place one GFX MicroSpin column into one Collection tube.

2. **Sample Binding**
    a. Centrifuge Caption buffer type 3-sample mix briefly to collect the liquid at the bottom of the tube.
    b. Transfer up to 800 µl Capture buffer type 3-sample mix onto the assembled GFX MicroSpin column and collection tube.
    c. Incubate at room temperature for 1 minute.
    d. Spin the assembled column and Collection tube at 16 000 x g for 30 seconds.
    e. Discard the flow through by emptying he Collection tube. Place the GFX MicroSpin column back inside the Collection tube.
    f. Repeat Sample Binding steps b. to e. as necessary until all the sample is loaded.

3. **Wash and Dry**
    a. Add 500 µl Wash buffer type 1 to the GFX MicroSpin column.
    b. Spin the assembled column and Collection tube at 16 000 x g for 30 seconds.
    c. Discard the Collection tube and transfer the GFX MicroSpin to a fresh DNase-free 1.5 ml microcentrifuge tube.

4. **Elution**
    a. Add 10-50 µl Elution buffer type 4 OR type 6 to the centre of the membrane in the assembled GFX MicroSpin column and sample Collection tube.
    b. Incubate the assembled GFX MicroSpin column and collection tube at room temperature for 1 minute.
    c. Spin the assembled column and sample collection tube at 16 000 x g for 1 minute to recover the purified DNA.
    d. Proceed to downstream application. Store purified DNA at -20 ^{o}C