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Note

Monitoring of four DNA extraction methods upstream of high-throughput sequencing of Anisakidae nematodes

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ABSTRACT

Different methods were evaluated to extract DNA from pooled nematodes belonging to *Anisakis*, *Contracaecum*, *Pseudoterranova* and *Hysterothylacium* genera isolated from edible fish. Pooled DNA extraction is the first and compulsory step to allow the identification of a large number of samples through high-throughput DNA sequencing with drastic time and cost reductions.

In 2010, about 20,000 cases of Anisakidosis were reported worldwide. The human disease occurs accidentally after consumption of third-stage larvae of nematodes belonging to the Anisakidae family such as *Anisakis simplex* and some *Pseudoterranova* species occurring in fish (EFSA, 2010; Mattiucci and Nascetti, 2008). The increase in reported cases is associated with changes in the consumption habits toward raw food, and a better diagnosis of the disease (Pravettoni et al., 2012). Nevertheless, knowledge of intermediate hosts, geographical distribution, taxonomy and the exact number of human cases of Anisakidosis is still incomplete.

The Fish-Parasites program (ANR-10-ALIA-004) has demonstrated that large numbers of Anisakidae nematodes were detected in fish organs in France (Seesao et al., in preparation). Pooled DNA extraction combined with the recent technology of high-throughput sequencing (HTS) was chosen to identify nematodes at the species level by targeting the *COX2* locus. Compared with traditional methods dealing with one sample at a time, HTS offers the advantage to be less time consuming and to reduce costs (Porazinska et al., 2009).

The yield and quality of genomic DNA (gDNA) extraction are crucial issues to be solved prior to HTS. Extraction of nematode gDNA is not straightforward (Lawton et al., 1998) because of their robust cuticle layer (460 nm-thick) which is resistant to chemical, enzymatic and mild physical disruptions (Dawkins and Spencer, 1989; McManus et al., 1985).

The aim was to develop a method to efficiently and evenly purify gDNA from *Anisakis*, *Contracaecum*, *Pseudoterranova* and *Hysterothylacium* nematodes. Several DNA extraction methods were adapted to large quantities of starting material and the quality and yield of DNA extractions were monitored (Fig. 1).

Nematodes were isolated from several fish species bought from fish retailers in Boulogne-sur-Mer (France). The nematodes belonging to the genera *Anisakis*, *Contracaecum*, *Pseudoterranova* and *Hysterothylacium* were morphologically identified according to Huang (1988) and separated. The worms were artificially mixed in a weight proportion resembling real conditions observed in formerly sampled fish: 75% of *Anisakis* (about 43–47 worms), 3% of *Contracaecum* (about one worm), 2% of

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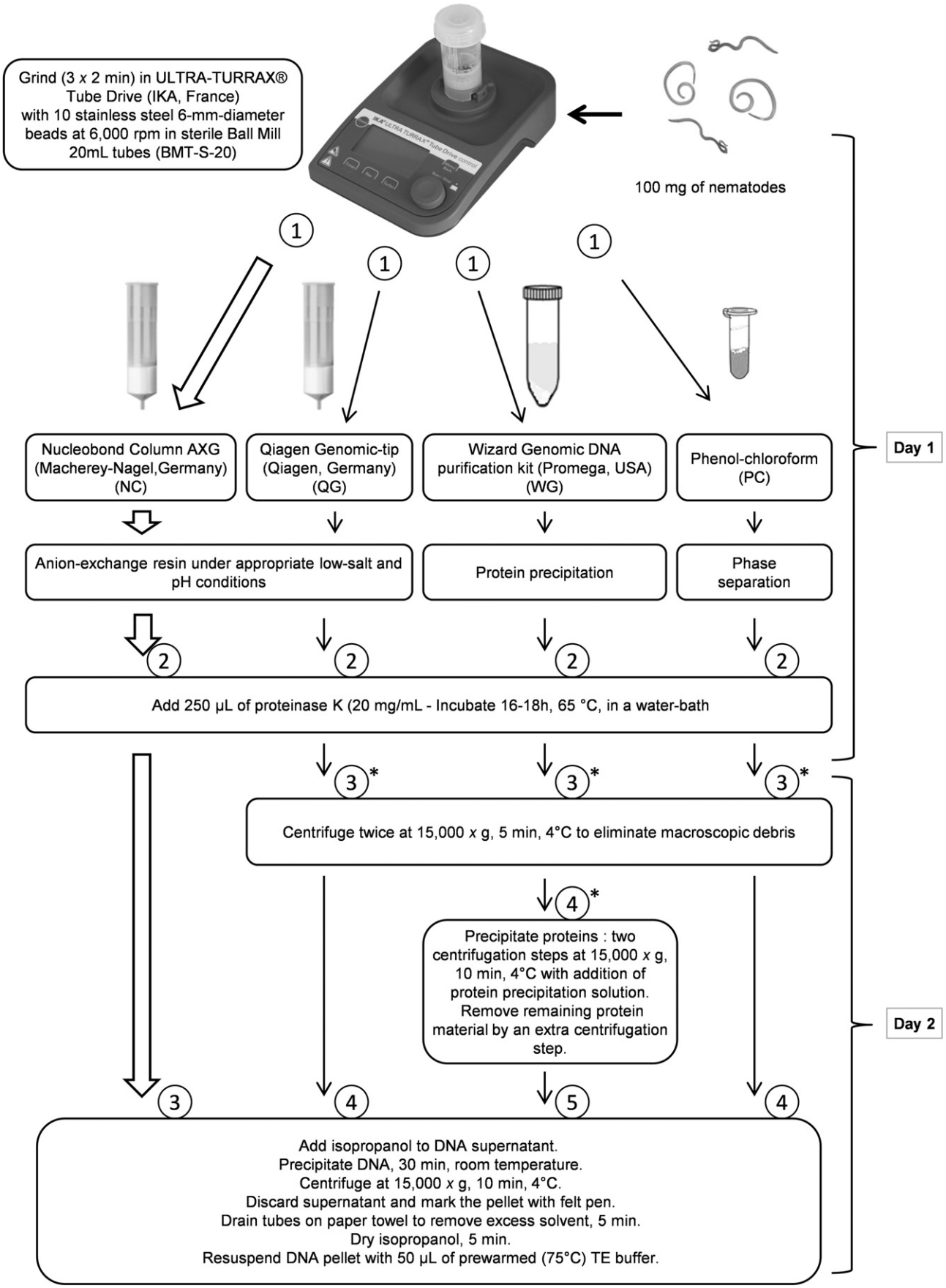
Grind (3 x 2 min) in ULTRA-TURRAX® Tube Drive (IKA, France) with 10 stainless steel 6-mm-diameter beads at 6,000 rpm in sterile Ball Mill 20mL tubes (BMT-S-20)
100 mg of nematodes
1
Nucleobond Column AXG (Macherey-Nagel,Germany) (NC)
Qiagen Genomic-tip (Qiagen, Germany) (QG)
Wizard Genomic DNA purification kit (Promega, USA) (WG)
Phenol-chloroform (PC)
Day 1
Anion-exchange resin under appropriate low-salt and pH conditions
Protein precipitation
Phase separation
2
Add 250 µL of proteinase K (20 mg/mL - Incubate 16-18h, 65 °C, in a water-bath
3*
Centrifuge twice at 15,000 x g, 5 min, 4°C to eliminate macroscopic debris
4*
Precipitate proteins : two centrifugation steps at 15,000 x g, 10 min, 4°C with addition of protein precipitation solution. Remove remaining protein material by an extra centrifugation step.
Day 2
3
4
5
4
Add isopropanol to DNA supernatant.
Precipitate DNA, 30 min, room temperature.
Centrifuge at 15,000 x g, 10 min, 4°C.
Discard supernatant and mark the pellet with felt pen.
Drain tubes on paper towel to remove excess solvent, 5 min.
Dry isopropanol, 5 min.
Resuspend DNA pellet with 50 µL of prewarmed (75°C) TE buffer.

Table 1
Comparison of the quantity and quality of gDNA extracted from a nematode mixture consisting of four Anisakidae genera using four extraction methods. The cycle thresholds (Ct) of the *COX2* qPCR are also compared. The six DNA quantities were tested for each method in the *COX2* qPCR assay and ranged from 0.80 ng to 25 ng.

	DNA quantity (ng/μL, PicoGreen®)	DNA quality (Nanodrop™ 2000)		*COX2* qPCR
DNA extraction methods		$OD_{260\ nm}/OD_{280\ nm}$ ratios	$OD_{260\ nm}/OD_{230\ nm}$ ratios	$Ct_{0.80\ ng}$ to $Ct_{25\ ng}$
Nucleobond Column AXG kit (NC)	109.80	1.78	1.80	10.88–14.37
Wizard Genomic DNA purification kit (WG)	96.00	1.99	1.33	10.60–14.44
Qiagen Genomic-tip kit (QG)	50.90	1.74	0.50	15.02–19.27
Phenol–Chloroform method (PC)	36.69	1.95	1.31	18.88–21.13

Table 2
Comparing the repeatability of both selected DNA extraction methods. The quantity and quality of extracted nematode gDNA were monitored as well as the presence of inhibitors using the *COX2* qPCR. The six DNA quantities tested for qPCR ranged from 0.62 ng to 20 ng. The means (in bold) tagged with different letters [a] and [b] are significantly different with an error risk of 5% (Kruskall–Wallis statistical test).

	Values and means of DNA quantity (ng/μL, PicoGreen®)	Values and means of DNA quality (Nanodrop™ 2000)		*COX2* qPCR
DNA extraction methods		$OD_{260\ nm}/OD_{280\ nm}$ ratios	$OD_{260\ nm}/OD_{230\ nm}$ ratios	$Ct_{0.62\ ng}$ to $Ct_{20\ ng}$
Nucleobond Column AXG kit (NC)	103.76	2.09	1.32	10.90–14.48
	93.20	1.88	0.97	(n = 10)
	98.59	2.01	1.00	
	91.45	2.00	0.99	
	109.34	1.83	1.12	
	102.32	2.06	0.96	
	101.65	1.81	1.02	
	101.60	1.96	1.33	
	112.64	1.85	1.02	
	100.67	2.02	0.96	
	101.52 ± 6.41[a]	**1.95 ± 0.10[a]**	**1.07 ± 0.14[a]**	
	(n = 10)	(n = 10)	(n = 10)	
Wizard Genomic DNA purification kit (WG)	80.99	1.84	0.76	11.73–15.40
	90.80	1.83	0.72	(n = 9)
	86.82	1.82	0.68	
	61.23	1.87	0.85	
	104.93	1.86	0.80	
	85.20	1.73	0.61	
	46.25	1.86	0.74	
	69.84	1.81	0.68	
	64.11	1.83	0.72	
	76.68 ± 17.89[b]	**1.83 ± 0.04[b]**	**0.73 ± 0.07[b]**	
	(n = 9)	(n = 9)	(n = 9)	

Pseudoterranova (about one worm) and 20% of *Hysterothylacium* (about one worm), the number of worms depending on their respective size. Hundred milligrams of the mixed nematode material was used to assay the gDNA extraction performance of four selected methods: Wizard Genomic (WG) DNA purification kit (Promega, USA), Nucleobond Column (NC) AXG kit (Macherey-Nagel, Germany), Qiagen Genomic-tip (QG) kit (Qiagen, Germany) and Phenol–Chloroform (PC) standard method. The essentials steps of the four nematode gDNA extraction protocols are displayed in Fig. 1. The grinding step using the ULTRA-TURRAX® Tube Drive workstation (IKA, France) was common to all protocols. It was chosen for its efficiency, speed, easiness to handle and its disposable and hermetic tubes that avoid cross-sample contaminations.

The gDNA quality and quantity were respectively measured using the NanoDrop™ 2000 spectrophotometer (Thermo Scientific, France) and the Quant-iT™ PicoGreen® dsDNA Assay Kit on a fluorescence microplate reader (Tecan GENios, Tecan Genios, Switzerland). To detect the presence of inhibitors, a quantitative PCR (qPCR) (RotorGene, Corbett Life Science, Australia) targeting a fragment of the Anisakidae *COX2* gene was performed using specific primers (F-univ-nem 5′-GGT GTT CTT TCT TTT GTT TCT G-3′ and R-univ-nem 5′-ATA AAA CTA TGG TTA GCC CCA C-3′). This primer pair is commonly used in our laboratory and evenly amplifies a 530-pb fragment of the *COX2* gene from the four parasite genera. The PCR thermocycling program was as follows: primary denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 20 s, 45 °C for 15 s and 72 °C for 45 s. Quantitative PCR was performed with a Brilliant II SYBR® Green qPCR Mastermix (Stratagene, USA) with the following mix formulation for a final volume of 15 μL: Brilliant II 2X Mix, F-univ-nem and R-univ-nem primers 400 nM, and DNA sample 2 μL. To proceed with melting curves, temperature ramping was set from 47 °C to 95 °C, rising by 1 °C every 5 s.

The NC and WG kits gave the best results compared to the other techniques. The purity of gDNA was good with $OD_{260/280}$ ratios close to or above 1.80 for all kits (Table 1). For the NC kit, the $OD_{260/230}$ ratio was the highest (1.80) compared to the other kits and attested the removal of most of solvent contaminations. Ct values demonstrated less inhibitors using the NC and WG kits in comparison to the QG and PC kits. Moreover, both NC and WG kits gave better results when extracting *Contracaecum* DNA, which is harder to extract with good yield due to its small size and probably complex cuticle structure. Genomic DNA of the same sample mix, containing the four Anisakid genera, was extracted ten times with both NC and WG adapted methods to evaluate their repeatability (Table 2).

Fig. 1. Comparison of different nematode gDNA extraction protocols. Four kits based on 3 different principles were assayed. Some steps were modified to be adapted to nematode DNA extraction and are depicted on the figure: longer proteinase K incubation time (step 2), 250 μL of proteinase K (20 mg/mL) added to 100 mg ground nematodes (step 2), repeated lysate centrifugation before extraction (step 3*), two protein precipitation steps (step 4*), and drying of DNA pellet and suspension in prewarmed TE buffer (last step). The Nucleobond column AXG kit was selected to be used in downstream HTS application in the frame of the Fish-Parasites program (thick arrow).

Individual DNA quantity values fell in a narrower range for NC (91.45 ng/μL–109.34 ng/μL) than for WG (46.25 ng/μL–104.93 ng/μL) kits. The lower standard deviation (SD) of the corresponding averaged values for the NC kit (SD = 6.41 ng/μL) also strengthens the steadier yields of the gDNA extracted by the NC kit compared to the WG (SD = 17.89 ng/μL) kit (Table 2). The averaged yield of extracted DNA was also significantly higher with the NC (101.52 ng/μL) than with the WG (76.68 ng/μL) kit (Table 2). For WG, one sample has been discarded because the DNA quantity was less than 2 fold the standard deviation.

In terms of quality, both $OD_{260\ nm/280\ nm}$ and $OD_{260\ nm/230\ nm}$ ratios were significantly better for NC than for WG. Both kits were efficient at purifying gDNA from proteins but NC was better at removing any remaining solvent traces thanks to the anion-exchange resin (Tables 1 and 2). Quantitative PCR was conducted on a pool of the 9 or 10 repeats for each kit. Ct of NC showed slightly less inhibitors than WG (Table 2).

In our study, the HTS downstream application required as good quality and quantity of nematode gDNA as possible. In addition, the complex structure of the nematode cuticle rendered the gDNA purification difficult. This is why some modifications to the classical tissue protocols such as the grinding step, the addition of proteinase K for efficient tissue lysis, the longer incubation time and the elution with pre-warmed TE were implemented (Fig. 1). The Nucleobond Column AXG (Macherey-Nagel, Germany) was selected amongst the four tested gDNA purification methods because it allowed the best purification of nematode gDNA with the best and most repeatable yield at a lower cost (thick arrow, Fig. 1).

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