PCR for detection of Campylobacter

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TemaNord 2006:527

© Nordic Council of Ministers, Copenhagen 2006

ISBN 92-893-1320-X

Copies: Print-on-Demand

This publication can be ordered on www.norden.org/order. Other Nordic publications are available at www.norden.org/publications

Printed in Denmark

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The Nordic Food Policy Co-operation

The Nordic Committee of Senior Officials for Food Issues is concerned with basic Food Policy issues relating to food and nutrition, food toxicology and food microbiology, risk evaluation, food control and food legislation. The co-operation aims at protection of the health of the consumer, common utilisation of professional and administrative resources and at Nordic and international developments in this field.

Nordic Environmental Co-operation

The Nordic Environmental Action Plan 2005-2008 forms the framework for the Nordic countries' environmental co-operation both within the Nordic region and in relation to the adjacent areas, the Arctic, the EU and other international forums. The programme aims for results that will consolidate the position of the Nordic region as the leader in the environmental field. One of the overall goals is to create a healthier living environment for the Nordic people.

Nordic co-operation

Nordic co-operation, one of the oldest and most wide-ranging regional partnerships in the world, involves Denmark, Finland, Iceland, Norway, Sweden, the Faroe Islands, Greenland and Åland. Co-operation reinforces the sense of Nordic community while respecting national differences and similarities, makes it possible to uphold Nordic interests in the world at large and promotes positive relations between neighbouring peoples.

Co-operation was formalised in 1952 when *the Nordic Council* was set up as a forum for parliamentarians and governments. The Helsinki Treaty of 1962 has formed the framework for Nordic partnership ever since. The *Nordic Council of Ministers* was set up in 1971 as the formal forum for co-operation between the governments of the Nordic countries and the political leadership of the autonomous areas, i.e. the Faroe Islands, Greenland and Åland.

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Preface

An effective control of *Campylobacter* in the primary production, manufacturing and sale of poultry products necessitate knowledge about the presence of the microorganism in the various steps of production. The aim of this project was to disseminate the knowledge of an already developed PCR-method for detection of *Campylobacter* in the Nordic countries. The PCR-method, based on the most recent technology (real-time PCR), had already been tested on a large amount of samples from the poultry production chain, and a national validation of the method carried out at the Danish Institute for Food and Veterinary Research, Department of Microbiological Food Safety, the promoters of the present project.

Real-time PCR is much easier and less work demanding than traditional cultivation. In the case of *Campylobacter* this means that the time of analysis can be reduced from 4-5 days to only 3 hours for faecal samples, and a little more than a day for meat samples. The described PCR-method has been successfully implemented at the Danish poultry producer Danpo. They are very satisfied with the robustness of the method, and are currently using it on a routine basis to declare chickens and chicken products *Campylobacter*-free.

Automated PCR-methods, like real-time PCR, are more easily transferred from expert laboratories to end-user laboratories, however a thorough validation through a collaborative trial will facilitate the implementation in the food industry and control laboratories in Scandinavia and Europe. In the present project the implementation of a validated, open-formula (non-commercial) and very user friendly real-time PCR method was carried out through a technology transfer trial with participants from Sweden, Iceland and Finland. The trial was designed according to the guidelines given by NordVal. The trial comprised testing the real-time PCR method on 3 matrices (chicken meat from retail, cloacae swab samples from the slaughter line and shoe cover samples from the rearing houses) spiked with *Campylobacter* in 3 different levels. Based on the results obtained in the trial it was determined that the method was suitable for standardisation, and a standard in reference to the Nordic Committee on Food Analysis was proposed.

The project was supported by a grant from the Nordic Council of Ministers (grant No.: 681048-05317).

The project was coordinated by Mathilde Josefsen, Danish Food and Veterinary Research Institute, Denmark.

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Summary (English)

In 1999 campylobacteriosis became the single most important cause of gastrointestinal disease in Denmark. An intervention strategy applied in Denmark includes, among other things, the implementation of strategic slaughter of broilers. Using real-time PCR for screening the broiler flocks for presence of *Campylobacter* would be advantageous for several reasons. Real-time PCR is more sensitive and specific than gel-based PCR, it is less work demanding, and the number of manual handling steps is reduced. Last, but not least, the risk of carry-over contamination is markedly reduced since the PCR products are not exposed to the environment. This is a very relevant advantage, since carry-over contamination could be problematic in a routine laboratory with a high put through on the same test.

The main aim of the present project was to disseminate the knowledge of an already validated real-time PCR method for detection of thermotolerant *Campylobacter* in samples from the chicken production line.

The dissemination part of this project was carried out through a technology transfer trial with participation of central Nordic laboratories. The participating laboratories were assisted in implementing the real-time PCR method, and received samples, reagents and media for enrichment, DNA extraction and PCR. Detailed protocols on sample preparation and real-time PCR were prepared for each participant corresponding to their in-house real-time PCR equipment.

Finally, based on the validation, and the results obtained in the technology transfer trial a standard proposal for the Nordic Committee on Food Analysis for detection of thermotolerant *Campylobacter* by real-time PCR was prepared.

Summary (Danish)

I 1999 var campylobacteriose den vigtigste årsag til gastrointestinale infektioner i Danmark. En interventions strategi anvendt i Danmark inkluderer blandt andet implementeringen af strategisk slagting af kyllingeflokke. At bruge real-time PCR til at screene kyllingeflokkene for tilstedeværelsen af thermotolerante *Campylobacter* ville være fordelagtig af flere grunde. Real-time PCR er mere følsom og specifik end gel-baseret PCR, det er mindre arbejdskrævende, og antallet af manuelle håndterings-trin er reduceret. Sidst, men ikke mindst, er risikoen for carry-over kontamination betydeligt reduceret, eftersom det omgivende miljø ikke eksponeres for PCR produkterne. Dette er en meget relevant fordel, da carry-over kontamination kunne blive problematisk i et rutine laboratorium med et højt prøveantal kørt gennem samme test.

Hovedformålet med projektet var at udbrede kendskabet til en allerede valideret real-time PCR metode til detektion af thermotolerante *Campylobacter* i prøver fra fjerkræproduktionen.

I projektet blev udbredelsen af metoden gennemført via en teknologioverførsels-ringtest med deltagelse af centrale nordiske laboratorier. De deltagende laboratorier blev assisteret i at implementere real-time PCR metoden, og modtog prøver, reagenser og opformeringsmedier til DNA ekstraktion og PCR. Detaljerede protokoller for prøveforberedelse og real-time PCR blev udarbejdet for hvert deltagende laboratorium svarende til deres real-time PCR udstyr.

Endelig blev der i projektet udarbejdet et standardforslag til den Nordiske MetodikKomité for Næringsmidler (NMKL) for detektion af thermotolerante *Campylobacter* med real-time PCR, baseret på valideringen og de resultater der blev opnået i teknologioverførsels-ringtesten.

1. Introduction

In 1999 campylobacteriosis became the single most important cause of gastrointestinal disease in Denmark. In 2004, 3724 cases were reported, corresponding to an incidence of approx. 70 cases per 100,000 inhabitants. The previous two years a decline was observed in the number of *Campylobacter* infections in Denmark, but the numbers reported for 2004 unfortunately reversed this tendency. The major source of human campylobacteriosis is believed to be handling and consumption of poultry and poultry products, and cross contamination from these to other ready-to-eat food items (Anon., 2005(a)).

In 2003 a voluntary intervention strategy aimed at reducing the number of cases of human campylobacteriosis was implemented. The strategy was based on experience from the other Nordic countries, results from research projects in the primary production and results from risk assessment studies. The efforts were directed on to three levels:

- 1) Reducing the prevalence of *Campylobacter* infected broiler flocks by improving the bio security in and around the poultry rearing houses
- 2) Reducing the number of *Campylobacter* in broiler meat after slaughter by allocating negative flocks to production of fresh chilled products and positive flocks to production of frozen products
- 3) Preventing cross contamination in domestic kitchens by education of, especially young, consumers.

The significant decrease in the number of human cases in 2002 and 2003 coincides with the implementation of the voluntary intervention strategy. According to the Annual Report on Zoonoses in Denmark (2004), it is likely that point 2) the allocation of negative and positive flocks to different products, contributed to this reduction (Anon., 2004(b)).

Determining the *Campylobacter* status of the broiler flocks using real-time PCR would be advantageous for several reasons. Real-time PCR is more sensitive and specific than gel-based PCR, it is less work demanding, and the number of manual handling steps is reduced. Last, but not least, the risk of carry-over contamination is markedly reduced since the PCR products are not exposed to the environment. This is a very relevant advantage, since carry-over contamination could be problematic in a routine laboratory with a high put through on the same test.

2. The project “PCR for detection of *Campylobacter*”

The main aim of this project was to disseminate the knowledge of an already validated real-time PCR method for detection of thermotolerant *Campylobacter* in samples from the chicken production line. The method was developed and validated at the Danish Institute for Food and Veterinary Research (DFVF) in a previous project funded by The Directorate for Food, Fisheries and Agri Business (Denmark). The method was validated according to the NordVal criteria (Anon., 2004(a)) on three matrices; chicken neck skin, faeces on cloacae swabs sampled at the slaughter line, and faeces on disposable shoe covers collected in the rearing houses. The method was approved as an alternative microbiological method for detection of thermotolerant campylobacters in chicken neck skin and cloacae swab samples, but not on shoe cover samples.

The first task of this project was therefore to optimize the method on shoe cover samples, in order for it to comply with the validation criteria.

The dissemination part of this project was carried out through a technology transfer trial with participation of three central laboratories in Sweden, Finland and Iceland. A Norwegian laboratory had to decline participation. The participating laboratories were assisted in implementing the real-time PCR method, and received samples, reagents and media for enrichment, DNA extraction and PCR. Detailed protocols on sample preparation and real-time PCR were prepared for each participant corresponding to their in-house real-time PCR equipment.

Finally, based on the NordVal approval, and the results obtained in the technology transfer trial a standard proposal for the Nordic Committee on Food Analysis for detection of thermotolerant *Campylobacter* by real-time PCR was prepared.

The project was divided into four parts:

- Optimization of the method for shoe cover samples
- National validation of the method on shoe cover samples
- Technology transfer trial
- Standard proposal for the Nordic Committee on Food Analysis

2.1 Optimization

In the first collaborative trial, the real-time PCR method failed to detect any of the shoe cover samples regardless of the level of spiking. Detection of *C. jejuni* in the shoe cover samples was possible at DFVFs laboratory at both low and high spiking levels.

Post-trial investigations have shown that the reason for this discrepancy lies in the DNA-extraction procedure. The extraction protocol provided to the participating laboratories was modified for compatibility with several DNA-extraction platforms, and this approach resulted in a markedly reduced amount of total DNA recovery. The shoe cover samples is possibly more PCR inhibitory and yield less DNA than the other sample matrices, and it is likely that these features were the cause of the absence of positive signals.

In order to enhance the DNA extraction from shoe cover samples different approaches were examined. The effect of washing the cells before DNA extraction was evaluated. This did not contribute to an enhanced amount of DNA extracted from the samples. The automated DNA extraction in the KingFisher was systematically altered in order to evaluate the effect of the different steps of the extraction procedure. However, the original program (see www.pcr.dk for details) was found to give the highest DNA yield, and hence no alterations were made in the programme. Likewise, the thermal profile of the real-time PCR run was altered systematically to see if better results could be achieved by different temperatures, but this approach did not give better results.

Finally, the amount of paramagnetic particles in the DNA extraction was increased, and this gave a markedly higher DNA yield and acceptable real-time PCR results.

In the second collaborative trial on shoe covers, the amount of paramagnetic particles was more than tripled (from 20 µl per sample to 75 µl per sample), rectifying the problem with DNA extraction from this matrix. Shoe cover samples contain substantial amounts of extraneous material, and the results indicate that clotting of the magnetic particles hindered the absorption of DNA.

2.2 Validation

A new collaborative trial involving eight national laboratories was performed to evaluate the robustness and reproducibility of the real-time PCR method testing identical shoe cover samples.

The collaborative trial was designed and conducted according to the recommendations from NordVal (Anon., 2004(a)).

The eight participating laboratories received pellets from 6 coded 1-ml cloacae swab samples. The samples were spiked in duplicate with *C.*

jejuni CCUG 11284 at three levels, making it possible to assess the usefulness of the method at various infection levels. The shipment included a positive (1 µg/ml *C. jejuni* CCUG 11284) and a negative (1 µg/ml *Arco-bacter butzleri* CCUG 30485) DNA control, a ready-to-use PCR mixture with added internal amplification control (IAC), and reagents for a magnetic-based DNA extraction. To minimize any inter-laboratory variability (not attributable to the method performance), DFVF supplied all the reagents necessary. Each participant received a detailed protocol describing the DNA extraction, real-time PCR setup, real-time PCR run, and data analysis, and a reporting form to record the obtained PCR results to return to DFVF. The participants were also asked to return a file containing the real-time PCR runs.

Samples.

The shoe cover samples for the collaborative trial were collected in the rearing houses and shipped at ambient temperature to DFVF. On arrival, the shoe cover samples were weighed and added to 10 x w/v physiological saline and homogenized for 60 sec. in a stomacher.

From the samples, 1-ml aliquots were drawn, spiked with 0, 100–500, or 1000–2000 CFU/ml, and centrifuged at $16,000 \times g$ for 7 min at 4 °C. The supernatant was discarded and the pellet kept at -80 °C until shipped on ice to the trial participants.

The *Campylobacter* status of all samples was confirmed at DFVF by the reference culture method according to ISO 10272-1 (Anon., 2002) and NMKL 119 (Anon., 2005(b)) prior to and after spiking. The stability of the samples was examined using real-time PCR immediately after spiking, prior to commencement of the collaborative trial, and during the period of analysis, to verify the continued detection of *Campylobacter*. The possible detrimental effect of shipping time at ambient temperature on the real-time PCR results was investigated and no detrimental effect was found.

Participating laboratories.

At the participating laboratories, DNA was extracted from the samples using the Magnesil KF, Genomic System (Promega, Madison, WI, USA) on an automated DNA extraction platform of the laboratories choice, but following the KingFisher protocol. Briefly, the sample pellet was re-suspended in lysis buffer and transferred to a 96-well plate (Thermo Lab-systems) containing magnetic particles, washing buffers, and elution buffer. The DNA extraction program consisted of two salt-buffer washing steps and two alcohol-buffer washing steps, followed by a final elution step (for a detailed protocol see www.pcr.dk, Innovations PCR). A total of 5 µl of the extracted DNA was used as template in the real-time PCR.

The real-time PCR at the participating laboratories was performed on Mx3000 or Mx4000 Real-time PCR Systems (Stratagene, La Jolla, CA,

USA), ABI-PRISM 7000 or 7900 (Applied Biosystems, Foster City, CA, USA), RotorGene 3000 (Corbett Research, Mortlake, Australia), or an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). The participating laboratories were asked to use the blank (NTC), the process blank (a *Campylobacter*-negative sample processed throughout the entire protocol), and the negative control to assign the cut-off line, and report back the threshold cycle (Ct) values.

Data analysis.

The test reports and the real-time PCR analyses from the participating laboratories were carefully evaluated on return to the expert laboratory, and the results were approved for inclusion in the statistical analysis, unless they fell into one of the following two categories: (i) obvious performance deviation from the protocol, and/or (ii) presence of target amplicons in the negative control results, indicating cross contamination at the participating laboratory.

The results obtained in the collaborative trial were analyzed according to the recommendations from NordVal. The relative specificity was calculated for the unspiked samples by the equation: $(1 - (FP/N-)) \times 100\%$, where N- refers to the total number of unspiked samples. The relative sensitivity was calculated for each level of spiking by the equation: $(TP/N+) \times 100\%$, where N+ refers to the number of spiked samples. The relative accuracy was calculated for all levels of spiking by the equation: $((PA + NA + FP)/N) \times 100\%$, where N refers to the number of samples tested.

Results

In agreement with the predefined criteria, results from all eight participating laboratories were included in the statistical data analysis (Table 1).

Table 1. Real-time PCR results for the detection of thermotolerant *Campylobacter* in spiked shoe cover samples.

Spiking level [CFU]	Threshold cycle (Ct) values							
	Participant no.							
	1	2	3	4	5	6	7	8
0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
	29.2	40.0	40.0	40.0	40.0	40.0	40.0	40.0
100-500	26.0	25.3	26.0	20.0	27.1	20.5	28.0	27.4
	26.8	26.1	26.0	22.1	26.5	21.1	26.9	27.8
1000-2000	24.1	23.0	23.0	18.2	24.2	18.4	24.9	25.7
	23.5	23.5	23.3	18.7	24.7	- ^a	22.7	24.7

^aIn accordance with ISO/DIS 20838:2003 (Anon., 2003), this result has been withdrawn from the data analysis due to lack of PCR response from the internal amplification control (IAC).

The relative specificity, sensitivity, and accuracy were 94%, 100%, and 100%, respectively. The considerable difference in the Ct-values obser-

ved among the participating laboratories for the same samples can be attributed to variation in the sensitivity of the real-time PCR platforms used. It has been shown that transferring a PCR method from one type of real-time instrument to another can result in a substantial shift in Ct-values (Josefsen et al., 2004).

The samples tested in the collaborative study were not naturally infected. Because the concentrations of the target microorganism in naturally infected samples are unknown, it would have been difficult to assess the method performance on samples with low levels of thermotolerant *Campylobacter*.

2.3 Technology transfer trial

2.3.1 Finding an alternative manual DNA extraction method

The first challenge in designing the technology transfer trial was finding an alternative DNA extraction procedure to the automated one performed on KingFisher or equivalent equipment. The participants in the trial were not expected to have automated DNA extraction equipment in-house, and in general it would not be suitable to have a method locked to this expensive equipment.

For this reason several manual DNA-extraction methods and kits were tested and the results obtained by these compared to the results obtained on parallel samples purified on the KingFisher platform.

2.3.1.1 DNA extraction by ShortPrep foodproof 1 Kit

DNA from the samples was extracted by the ShortPrep foodproof I Kit (Roche, Germany). The kit is designed for the rapid preparation of bacterial DNA for direct use in PCR.

The following procedure was applied for the DNA extraction from 1-ml shoe cover samples spiked in duplicate with *C. jejuni* CCUG 11284 at the level 0, 100-500 and 1000-2000 CFU/ml.

- The samples were pelleted by centrifugation at $16,000 \times g$ for 7 min at 4°C.
- The ShortPrep tubes were centrifuged at $500 \times g$ for 60 sec.
- 50 µl of sterile water was added to the sample pellet and mixed by vortexing.
- The pellet was transferred to the ShortPrep tubes and mixed by vortexing.
- The ShortPrep tubes were incubated for 10 min at 95-100°C.
- The ShortPrep tubes were left at room temperature for 1 min and mixed by vortexing.

- The ShortPrep tubes were centrifuged at 8000 x g for 1 min, and the supernatant transferred to a sterile Eppendorf tube.
- 5 µl was used as template for real-time PCR.

No *Campylobacter*-positive results were obtained by the ShortPrep foodproof I Kit in real-time PCR. There was no signal present from the internal amplification control in these samples either, indicating that the samples contain PCR inhibitory substances.

2.3.1.2 DNA extraction by NucleoSpin Tissue Kit

With the NucleoSpin Tissue (Macherey-Nagel, Germany) method, genomic DNA can be prepared from bacteria cells. Lysis is achieved by incubation of the sample material in a proteinase K solution / SDS solution. Appropriate conditions for binding of DNA to the silica membrane in the NucleoSpin Tissue columns are created by the addition of chaotropic salts and ethanol to the lysate. The binding process is reversible and specific to nucleic acids. Contaminations are removed by subsequent washing with two different buffers. Pure genomic DNA is finally eluted under low ionic strength conditions in a slightly alkaline elution buffer.

The following procedure was applied for the DNA extraction from 1-ml shoe cover samples spiked in duplicate with *C. jejuni* CCUG 11284 at the level 0, 100-500 and 1000-2000 CFU/ml.

- The samples were pelleted by centrifugation at 16,000 × g for 7 min at 4°C.
- The sample pellet was re-suspended in 200 µl buffer T1.
- 25 µl of proteinase K was added and the sample mixed by vortexing.
- 200 µl of buffer B3 was added and the sample mixed by vortexing.
- The sample was incubated at 70°C for 10 min.
- 210 µl of ethanol (96-100%) was added and the sample mixed by vortexing.
- The NucleoSpin tube was placed in the collection tube and the sample was added.
- The tube was centrifuged at 11,000 x g for 1 min, and the flow thorough discarded. If the sample was not drawn completely through the matrix, the centrifugation step was repeated.
- 500 µl of buffer BW was added.
- The tube was centrifuged at 11,000 x g for 1 min, and the flow thorough discarded.
- 600 µl of buffer B5 was added.
- The tube was centrifuged at 11,000 x g for 1 minute, and the flow thorough discarded.
- The tube was centrifuged at 11,000 x g for 1 minute, and the flow thorough discarded.

- The NucleoSpin tube was placed in an Eppendorf tube, and 100 µl of 70°C hot buffer BE was added. The tube was left at the table for 1 min.
- The tube was centrifuged at 11,000 x g for 1 min, and the NucleoSpin tube removed.
- 5 µl was used as template for real-time PCR.

The DNA extracted by the NucleoSpin Tissue Kit gave very good real-time PCR results. The Ct-values were as good or better than when the DNA from the samples had been extracted on the KingFisher.

2.3.1.3 DNA extraction by the Chelex method

Chelex 100 chelating resin (BioRad, US) has a high affinity for polyvalent metal ions. The resin is composed of polymers acting as chelating groups binding PCR inhibitors and DNA degrading substances. A very simple and economic pre-PCR boiling procedure should produce PCR quality DNA.

The following procedure was applied for the DNA extraction from 1-ml shoe cover samples spiked in duplicate with *C. jejuni* CCUG 11284 at the level 0, 100-500 and 1000-2000 CFU/ml.

- The samples were pelleted by centrifugation at 16,000 × g for 7 min at 4°C.
- 1.5 g Chelex 100 resin was dissolved in 25 ml of distilled water and left under constant stirring.
- 300 µl of this was added to the sample.
- The sample was incubated at 56°C for 15 to 20 min, and mixed by vortexing every 5 min.
- The sample was mixed by vortexing for 10 sec and incubated for 8 min at 100°C.
- The sample was mixed by vortexing for 10 sec and placed on ice for 5 min.
- The sample was centrifuged at 14,000 x g for 5 min.
- 100 µl of the supernatant was transferred to a sterile Eppendorf tube, and 5 µl of this used as template in real-time PCR.

No *Campylobacter*-positive results were obtained by the Chelex method in real-time PCR. There were signal present from the internal amplification control in these samples, indicating that the concentration of target DNA was too low.

2.3.1.4 DNA extraction by the DNeasy Tissue Kit

The DNeasy Tissue Kit is designed for rapid isolation of total DNA from a variety of sample sources including bacteria. The purified DNA is free

of contaminants and enzyme inhibitors and is ideal for all PCR applications.

The following procedure was applied for the DNA extraction from 1-ml shoe cover samples spiked in duplicate with *C. jejuni* CCUG 11284 at the level 0, 100-500 and 1000-2000 CFU/ml.

- The samples were pelleted by centrifugation at $16,000 \times g$ for 7 min at 4°C.
- The pellet was dissolved in 180 µl lysis buffer (Promega, Magnesil KF, Genomic System).
- The sample was incubated for a minimum of 30 min at 37°C.
- 25 µl proteinase K was added and afterwards 200 µl of buffer AL, the sample was mixed by vortexing.
- The sample was incubated for 30 min at 70°C.
- 200 µl of ethanol (96-100%) was added, and the sample mixed by vortexing.
- The sample was transferred to a DNeasy mini column with collection tube.
- The tube was centrifuged at 8,000 rpm for 1 min and the mini column transferred to a new collection tube.
- 500 µl of buffer AW1 was added and it was centrifuged at 8,000 rpm for 1 min.
- The mini column was transferred to a new collection tube and added 500 µl of buffer AW2.
- The tube was centrifuged at maximum speed for 3 min, and the mini column transferred to an Eppendorf tube.
- 100 µl of buffer AE was added and the tube left for 1 min before centrifuged at 8,000 rpm for 1 min.
- Another 100 µl of buffer AE was added and the tube centrifuged again, collecting the flow through in the same Eppendorf tube.
- 5 µl of this was used as template in real-time PCR.

This DNA extraction method did not give consistent results. Some were comparable to the ones obtained by the automated DNA extraction on KingFisher, and some samples gave no positive *Campylobacter* responses in real-time PCR.

2.3.1.5 DNA extraction by QIAamp DNA stool Mini Kit

The kit provides fast and easy purification of total DNA from fresh or frozen stool samples. The purified DNA is ideal for reliable use in PCR and other downstream enzymatic reactions.

The following procedure was applied for the DNA extraction from 1-ml shoe cover samples spiked in duplicate with *C. jejuni* CCUG 11284 at the level 0, 100-500 and 1000-2000 CFU/ml.

- The samples were pelleted by centrifugation at $16,000 \times g$ for 7 min at 4°C.
- 1,4 ml of buffer ASL was added to the pellet and the sample was mixed by vortexing for 1 min, or until the pellet was completely dissolved.
- The sample was incubated for 5 min at 95°C and mixed by vortexing for 15 sec.
- The sample was centrifuged at maximum speed for 1 min.
- 1,2 ml of the supernatant was transferred to a sterile 2-ml Eppendorf tube, and the pellet was discarded.
- One inhibitEX was added and the sample was immediately mixed by vortexing for 1 min, or until the tab was completely dissolved.
- The sample was incubated at room temperature for 1 min.
- The sample was centrifuged at maximum speed for 3 min.
- The supernatant was transferred to a sterile Eppendorf tube, and the pellet discarded.
- The sample was centrifuged at maximum speed for 3 min.
- 15 µl of proteinase K was added to a sterile Eppendorf tube and 200 µl of the supernatant followed by 200 µl of buffer AL was added.
- The sample was mixed by vortexing for 15 sec.
- The sample was incubated for 10 min. at 70°C.
- 200 µl of ethanol (96-100%) was added and the sample mixed by vortexing.
- The sample was transferred to a QIAamp spin column with collection tube, and centrifuged at maximum speed for 1 min. If the sample was not drawn completely through the matrix, the centrifugation step was repeated.
- The spin column was placed in a new collection tube and 500 µl of buffer AW1 was added.
- The sample was centrifuged at maximum speed for 1 min.
- The spin column was placed in a new collection tube and 500 µl of buffer AW2 was added.
- The sample was centrifuged at maximum speed for 3 min.
- The spin column was placed in a new collection tube and centrifuged at maximum speed for 1 min.
- The spin column was placed in a new Eppendorf tube and 200 µl of buffer AE was added.
- The tube was left at room temperature for 1 min, and centrifuged at maximum speed for 1 min.

This DNA extraction method did not give consistent results. Some were comparable to the ones obtained by the automated DNA extraction on KingFisher, and some samples gave no positive *Campylobacter* responses in real-time PCR.

2.3.1.6 Choice of manual DNA extraction method

Of the five alternative DNA extraction methods investigated only the NucleSpin Tissue Kit gave as good results as automated DNA extraction on KingFisher. This method was therefore chosen for the technology transfer trial.

2.3.2 Sample preparation for the technology transfer trial

Chicken samples: Certified *Campylobacter*-free chickens were purchased and split into 25 g samples of neck skin/meat. The *Campylobacter*-status of one sample from each chicken was investigated by the traditional microbiological reference method of NMKL (Anon., 2005(b)), in order to verify that no *Campylobacter* were present in the samples. The 25 g samples were kept at -20°C until shipped to the technology transfer trial participants.

Cloacae swab samples: The cloacae swabs were provided by the Danish Chicken producer Danpo A/S. Twenty cloacae swabs were pooled in a filtered stomacher bag and 30 ml of physiological saline was added. The bags were homogenised manually for 60 sec and left at the table at room temperature for 10 min. The *Campylobacter*-status every sample batch was investigated by the traditional microbiological reference method of NMKL (Anon., 2005(b)), in order to verify that no *Campylobacter* were present in the samples. The samples were split into 1-ml sample volumes and spiked with three levels of *C. jejuni* CCUG 11284: 0, 100-500 and 1000-2000 CFU/ml. The samples were pelleted by centrifugation at 16,000 x g for 7 min at 4°C, and kept at -20°C until shipped to the technology transfer trial participants.

The *Campylobacter* content of the spiked samples was verified by the traditional microbiological reference method of NMKL (Anon., 2005(b)).

Shoe cover samples: the Danish Chicken producer Danpo A/S provided the shoe cover samples. The samples were transferred to a filtered stomacher bag and added 1:10 (w/v) physiological saline. The samples were homogenised in a stomacher for 30 sec, and split into 1-ml sample volumes. The 1-ml samples were spiked with three levels of *C. jejuni* CCUG 11284: 0, 100-500 and 1000-2000 CFU/ml, and pelleted by centrifugation at 16,000 x g for 7 min at 4°C. The samples were kept at -20°C until shipped to the technology transfer trial participants.

The *Campylobacter* content of the spiked samples was verified by the traditional microbiological reference method of NMKL (Anon., 2005(b)).

2.3.3 Preparation of guidelines for the technology transfer trial

A general protocol describing the technology transfer trial was prepared. The protocol described in detail the enrichment, DNA extraction, PCR set-up and the reporting of obtained results. The protocol looked as shown below:

Protocol for the *Campylobacter* real-time PCR transfer trial

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Real-time PCR transfer trial

Aim:

The aim of this trial is to transfer the technology of a real-time PCR method for detection of thermotolerant *Campylobacter* to three Nordic laboratories.

Time schedule:

The test materials will be shipped by ordinary mail from DFVF on the 20/2-2006.

Report your results by returning your result form and your real-time analysis on a disc/CD-rom by ordinary mail at latest on: 3/3-2006 to: DFVF, Bülowsvej 27, 1790 Copenhagen V. Att.: Mathilde Josefsen, or by E-mail to mjo@dfvf.dk.

Participants:

Partner: Name and Institution	E-mail
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Eyjólfur Reynisson. Icelandic Fisheries Laboratories, Skulagata 4, 101 Reykjavik, Iceland.	eyolfur@rf.is

General information:

The real-time PCR transfer trial will be performed on DNA from spiked samples from the chicken production, applying ready-to-use PCR mix. The samples have the following origin: cloacae swabs collected at the abattoir, shoe cover samples collected in the rearing houses and meat from chickens at retail.

This trial comprises testing of 18 samples (6 cloacae swabs, 6 shoe cover samples and 6 chicken meat samples enriched in Bolton broth). **All materials needed to participate in the trial will be shipped to the participants.**

The 12 cloacae swabs and shoe cover samples you will receive as pellet from 1 ml samples, and they will be **ready** for DNA extraction.

The three chicken meat samples are to be transferred to Bolton broth and two of these are to be spiked with *C. jejuni*. The meat samples should be incubated for 48 hours in total. After 24 hours samples should be drawn for DNA extraction and PCR. The meat samples are to be further incubated for another 24 hours followed by streaking onto mCCDA plates. These selective plates are to be incubated for 48 hours, *Campylobacter* growth subcultured on blood agar plates and confirmed by microscopy.

The DNA extraction should be performed using NucleoSpin Tissue columns, and the real-time PCR carried out on the extracted DNA. You will receive ready-to-use PCR mix, so after you have aliquoted this into the PCR tubes, you just add the extracted DNA and run the PCR.

Sample preparation on DFVF:

All samples were confirmed *Campylobacter*-negative using traditional culture methods (NMKL 119, 1990, 2nd Ed).

The cloacae swabs were washed in 0.9% NaCl.

The disposable shoe covers samples were weighed and washed in ten times volume of 0.9% NaCl and homogenized using a stomacher.

These samples were spiked at 3 levels with 100 µl inoculums (2 samples at each level) to a final concentration of: 0 (unspiked), 100-500, and 1000-2000 CFU/ml. The chicken meat samples are provided to the participating laboratories in samples of 25 g.

The materials shipped to the participating laboratories:

Content of package	Designation	Volume	Store at
12 faecal samples	Vial 1-12	Pellet	-20°C
3 chicken meat samples	13-15	25 g	
1 vial with ready-to-go PCR mix	Vial 17	510 µl	
1 vial with <i>C. jejuni</i>	16	Lyophilized	Room temperature
NucleoSpin Tissue columns	NST	20	
NucleoSpin Tissue collecting tubes	-	20	
Caps for Eppendorf tubes	-	20	
Buffer T1	T1	4.5 ml	
Buffer B3	B3	4.5 ml	
Buffer B5	B5	13 ml	
Buffer BW	BW	11 ml	
Buffer BE	BE	2.5 ml	
Ethanol (96%)	EtOH 96%	6 ml	
Proteinase K	Prot. K	600 µl	5°C
Bolton broth	Bolton	700 ml	
Buffered peptone water	BPW	100 ml	
mCCDA plates	mCCDA	3	
Blood agar plates	BA	3	
Brain Heart Infusion broth	BHI	1 ml	
1 vial with sterile water (for NTC)	Vial 18	50 µl	
1 vial with negative control DNA	Vial 19	20 µl	
1 vial with positive control DNA	Vial 20	20 µl	

Protocol:

1. Receiving the transfer trial shipment

The package sent to you will contain: samples, reagents for DNA extraction and real-time PCR, a detailed protocol and an instruction for the real-time PCR instrument of your laboratory.

Please make sure to store the samples and reagents as shown in the Table on page 3, and read the protocol carefully before performing the sample enrichment, DNA extraction and real-time PCR.

2. Enrichment of chicken meat samples

- Thaw the three chicken meat samples (13-15).
- Aseptically remove the cap and the rubber lid from the vial containing *C. jejuni* (16). Add 1 ml of BPW, wash the inner side of the vial with a pipette and transfer the content to a bottle/flask containing 75 ml of BPW. Repeat this washing procedure another four times. Now you have 80 ml of a BPW suspension containing approx. 100 CFU/ml of *C. jejuni*.
- Transfer each of the three chicken meat samples into a 250 ml bottle with screw cap containing 225 ml of Bolton broth, and name the bottles 13, 14 and 15.
- Add 90 µl of the BPW/*C. jejuni* suspension to bottle 14, and 900 µl to bottle 15, and shake them gently.
- The bottles (13-15) are incubated at $41.5 \pm 1.0^{\circ}\text{C}$ for 48 ± 4 hours in a microaerobic atmosphere by placing the bottle - with cap unscrewed – in an incubator or jar with microaerobic atmosphere *or alternatively* by fastening the cap of the bottle tightly (using this technique the headspace in the bottle must not exceed 1/4 of the total volume).
- After 24 ± 2 hours draw 2 x 1 ml samples from each bottle (13-15). The samples should be drawn from just below the surface of the broths. Centrifuge the drawn 1 ml samples at $16\,000 \times g$ for 7 minutes and discard the supernatant. Unless you wish to perform the DNA extraction immediately, freeze the sample pellets at -20°C .

Name the drawn 1 ml samples as shown:

From enrichment 13: **13** and **14**

From enrichment 14: **15** and **16**

From enrichment 15: **17** and **18**

- After 48 ± 4 hours transfer approx. 10 µl of inoculum onto the surface of a mCCDA plate with a sterile loop. The inoculum should be drawn from just below the surface of the broths. Incubate the plates at $41.5 \pm 1.0^{\circ}\text{C}$ for 48 ± 4 hours in a microaerobic atmosphere.
- After incubation, inspect the plates for visible growth. Typical colonies of *Campylobacter* are flat or convex with a greyish or white colour and a glossy surface. The sizes of the colonies vary with the surface moist on the plates.
- For confirmation, 5 *Campylobacter* like colonies (from plates with less than 5 colonies all are examined) are subcultured on blood agar plates, which are incubated at $41.5 \pm 1.0^{\circ}\text{C}$ for 24 ± 3 hours.

- Suspend colony material in BHI on a slide. Cover with a cover slip and examine immediately under the microscope using phase contrast and 1000 x magnification. *Campylobacter* are highly motile, slender rods with spiral morphology. Darting or corkscrew-like movements characterize motility.

3. DNA extraction

DNA is to be extracted from the pellet of the 6 cloacae swab, 6 shoe cover and 6 enriched chicken meat samples. The cloacae swab and shoe cover samples you received ready for DNA extraction, the chicken meat samples you prepared your self from the 24-h enrichments.

- Resuspend the sample pellets in 200 µl buffer T1.
- Add 25 µl of proteinase K and mix by vortexing.
- Add 200 µl buffer B3 and mix by vortexing.
- Incubate in water bath at 70°C for 10 minutes (remember to cap the tubes).
- Add 210 µl ethanol (96-100%) and mix by vortexing.
- Label the NucleoSpin tubes (NST), and place them in the collecting tubes and add the sample.
- Centrifuge at 11 000 x g for 1 minute, and discard the flow-through. If the sample is not drawn completely through the matrix repeat the centrifugation step and discard the flow-through.
- Place the NST in the collecting tube again, and add 500 µl buffer BW.
- Centrifuge at 11 000 x g for 1 minute, and discard the flow-through.
- Place the NST in the collecting tube again, and add 600 µl buffer B5.
- Centrifuge at 11 000 x g for 1 minute, and discard the flow-through.
- Place the NST in the collecting tube again, and centrifuge at 11 000 x g for 1 minute and discard the flow-through.
- Place the NST in an Eppendorf tube. Add 100 µl 70°C hot buffer BE, and leave it on the table for 1 minute.
- Centrifuge at 11 000 x g for 1 minute and discard the NST.
- 5 µl extracted DNA is used as template for the real-time PCR.

4. PCR setup

- Thaw vial 17 with real-time PCR mix at room temperature (do not expose to light).
- Take 21 PCR tubes/wells for the set up.
- Add 20 µl of the real-time PCR mix to each of the 21 PCR tubes/wells.
- Add 5 µl of water (vial 18) to tube 1. This is the no template control (NTC).
- Add 5 µl of DNA from the coded samples (vial 1 to 18) to tube 2-19.
- Add 5 µl of the negative control (vial 19) to tube 20.
- Add 5 µl of the positive control (vial 20) to tube 21.
- Close the tubes and place them in the real-time PCR machine.

5. Real-time PCR

Please refer to the detailed real-time PCR machine instructions included in the shipment.

6. Reporting the results

Please fill in the enclosed result form and copy your real-time analysis to a CD-rom/disc. Send it all by ordinary mail to: DFVF, Bülowsvej 27, 1790 Copenhagen V, Att.: Mathilde Josefsen, or by e-mail to mjo@dfvf.dk

Result form

PCR results:

Tube no.	Sample	Result (pos./neg.)	FAM (Ct-value)	JOE/HEX (Ct-value)
1	Non template control			
2	Sample 1			
3	Sample 2			
4	Sample 3			
5	Sample 4			
6	Sample 5			
7	Sample 6			
8	Sample 7			
9	Sample 8			
10	Sample 9			
11	Sample 10			
12	Sample 11			
13	Sample 12			
14	Sample 13			
15	Sample 14			
16	Sample 15			
17	Sample 16			
18	Sample 17			
19	Sample 18			
20	Negative control DNA			
21	Positive control DNA			

Culture results:

Sample No.	Growth identified in 25 g sample (+/-)	Confirmation of isolates as thermotolerant <i>Campylobacter</i> in isolate no.: (+/-)	Thermotolerant <i>Campylobacter</i> detected in 25 g sample (+/-)

			1	2	3	4	5		
	13								
	14								
	15								

Individual guidelines for the participants in-house real-time PCR equipment was prepared as follows:

Real-time PCR on Mx3000/3005 (Software version MxPro)

1. Turn on the Mx3000/3005 and the computer.
2. Open the Mx3000/3005 programme by pressing the icon:
3. Under File/New choose Quantitative PCR, click off the square turn lamp on for warm-up [OK].

4. Under Plate Setup mark the wells in use with the mouse.
5. Under Well type choose <UNKNOWN>.
6. Under Collect fluorescence data click off in the squares FAM and HEX. Reference dye should be set to <NONE>
7. Mark the well containing the NTC, and change it to NTC under Well type. Mark the well containing the negative DNA control, and change it to FAM Negative control under Well type. Mark the well containing the positive DNA control, and change it to FAM positive control under Well type:

8. Name the samples according to the transfer-trial protocol by double clicking on the individual wells and filling in the names as shown on the picture:

Well Information

Well annotation

Name:

Comments:

Assign assays within selected wells

Dye	Assay	Assay Color
CY5		
CY3		
ROX		
HEX		
FAM		

☐ Use for all wells

9. Choose Thermal Profile Setup, set segment 1 to 95°C for 3 min;

segment 2 to 95°C for 15 sec, 58°C for 1 min and 72°C for 30 sec. Segment 2 should be repeated 40 times with Endpoint data collection in the annealing at 58°C (draw the magnifying glass called END to the annealing step with the mouse), as shown in the picture:

10. Choose Start Run. Save the file and run the PCR.

11. When the PCR has finished, choose Analysis, Analysis Selection/setup and mark the wells in use. In the box "Algorithm Enhancements" click off Adaptive baseline and moving average.

12. Under Results, "Area to analyze" choose Amplification Plots. In the square Fluorescence choose dR. In the bottom of the amplification plot there are two functions (Assays shown and Well types shown), and here you choose what to look at. Begin by choosing FAM in Dyes shown, and all types in Well types shown, as shown in the

picture:

13. Double click on the Y-axis (fluorescence dR) and convert it to log scale by pressing Log [OK]:

14. Put the threshold (blue line) above the background noise and known negative samples (NTC and negative DNA control). Double click on the Y-axis and convert it back to linear form and print.
15. In "Area to Analyze" choose Text report and print it.
16. Begin again from point 12, but this time look at HEX and put the threshold above background noise.

Real-time PCR on RotorGene 3000 (Software version 5)

- Turn on the RotorGene 3000 and the computer.
- Place the 36-sample rotor head in the rotor.
- Place the samples in the rotor head, and fill the rotor head up with empty tubes. ALWAYS wear powder-free gloves handling PCR tubes.
- Click on the icon "RotorGene".
- Under "New Experiment" choose "New Experiment" and press "New".
- The New Experiment Wizard should be filled in as shown:

- Press "Next".
- Under Temperature Profile press "Edit Profile", mark Denature as shown and fill in the following:

- Under Cycling, mark the first step (95 deg. for 15 sec) as shown and fill in the following:

- Under Cycling, mark the second step (58 deg. for 60 sec) as shown and fill in the following:

NB! Press "Acquiring to Cycling A" and transfer FAM and JOE to Acquiring Channels as shown, and press "OK":

- Press "OK".
- Under Channel Setup press "Calibrate", press "Calibrate Acquiring" and mark "Perform Calibration before 1st Acquisition" as shown:

- Press "Close".
- Press "Next" followed by "Start Run".
- Save the file.
- Under Edit Samples choose according to the samples:

Type: "Sample/NTC/Positive Control/Negative Control"

Name: Name the samples and controls

C: Give the different samples colours:

- Press "Finish".

Data analysis

1. Press "Analysis", choose Quantitation as shown and double click on Cycling A.FAM.

2. Press OK if a text message regarding threshold appears.
3. Close the small Analysis window (by pressing the cross in the upper right corner).
4. Now the FAM-graphs occur. Press "Dynamic Tube" and "Slope Correct" as shown in the picture. Press "Ignore First" and type in 1. Cycle:

5. In the square CT Calculation: "Eliminate Cycles before" should be set to 5.

6. Threshold of FAM is put down manually above the background noise and the known negative samples (Neg. DNA control and NTC). This is done by marking only the negative samples, pressing the red arrow behind the square Threshold with the mouse, and thereafter putting the threshold on the graph with the mouse.

7. Press "Quant. Settings" and set the NTC Threshold at 5% as shown and press "OK":

8. Press "Reports", choose Full Report and press "OK" followed by "Print". When the data for FAM is printed close the Preview window.
9. Begin again at point 1, but double click at Cycling A.JOE and continue following the same procedure until point 6.
10. Threshold for JOE is put down manually (as for FAM) above background noise as shown in the picture:

11. Repeat point 7 where the NTC Threshold is set to 5%.
12. Press "Reports", choose Full Report and press "OK" followed by "Print". When the data for JOE is printed close the Preview window.
13. Save the data on a disc/CD rom and send it to DFVF together with the result form, or by e-mail to mjo@dfvf.dk.

Real-time PCR on iCycler iQ (Software version 3.0a)

- Turn on the iCycler, and the computer.
- Open the iCycler-program.
- Under the module *Library*, in the section View Protocol, Press Create a new protocol:

- Now you are working in the module *Workshop*.
- Under the section Edit Protocol, fill in the following thermal profile: 3 minutes at 95°C, followed by 40 cycles consisting of 15 seconds at 95°C, 60 seconds at 58°C and 30 seconds at 72°C. In the area Select data collection step(s) choose Step 2 in Cycle 2. Under Protocol Filename, fill in the name (name.tmo) and press Save this protocol. The screen shot should look as shown below:

Protocol Filename: Campylobacter ringtest.tmo Selected Plate Setup: Template.pts
 All changes must be saved before running.
 Selected Plate Setup is loaded in the Edit Plate Setup tab

Show options:

☐ Initial Hold ☐ Increment Time
☐ Gradient ☐ Decrement Time
☐ Melt Curve ☐ Ramping
☐ Increment Temperature ☐ Cycle Description
☐ Decrement Temperature ☐ Step Process

Select data collection step(s):

☐ Cycle 1
☒ Step 1
☐ Cycle 2
☒ Step 1
☒ Step 2
☐ Step 3

Cycle	Repeats	Step	Dwell Time	Setpoint	Melt Curve	Temp	Time	Time
1	1	1	00:00	9.5				
2	40	1	00:15	9.5				
		2	01:00	5.8				
		3	00:30	7.2				

Running: Instrument not in Remote 23-11-2005 21:24

- Under the section Edit Plate Setup, choose the section Samples: Whole Plate Loading and mark the sample types in the 21 wells.
- The 21 samples are named by marking the individual samples, and typing their name in the square Sample Identifier.
- Choose the section Select and load fluorophores. Under point 1. "Select or deselect a fluorophore" choose FAM-490. Under point 2. "Assign a colour" choose a colour, and mark the 21 wells in use. Go to point 1. again and choose HEX-530, go to point 2. and choose another colour, and again mark the 21 wells in use:

- Name the plate setup (name.pts) in the square Plate Setup Filename and save it by pressing Save this plate setup.
- Press Run with selected protocol.
- Choose the section Run Prep, set the Sample volume to 25, Select run purpose for PCR Quantification/Melt Curve and press Begin run.

Data analysis

- If you analyze your data immediately after the PCR run go to the next step, otherwise choose the section View Post-run Data in the Library. Mark the file in question and press Analyze Data.
- Choose the section PCR Quantification. Under Threshold cycle calculation choose Auto calculated for both Baseline cycles and Threshold position.
- Check that the Threshold line is placed above the known negative samples (NTC and negative control). If this is not the case, move the threshold line either by pulling it manually with the mouse, or by clicking User defined under Threshold position and typing in the wanted position.
- The Ct values will be listed in the squares to the right of the run diagram.

2.3.4 Performing the technology transfer trial

The test material sent to the three participating laboratories consisted of 15 samples (6 cloacae swabs, 6 shoe cover samples and 3 chicken neck skin/meat samples). The shipment further included all reagents needed for the DNA extraction, the real-time PCR, the cultivation of samples and further more a protocol describing the technology transfer trial and a specific guideline for the real-time PCR instrument of the laboratory.

The trial material was shipped by ordinary mail as parcel post with ice packages from DFVF at 20/2-2006.

The 12 faecal samples were shipped as pellet from 1 ml samples, already spiked with *C. jejuni* as described under sample preparation, and ready for DNA extraction. The participants were asked to transfer the three chicken samples to Bolton broth, and spike two of these with *C. jejuni*. The samples should be enriched for 48 hours in total, and samples for DNA extraction followed by real-time PCR should be drawn after 24 hours of enrichment. After the 48 hours the samples should be plated on to mCCDA plates, and incubated further for 48 hours. The *Campylobacter* growth should be subcultured on to blood agar plates and confirmed by microscopy.

The participants were asked to perform the technology transfer trial according to the instructions shown in the protocol, and report back their results at latest on the 3/3-2006.

The participants were offered advice and guidance during the trial by phone or mail.

2.3.5 Results from the technology transfer trial

The results obtained by the participants in the trial are shown in the table below.

Sample	Spiking level ^a	Laboratory ^b					
		1		2		3	
		Ct [FAM]	Ct [VIC]	Ct [FAM]	Ct [VIC]	Ct [FAM]	Ct [VIC]
1	++	27.1	- ^c	26.2	24.4	34.7	28.0
2	++	28.6	-	23.2	24.5	-	27.8
3	-	-	-	-	24.4	26.7	27.9
4	-	-	-	-	24.9	-	27.7
5	+	30.8	-	26.4	24.6	29.9	27.7
6	+	32.5	-	28.4	24.7	-	27.9
7	-	24.5	-	-	24.6	-	27.9
8	-	-	-	-	24.5	-	27.7
9	++	31.1	-	27.1	24.8	34.3	28.2
10	++	27.1	-	23.1	24.5	26.4	27.8
11	+	29.8	-	28.4	24.6	37.7	27.9
12	+	31.3	-	25.7	24.2	29.6	27.6
13	-	-	-	-	24.8	-	27.9
14	-	-	-	-	24.9	-	27.7
15	+	25.0	-	-	25.0	19.2	26.9
16	+	24.0	-	30.5	24.7	17.9	25.2
17	++	23.0	-	26.1	24.3	27.2	27.8
18	++	21.2	-	26.1	24.5	27.2	27.9
NTC	-	-	-	-	24.9	-	28.7
Negative DNA		-	-	-	25.1	-	27.7
Positive DNA		20.6	-	18.0	23.0	20.7	27.1

^a -: Not spiked, +: Spiked with 100-500 CFU for faecal samples and 1-10 CFU *C. jejuni* for chicken meat samples, ++: Spiked with 1000-2000 CFU for faecal samples and 10-100 CFU *C. jejuni* for chicken meat samples.

^b 1: National Veterinary and Food Research Institute, Department of Bacteriology, EELA, Finland.

2: National Veterinary Institute of Sweden, Zoonosecenter, Sweden.

3: Icelandic Fisheries Laboratories, Iceland.

^c The samples were analysed using a commercially prepared standard master mix, and for this reason no internal amplification control was present.

The samples were coded as follows:

Cloacae swab samples (0 CFU/ml): 4 and 8.

Cloacae swab samples (100-500 CFU/ml): 6 and 11.

Cloacae swab samples (1000-2000 CFU/ml): 1 and 9.

Shoe cover samples (0 CFU/ml): 3 and 7.

Shoe cover samples (100-500 CFU/ml): 5 and 12.

Shoe cover samples (1000-2000 CFU/ml): 2 and 10.

Chicken neck skin samples: 13, 14 and 15.

The results reported by laboratory 1, National Veterinary and Food Research Institute in Finland, were obtained using a premixed master mix (2 x iQ Supermix) from BioRad. The samples were run the first time in the master mix provided by DFVF, but due to technical problems with their real-time PCR equipment no results were obtained from this run. Despite the fact that the PCR was therefore run under sub optimal conditions quite good results were obtained, indicating a good robustness of the method. As shown in the table only one unexpected result was obtained from sample 7. The sample was unspiked but a Ct-value of 24.5 was obtained. This could be due to cross contamination during DNA extraction, a mix up of samples either at DFVF or at the participating laboratory.

The results reported by laboratory 2, The National Veterinary Institute of Sweden, were as expected except for sample 15. This sample was a chicken neck skin sample spiked at the participating laboratory. Since the parallel sample 16 gave a Ct-value of 30.5, the spiking of the chicken neck skin enrichment seems to have been successful. There was obtained a signal from the internal amplification control for sample 15, indicating that the conditions for a PCR reaction were present. The problem therefore could be ascribed the DNA extraction that maybe failed in this particular sample, or simply that no DNA was transferred to the PCR tube during setup.

The results obtained by laboratory 3, The Icelandic Fisheries Laboratories, should be regarded with some reservation, since the shipment of the trial materials were several days late, and the parcel quite spoiled when arriving at its destination. However, the Icelandic partner was asked to go ahead with the trial anyway, and the results obtained were actually surprisingly good. Unexpected results were obtained from sample 2, 3 and 6. Two of the samples were spiked and suppose to give a positive PCR response but did not, and the last sample was unspiked, but resulted in a Ct-value of 26.7. The master mix and the spiked samples had at this point been at room temperature for several days, and the consequences of this are difficult to predict.

The overall conclusions drawn from this technology transfer trial was that the method seems quite easy to implement. The participating laboratories did not request any further assistance during the trial period, and found the method easy to work with. Further more it was concluded that shipping ring trial material by ordinary mail across borders is not an option. The shipments were delayed and mishandled at arrival. Despite this fact the outcome of the trial was quite successful, and based on these findings the real-time PCR method seems to be quite robust.

Since the method was already validated nationally, and the results from the technology transfer trial were satisfactory, it was decided in

collaboration with the Danish National Committee of the Nordic Committee on Food Analysis (NKML) to propose the method as a standard.

2.4 NMKL standard proposal

A standard proposal for the Nordic Committee on Food Analysis was prepared as follows:

THERMOTOLERANT *CAMPYLOBACTER*.

A REAL-TIME POLYMERASE CHAIN REACTION (PCR) METHOD FOR DETECTION IN CHICKEN NECK SKIN, FAECES ON CLOACAE SWABS AND ON SHOE COVERS.

This NMKL method is validated in a collaborative study.

1. SCOPE AND FIELD OF APPLICATION

This standard specifies a real-time PCR method for detection of human pathogenic thermotolerant *Campylobacter* (*C. jejuni*, *C. coli* and *C. lari*) in chicken neck skin, faeces on cloacae swabs and faecal samples collected on disposable shoe covers in chicken rearing houses.

The primers are targeting a sequence within the 16S rRNA gene, specific for *C. jejuni*, *C. coli* and *C. lari*, producing a 287 bp amplification product. The inclusivity and exclusivity of the primers has been determined to 100 and 97%, respectively. Thermotolerant *Campylobacter* are qualitatively determined with a detection limit of 1-10 CFU per 40 g chicken neck skin and 100-500 CFU per ml dilution of cloacae swabs and faecal samples collected on disposable shoe covers.

2. DEFINITION AND EPIDEMIOLOGICAL ASPECTS

In the following method description, the term “thermotolerant *Campylobacter*” will be referring to *C. jejuni*, *C. coli* and *C. lari*, at present the most important species associated with human campylobacteriosis.

Thermotolerant *Campylobacter* are presently one of the most common causes of human food borne infections. The majority of infections are sporadic. The sources are rarely identified, but raw milk, polluted water, contact with pet animals and insufficient cooked meat products (pork, beef and especially

poultry) are known to be associated with infection.

Thermotolerant *Campylobacter* are Gram-negative, oxidase-positive and catalase-positive curved or spiral shaped rods. Most cells are motile with a characteristic corkscrew motility by means of a single polar flagellum at one or both ends. Coccoid forms may occur in older cultures.

Thermotolerant *Campylobacter* are microaerobic and will grow in a microaerobic atmosphere. Most species grow at temperatures between 32 and 45°C, but some strains do not grow below 35°C.

3. REFERENCES

3.1 NMKL Report No. 5, 2nd ed., 1994: Quality Assurance Guidelines for Microbiological Laboratories.

3.2 Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens – Performance testing for thermal cyclers. ISO/TS 20866, 1st Ed., 2005.

3.3 Josefsen M.H., N.R. Jacobsen, and J. Hoorfar. 2004. Enrichment followed by quantitative PCR for rapid detection and as a tool for quantitative risk assessment of food borne thermotolerant *Campylobacters*. *Journal of Applied Environmental Microbiology*. 70:3588-3592.

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4. PRINCIPLE

For chicken neck skin samples this method is a combination of conventional enrichment and PCR, for faecal samples PCR is performed directly on the samples without prior enrichment.

The method has been validated for the following three samples matrices:

4.1 Chicken neck skin. Homogenised and enriched overnight

4.2 Cloacae swabs. Twenty rectal swabs homogenised with 30 ml of physiological saline

4.3 Disposable shoe covers. Homogenised in ten times (w/v) physiological saline

DNA extraction from the samples can be performed automatically using the Magnesil® KF, Genomic System (MD1460, Promega) on a KingFisher (ThermoLabsystems), a BeadRetriever (Dyna) or equivalent equipment, or manually using a NucleoSpin® Tissue Kit (740952.10, Macherey-Nagel) or equivalent.

The method includes an internal amplification control (IAC) to detect false negative responses, a process blank (a target-DNA negative sample processed throughout the entire protocol) to detect cross contamination during sample preparation, non-template controls (NTCs) to detect contamination of the PCR reagents, a positive DNA control, and a negative DNA control.

5. MEDIA AND REAGENTS

5.1 Diluent

5.1.1 Physiological saline

Sodium chloride	9 g
Dist. Water	1000 ml

5.2 Enrichment broth

5.2.1 Bolton broth (Oxoid CM983 or equivalent)

Meat peptone	10 g
Lactalbumin hydrolysate	5 g
Yeast extract	5 g
Sodium chloride, NaCl	5 g
Alpha-ketoglutaric acid	1 g
Sodium pyruvate	0.5 g
Sodium metabisulphite	0.5 g
Sodium carbonate	0.6 g
Haemin	10 mg
Cephoperazone	20 mg
Vancomycin	20 mg
Trimethoprim	20 mg
Amphotericin B	10 mg
Lysed blood	50 ml

Dist. water	1000 ml
-------------	---------

5.2.1.1 Bolton basal media

Bolton basal media is prepared according to the instructions of the manufacturer.

Sterilise in an autoclave at $121 \pm 2^\circ\text{C}$ for 15 ± 1 min. As soon as the autoclave has reached a temperature below 100°C , it is opened, and the screw caps are tightened to avoid inclusion of air.

After sterilisation, the pH at 25°C shall be 7.4 ± 0.2 .

In airtight bottles, the basal broth will keep for up to three weeks in the dark at $5 \pm 3^\circ\text{C}$.

5.2.1.2 Preparation of lysed blood

Use defibrinated or citrate stabilised blood from cattle or horse. The blood can be stored for 1 month at $5 \pm 3^\circ\text{C}$, provided no signs of deterioration occur.

The blood is lysed by freezing at $-20 \pm 5^\circ\text{C}$ overnight. Portions of lysed blood can be stored in tight vials at $5 \pm 3^\circ\text{C}$ for a few days.

5.2.1.3 Bolton broth selective supplement

A vial (Oxoid SR208E or equivalent) containing:

Cephoperazone	10 mg
Vancomycin	10 mg
Trimethoprim	10 mg
Amphotericin B	5 mg

Corresponds to 500 ml Bolton broth.

The supplement is dissolved according to the instructions of the manufacturer. Avoid frothing the solution.

5.2.1.4 Preparation of the complete Bolton broth

Bolton basal broth (5.2.1.1), supplement (5.2.1.3) and lysed blood (5.2.1.2) are mixed immediately prior to inoculations. The lysed blood is the last ingredient to be added. After tightening the screw caps to minimise uptake of oxygen, the complete broths are gently rotated.

Bolton broth is made by gently mixing the following ingredients:

Bolton basal broth (5.2.1.1)	100 ml
Bolton broth selective supplement (5.2.1.3)	1 ml
Lysed blood (5.2.1.2)	5 ml

The prepared media can be stored for 2 weeks if kept at $5 \pm 3^\circ\text{C}$.

5.3 Reagents for automated DNA extraction

5.3.1 Magnesil® KF, Genomic System (Promega)

Store at room temperature.

5.3.2 Ethanol (95-100 %)

Store at room temperature.

5.3.3 Isopropanol

Store at room temperature.

5.4 Reagents for manual DNA extraction

5.4.1 NucleoSpin® Tissue Kit (Macherey-Nagel)

Store at room temperature.

5.4.2 Ethanol (96-100%)

Store at room temperature.

5.5 Reagents for real-time PCR

5.5.1 Buffer (Roche)

10 x conc. PCR-buffer for *Tth* DNA polymerase containing 100 mM Tris-HCl, pH 8.9 (25 °C), 1 M KCl, 15 mM MgCl₂, 500 µg/ml bovine serum albumin (BSA), 0.5 % Tween 20 (v/v).

Store at -20°C.

5.5.2 DNA polymerase (Roche)

5 U/µl *Tth* DNA polymerase.

Store at -20°C.

5.5.3 MgCl₂

25 mM MgCl₂ solution in sterile water.

Store at 5°C.

5.5.4 dNTP with dUTP

12.5 mM solution of dNTP (2.5 mM each of dATP, dCTP, dGTP and 5.0 mM of dUTP).

Store at -20°C.

5.5.5 Bovine serum albumin

20 mg/ml BSA solution containing 50 mM Tris-HCl, 100 mM NaCl, 0.25 mM EDTA, 1 mM 2-mercaptoethanol, 50% glycerol (v/v), pH 7.5.

5.5.6 Glycerol

87% pure glycerol.

5.5.7 Primers

Forward primer (OT-1559):

5' CTG CTT AAC ACA AGT TGA GTA GG 3'

Reverse primer (18-1):

5' TTC CTT AGG TAC CGT CAG AA 3'

Quality: Reverse Phase-Fast Cartridge Purification or equivalent.

Store at -20°C.

5.5.8 *Campylobacter* and IAC probe

Target probe:

5'**FAM**-TGT CAT CCT CCA CGC GGC GTT GCT GC-**TAMRA** 3'

IAC probe:

5'**JOE**-TTC ATG AGG ACA CCT GAG TTG A-**TAMRA** 3'

Quality: Reverse Phase-Fast Cartridge Purification or equivalent.

5.5.9 Internal amplification control (IAC)

IAC 124 bp, (Lübeck et al., 2003a):

5'-CTG CTT AAC ACA AGT TGA GTA GGC AAC TCA GGT GTC CTC
ATG AAT TTG AAG ACA TAA ACA AGG GAC TGG TCT CCG TCC
CAA CCA AGA TCA TCC ATC TCC CGC TAT TCT GAC GGT ACC
TAA GGA A-3'

Quality: Hydrogel Purity Electrophoresis or equivalent.

6. APPARATUS AND EQUIPMENT

Real-time PCR thermal cycler

Plastic ware for real-time PCR

KingFisher (ThermoLabsystems), BeadRetriever (Dynal) or equivalent equipment

Plastic ware for KingFisher, BeadRetriever or equivalent equipment

Microcentrifuge

Micropipettes: 20 µl, 100 µl and 1 ml

Filter tips for micropipettes, sterile

Eppendorf tubes: 0.5 and 1.5 ml, sterile

NucleoSpin Tissue columns

NucleoSpin Collecting tubes

Incubator, 41.5 ± 1.0°C

Bottles, 100 ml with air tight screw.

Equipment for microaerobic incubation. Jars or incubators in which a microaerobic atmosphere composed by 5-10 % oxygen (O₂), 70-85 % nitrogen (N₂) and 10-20 % carbon dioxide (CO₂) can be established.

NOTE: Commercially available equipments like e.g. Anoxomat (MART) and Campylobacter Gas Generating Kits (Oxoid) may be used.

7. PROCEDURE

7.1 Enrichment and sample preparation

7.1.1 Chicken neck skin

Dilute 40 g of raw chicken neck skin in 1:1 (w/v) physiological saline (5.1.1) and homogenise it for 1 min in a stomacher. Transfer 10 ml of this solution to 90 ml of Bolton broth (5.2.1), and incubate at $41.5 \pm 1.0^{\circ}\text{C}$ in a microaerobic atmosphere for 24 ± 3 hours. Remove 1 ml for DNA extraction. Centrifuge the sample at $16\,000 \times g$ for 7 min., and discard the supernatant. Keep the sample pellet at $-20.0 \pm 0.1^{\circ}\text{C}$ until performing DNA extraction.

7.1.2 Cloacae swabs

Homogenize twenty cloacae swabs in a plastic bag with 30 ml physiological saline (5.1.1), and remove 1 ml for DNA extraction. Centrifuge the sample at $16\,000 \times g$ for 7 min., and discard the supernatant. Keep the sample pellet at $-20.0 \pm 0.1^{\circ}\text{C}$ until performing DNA extraction.

7.2.3 Shoe covers

Wash the shoe covers in ten times (w/v) physiological saline (5.1.1), and homogenize in a stomacher for 1 min. Remove 1 ml for DNA extraction. Centrifuge the sample at $16\,000 \times g$ for 7 min., and discard the supernatant. Keep the sample pellet at $-20.0 \pm 0.1^{\circ}\text{C}$ until performing DNA extraction.

7.2 DNA extraction

7.2.1 Automated DNA extraction (KingFisher/ Bead- Retriever)

Before first use prepare the working solutions for DNA extraction by adding 110 ml 95-100 % ethanol (5.3.2) and 110 ml isopropanol (5.3.3) to the alcohol wash.

Re-suspend the sample pellet in 200 μl (KingFisher) or 300 μl (BeadRetriever) of lysis buffer and prepare the plates/racks for the automated DNA extraction as shown in Annex 1. Place the prepared plates/racks in the automated DNA extraction platform, and run the program shown in Annex 2 (KingFisher) or Annex 3 (BeadRetriever). Use 5 μl of the extracted DNA as template in the real-time PCR.

NOTE: Always include a process blank, i.e. a sample consisting only of lysis buffer when performing automated DNA extraction.

7.2.2 Manual DNA extraction (NucleoSpin Tissue Kit)

Before first use prepare the working solutions for DNA extraction by dissolving the lyophilized proteinase K in the proteinase buffer as indicated on the

bottle, by adding the indicated volume of ethanol 96-100% (5.4.2) to buffer B5, and by preparing buffer B3 by transferring buffer B1 to reagent B2 and mixing.

Re-suspend the sample pellet in 200 µl buffer T1. Add 25 µl proteinase K and mix by vortexing. Add 200 µl buffer B3 and mix by vortexing. Incubate at $70.0 \pm 0.2^\circ\text{C}$ in a water bath for 10 min. Add 210 µl 96-100 % ethanol (5.4.2) and mix by vortexing. Place the NucleoSpin Tissue column in a collecting tube and add the sample. Centrifuge the sample at 11 000 x g for 1 min. and discard the flow-through. Repeat this centrifugation step if the sample is not drawn completely through the matrix. Place the NucleoSpin Tissue column in the collecting tube again and add 500 µl buffer BW. Centrifuge the sample at 11 000 x g for 1 min. and discard the flow-through. Place the NucleoSpin Tissue column in the collecting tube again and add 600 µl buffer B5. Centrifuge the sample at 11 000 x g for 1 min. and discard the flow-through. Repeat the centrifugation step and discard additional flow-through. Place the NucleoSpin Tissue column in an Eppendorf tube and add 100 µl 70°C hot buffer BE. Leave the sample on the table for 1 min. Centrifuge the sample at 11 000 x g for 1 min. and discard the NucleoSpin Tissue column. Use 5 µl of the extracted DNA as template in the real-time PCR.

NOTE: Always include a process blank, i.e. a sample consisting only of buffer T1 when performing manual DNA extraction.

7.3 Real-time PCR analysis

7.3.1 Preparation of PCR mix

To avoid variations in the PCR reaction mix between individual tubes a master mix is prepared, i.e. a batch large enough to analyse all samples in the analysis. Always prepare a 10 % excess of master mix to ensure a sufficient amount for all samples.

Per sample:

7.75 µl	PCR grade water	
2.5 µl	10 x PCR Buffer	(5.5.1)
1.0 µl	12.5 mM dUTP	(5.5.4)
2.5 µl	25 mM MgCl ₂	(5.5.3)
2.0 µl	Glycerol	(5.5.6)
1.1 µl	10 pmol/µl Primer OT-1559	(5.5.7)
1.2 µl	10 pmol/µl Primer 18-1	(5.5.7)
0.25 µl	20 mg/ml BSA	(5.5.5)
0.2 µl	5 U/µl <i>Tth</i> DNA polymerase	(5.5.2)
0.25 µl	5 pmol/µl Target probe	(5.5.8)
0.25 µl	6 pmol/µl IAC probe	(5.5.8)
1.0 µl	IAC (~5 x 10 ³ copies)	(5.5.9)
5.0 µl	Template DNA	(7.2.1 / 7.2.2)
25.0 µl		

Dispense the mix in the real-time PCR tubes - 20 µl per tube, and add 5 µl of the extracted DNA (7.2.1 / 7.2.2). Include controls as described below (7.3.2).

7.3.2 Controls

Each real-time PCR analysis should include a positive control, a negative control, a process blank and two non-template controls (NTC).

7.3.2.1 Positive control

A solution containing DNA extracted from a *Campylobacter jejuni*, *C. coli* or *C. lari* strain corresponding to about 1.000-2.000 CFU/ml may be used as positive control.

7.3.2.2 Negative control

A solution containing DNA extracted from a non-Campylobacter, Gram-negative strain corresponding to about 1.000-2.000 CFU/ml may be used as negative control.

7.3.2.3 Process blank

A process blank consisting of 20 µl PCR mix added 5 µl lysis buffer/buffer T1 processed throughout the DNA extraction should be included.

7.3.2.4 Non-template controls

Two non-template controls consisting of 20 µl PCR mix added 5 µl PCR grade water should be included.

7.3.3 Real-time PCR run

The thermal cycler should be in compliance with the requirements for installation, performance and maintenance described in ISO/TS 20836.

Data should be collected in the annealing step from the probe fluorophores FAM (emission 520 nm) and JOE (emission 550 nm) in the real-time PCR thermal cycler.

Thermal profile:

Initial denaturation: 95°C for 3 min.

40 cycles: 95°C for 15 sec., 58°C for 1 min., 72°C for 30 sec.

After running the real-time PCR thresholds for FAM and JOE should be assigned manually above background noise and known negative samples.

8. REPORTING OF RESULTS

8.1 Positive samples

A sample is considered positive if a C_t value for FAM below 40 is obtained, and the controls are as expected. Due to competition of amplification between the *Campylobacter* target and the internal amplification control, absence of signal from the internal amplification control can occur in strong positive samples.

8.2 Negative samples

A sample is considered negative if a C_t value for FAM above 40 (no amplification has occurred) **AND** a C_t value for JOE below 40 is obtained. If the internal amplification control is not amplified in negative samples, the result may be false negative and the analysis should be repeated.

9. SAFETY PRECAUTIONS

Measures to be introduced in order to prevent contamination

Carry out the following procedures in separate rooms:

- DNA extraction
- Preparation of PCR mix
- Sample loading

It is crucial to keep working surfaces, test tube racks, pipettes, glassware, plastic ware etc. clean and free from bacteria and DNA. This can be achieved by using UV light and/or by chemical treatment (0.5 % sodium hypochlorite solution). Specially designed test tube racks and filter pipette tips may be used to prevent contamination.

The PCR mix includes addition of dUTP, enabling uracil-N-glycosylase treatment in case of carry-over contamination.

10. REFEREE OF THE METHOD

This NMKL method has been elaborated by Mathilde Josefsen and Jeffrey Hoorfar, Danish Institute of Food and Veterinary research. Bülowsvej 27, DK-1790 Copenhagen V, Denmark.

Annex 1. Preparation of plates/racks for KingFisher/ BeadRetriever

DNA extraction on Bead Retriever

RACK	Tube 1	2	3	4	5
Strip 1	300 µl Sample in lysis buffer (5.3.1) +75 µl beads (5.3.2)	500 µl Salt wash (5.3.3)	500 µl Al-cohol wash (5.3.4)	500 µl Al-cohol wash (5.3.4)	100 µl Nuclease-free water (5.3.7)
2	300 µl Sample in lysis buffer (5.3.1) +75 µl beads (5.3.2)	500 µl Salt wash (5.3.3)	500 µl Al-cohol wash (5.3.4)	500 µl Al-cohol wash (5.3.4)	100 µl Nuclease-free water (5.3.7)
3	300 µl Sample in lysis buffer (5.3.1) +75 µl beads (5.3.2)	500 µl Salt wash (5.3.3)	500 µl Al-cohol wash (5.3.4)	500 µl Al-cohol wash (5.3.4)	100 µl Nuclease-free water (5.3.7)
4	300 µl Sample in lysis buffer (5.3.1) +75 µl beads (5.3.2)	500 µl Salt wash (5.3.3)	500 µl Al-cohol wash (5.3.4)	500 µl Al-cohol wash (5.3.4)	100 µl Nuclease-free water (5.3.7)
5	300 µl Sample in lysis buffer (5.3.1) +75 µl beads (5.3.2)	500 µl Salt wash (5.3.3)	500 µl Al-cohol wash (5.3.4)	500 µl Al-cohol wash (5.3.4)	100 µl Nuclease-free water (5.3.7)

DNA extraction on KingFisher						
Plate	1	2	3	4	5	etc.
A	75 µl sample 1 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 2 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 3 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 4 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 5 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	
B	75 µl sample 1 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 2 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 3 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 4 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 5 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	
C	75 µl sample 1 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 2 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 3 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 4 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 5 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	
D	75 µl sample 1 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 2 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 3 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 4 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	75 µl sample 5 in lysis buffer (5.3.1) 25 µl beads (5.3.2)	
E	100 µl salt wash (5.3.3)	100 µl salt wash (5.3.3)	100 µl salt wash (5.3.3)	100 µl salt wash (5.3.3)	100 µl salt wash (5.3.3)	
F	100 µl alcoh. wash (5.3.4)	100 µl alcoh. wash (5.3.4)	100 µl alcoh. wash (5.3.4)	100 µl alcoh. wash (5.3.4)	100 µl alcoh. wash (5.3.4)	
G	100 µl alcoh. wash (5.3.4)	100 µl alcoh. wash (5.3.4)	100 µl alcoh. wash (5.3.4)	100 µl alcoh. wash (5.3.4)	100 µl alcoh. wash (5.3.4)	
H	100 µl nucle- ase- free water (5.3.7)	100 µl nucle- ase- free water (5.3.7)	100 µl nucle- ase- free water (5.3.7)	100 µl nucle- ase- free water (5.3.7)	100 µl nucle- ase- free water (5.3.7)	

One sample is divided into 4 wells (A1, B1, C1 and D1).

Annex 2. Programme for automated DNA extraction on KingFisher

Protocol DEMO_gDNA on KingFisher

[PROTOCOL PROPERTIES]

Name = DEMO_gDNA

Instrument type = KingFisher

Creator = ThermoLabsystems

Description = KingFisher protocol for Isolation of genomic DNA from blood, cells and tissue

Kit = Magnesil® KF, Genomic System, MD1460, Promega

Plate layouts = Genomic DNA purification

[PLATE LAYOUTS]**Genomic DNA purification**

Plate type = KingFisher plate 100 µl

Plate change message = Press start

A:

- volume = 10, name = Blood
- volume = 75, name = Lysis buffer
- volume = 16, name = magnetic particles

B:

- volume = 10, name = Blood
- volume = 75, name = Lysis buffer

C:

- volume = 10, name = Blood
- volume = 75, name = Lysis buffer

D:

- volume = 10, name = Blood
- volume = 75, name = Lysis buffer

E:

- volume = 100, name = Washing buffer

F:

- volume = 100, name = 70% EtOH

G:

- volume = 100, name = 70% EtOH

H:

- volume = 40, name = Distilled water

[STEPS]**BIND**

Step parameters

Name = Incubating

Well = A, Genomic DNA purification

Beginning of step:

Release = Yes, time = 0s, speed = Fast

Bind parameters:

Bind time = 3min 0s, speed = Bottom very fast

End of step:

Collect beads = Yes, count = 3

BIND

Step parameters

Name = Incubating

Well = B, Genomic DNA purification

Beginning of step:

Release = Yes, time = 0s, speed = Fast

Bind parameters:

Bind time = 3min 0s, speed = Bottom very fast

End of step:

Collect beads = Yes, count = 3

BIND

Step parameters

Name = Incubating

Well = C, Genomic DNA purification

Beginning of step:

Release = Yes, time = 0s, speed = Fast

Bind parameters:

Bind time = 3min 0s, speed = Bottom very fast

End of step:

Collect beads = Yes, count = 3

BIND

Step parameters

Name = Incubating

Well = D, Genomic DNA purification

Beginning of step:

Release = Yes, time = 0s, speed = Fast

Bind parameters:

Bind time = 3min 0s, speed = Bottom very fast

End of step:

Collect beads = Yes, count = 3

WASH

Step parameters

Name = Washing
 Well = E, Genomic DNA purification
Beginning of step:
 Release = Yes, time = 0s, speed = Fast
Wash parameters:
 Wash time = 15s, speed = Bottom fast
End of step:
 Collect beads = Yes, count = 4

WASH

Step parameters
 Name = Washing
 Well = F, Genomic DNA purification
Beginning of step:
 Release = Yes, time = 0s, speed = Fast
Wash parameters:
 Wash time = 15s, speed = Bottom fast
End of step:
 Collect beads = Yes, count = 4

WASH

Step parameters
 Name = Washing
 Well = G, Genomic DNA purification
Beginning of step:
 Release = Yes, time = 0s, speed = Fast
Wash parameters:
 Wash time = 15s, speed = Bottom fast
End of step:
 Collect beads = Yes, count = 4

DRY

Step parameters
 Name = EtOH evaporation
 Well = G, Genomic DNA purification
 Dry time = 600s
 Tip position = Outside well

ELUTION

Step parameters
 Name = Eluting
 Well = H, Genomic DNA purification
Beginning of step:
 Release = Yes, time = 0s, speed = Fast
Elution parameters:

Elution time = 600s, speed = Bottom fast

Pause parameters:

Pause for manual handling = No

Remove beads:

Remove beads = Yes, collect count = 3, disposal well = E

Annex 3. Programme for automated DNA extraction on BeadRetriever

Protocol DEMO_gDNA_mL on BeadRetriever

[PROTOCOL PROPERTIES]

Name = DEMO_gDNA_mL

Instrument type = KingFisher mL

Creator = ThermoLabsystems

Description = KingFisher mL protocol for isolation of genomic DNA from blood, cells and tissue

Kit = Magnesil® KF, Genomic System, MD1460, Promega

Plate layouts = Genomic DNA

[PLATE LAYOUTS]**Genomic DNA**

Plate type = BeadRetriever tubes 1000 µl

Plate change message = Press start

A:

- volume = 100, name = Blood

- volume = 750, name = Lysis buffer

- volume = 40, name = Magnetic particles

B:

- volume = 1000, name = Washing buffer

C:

- volume = 1000, name = 70% EtOH

D:

- volume = 1000, name = 70% EtOH

E:

- volume = 100, name = High quality water

[STEPS]**BIND****Step parameters**

Name = Incubating

Well = A, Genomic DNA

Beginning of step:

Premix = Yes

Bind parameters:

Bind time = 600s, speed = Fast dual mix

End of step:

Collect beads = Yes, count = 3

WASH

Step parameters

Name = Washing

Well = B, Genomic DNA

Beginning of step:

Release = Yes, time = 5s, speed = Fast

Wash parameters:

Wash time = 30s, speed = Fast dual mix

End of step:

Collect beads = Yes, count = 2

WASH

Step parameters

Name = Washing

Well = C, Genomic DNA

Beginning of step:

Release = Yes, time = 5s, speed = Fast

Wash parameters:

Wash time = 30s, speed = Fast dual mix

End of step:

Collect beads = Yes, count = 2

WASH

Step parameters

Name = Washing

Well = D, Genomic DNA

Beginning of step:

Release = Yes, time = 5s, speed = Fast

Wash parameters:

Wash time = 30s, speed = Fast dual mix

End of step:

Collect beads = Yes, count = 2

DRY

Step parameters

Name = EtOH evaporation

Well = D, Genomic DNA

Dry time = 600s

Tip position = Outside well

ELUTION

Step parameters

Name = Eluting

Well = E, Genomic DNA

Beginning of step:

Release = Yes, time = 0s, speed = Fast

Elution parameters:

Elution time = 600s, speed = Medium

Pause parameters:

Pause for manual handling = No

Remove beads:

Remove beads = Yes, collect count = 2, disposal well = B

The proposal has been presented to several Nordic contact persons under NMKL, and is currently being discussed in the Nordic National Committees. Hopefully the proposal will be accepted as a standard method.

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