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Feasibility Testing for Sample Storage and Transport for Nucleic Acid Assays

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This study was conducted for Mobidiag Oy as part of their part of a project developing new technologies for potential products. The aim of was to develop stool sample tube for detection of pathogens by PCR method. Purpose was to develop a sample tube that inactivates the pathogens from the stool sample and stabilizes samples nucleic acids.

PCR is slowly becoming a norm, as concerns detecting pathogenic organisms. PCR is less time consuming and less expensive than traditional culture-based methods. It is highly precise method and it is becoming increasingly used for analysis detecting pathogens from stool and other biological specimen in clinical laboratories.

Feasibility testing of the sample tube was made by series of different methods to get data of the possible composition of the sample tube and its capabilities needed for the sample tube. Data gathered for sample tube in this study were: NA stability, nucleic acid binding, microbial inactivation, detergent optimization and inhibition testing.

Results gave some good candidates for composition of the sample tube and the compositions microbial inactivation attributes. NA stability testing is still ongoing to gather reliable data for the NA stability. Compositions also were found to inactivate pathogens, which is important attribute for sample tube. Gathered results of this study will be utilized for further developing the sample tube in the future.

Keywords	PCR, stool sample, feasibility testing

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Opinnäytetyö suoritettiin osana Mobidiag Oy:n projektia uusien tuotteiden kehittämiseen. Opinnäytetyön tavoitteena oli tutkia ja kehittää ulostenäyteputkea, jota voidaan käyttää patogeenien detektointiin PCR-metodilla. Tarkoituksena oli saada aikaiseksi näyteputki, joka inaktivoi patogeeneja ulostenäytteestä ja stabiloi näytteen nukleiinihapot.

PCR-metodista on tullut normi patogeenisten organismien detektoinnissa. PCR-metodi on vähemmän aikaa kuluttavaa ja halvempaa kuin perinteiset viljelmätestit. Se on myös hyvin tarkka metodi, joka on tullut kasvavasti merkittäväksi metodiksi patogeenien detektointiin ulostenäytteistä ja muista biologisista näytteistä kliinisissä laboratorioissa.

Näyteputken soveltuvuustestaus tehtiin suorittamalla erilaisia analyyseja, jotta saataisiin dataa näyteputken mahdollisesta koostumuksesta ja sen tarvittavista ominaisuuksista. Dataa näyteputkea varten kerättiin nukleeiinihappojen stabiilisuudesta, mikrobiologisesta inaktivaatiosta, detergenttien ominaisuuksista ja inhibition testauksesta.

Tulokset antoivat osviittaa näyteputken mahdollisesta koostumuksesta ja koostumuksen mikrobiologisesta inaktivaatiokyvystä. NA-stabiilisuustestaus on vielä kesken, joten tuloksia koostumuksen stabiilisuusominaisuuksista ei voida pitää luotettavina, mutta suuntaa antavina. Näyteputken kompositiot inaktivoivat myös patogeeneja, joka on hyvin tärkeä ominaisuus komposition kannalta. Opinnäytetyössä saatuja tuloksia voidaan käyttää hyväksi näyteputken kehittämisessä tulevaisuudessa.

Keywords	PCR, ulostenäyte, soveltuvuustestaus

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Appendix 2. Instruction manual, Quick-DNA Fecal/Soil Microbe Miniprep Kit

Appendix 3. Amplidiag Bacterial GE PCR Quick Guide

Abbreviations

CFU	Colony-forming unit. Unit used to present the number of colonies of bacteria or fungi in sample.
CLED agar	Cystine lactose electrolyte deficient agar. CLED agar is used to isolate urinary organisms. Lactose-positive bacteria form yellow colonies on this agar.
Cq	Quantification cycle. Cq value is the cycle were amplification of the target curve is maximal.
EHEC	Enterohamerrhagic Escherichia coli. One of the bacteria that causes food poisoning.
MRSA	Methicillin-resistant Straphylococcus aureus. Strand of S. aureus bacteria that is resistant to common antibiotics. MRSA infects people with weakened immune system.
NA	Nucleic acid. Biomolecules that consist of different RNA and DNA.
PBS	Phosphate buffered saline. PBS is water based salt solution that is used for variety of biological analysis.
RT	Room temperature.
TE buffer	Buffer that contains EDTA and Tris (tris(hydroxymethyl)aminomethane). TE buffer is commonly used in molecular biology laboratories as it solubilizes DNA and RNA.

1 Introduction

Infectious diarrhea is a major factor of morbidity worldwide, especially in developing world. Infectious diarrhea is especially dangerous for infants. There are estimated 2 to 4 billion cases of infectious diarrhea around the world annually. These cases are caused by different bacteria, viruses and parasites for example pathogenic strands of *Escherichia coli, Clostridium difficile, Salmonella spp, Entamoeba histolytica*, Norovirus and Rotavirus. There has been improvements of hygiene and public health, as well as the treatment and the detection of these pathogens, but the risk of contracting infectious diarrhea remains very high [1].

PCR is slowly becoming norm as concerns detecting these pathogenic organisms. PCR is less time consuming and less expensive than traditional culture-based methods. PCR is highly precise method and it is becoming increasingly used analysis detecting pathogens from stool and other biological specimen in clinical laboratories [2].

This study was conducted for Mobidiag Oy as part of their part of a project developing new technologies for potential products. The laboratory work was carried out mainly in Mobidiag's R&D laboratory. The aim of was to develop stool sample tube for detection of pathogens by PCR method. Purpose was to develop a sample tube that inactivates the pathogens from the stool sample and stabilizes samples DNA and RNA. Sample tube also needs to be compatible with Mobidiags Novodiag product.

2 Different Biological Specimens

There is a variety of different kind of biological specimens that can be analysed from patients. The sample of interest must be extracted, stored and transported correctly so that sample can be analysed without faults. Processing of the sample, goal of the analysis, storing of the sample and the compatibility with further analyses must be taken into the consideration when choosing the sample type. Some of the most commonly analyzed specimens are: blood (whole blood or different fractions of blood), feces, saliva and buccal cells, tissue, urine and hair samples [3, 27].

Specimens are extracted from patient with a specific protocol and then either stored or analyzed. Depending on the sample, method and how the sample is stored, the sample can be stored in different temperatures for different amount of time. For example, blood samples do not need rapid processing after collection. However, with other biological specimen the time between collection of the sample and the analysis of the sample should be minimized.

Depending on the specimen or the further analysis of the sample, specimens can be stored in different conditions. Usually, to ensure the DNA stability, specimen should be stored from -0.5 °C to -196 °C [3, 32-33].

3 Requirements of Sample Tube

Sample tube developed in this study is supposed to be swab tube for fecal samples. Tube contains 2 ml of liquid composition that must inactivate pathogenic organisms and ensure the stability of NA. Pathogenic inactivation is important to make sure that the sample is safe to handle, transport and process. Sample tube also must stabilize DNA and RNA for further analysis and storage of samples. The sample tube needs to contain components that do not inhibit the further PCR method or destroy the DNA of the sample. This particular sample tube should be also compatible with molecular systems. Some of the specific concentrations of components and compositions cannot be specified in this study. Compositions consists of guanidinium thiocyanate, detergents and number of other components to ensure the sample preservation and further analysis.

3.1 Inactivation of Pathogens

Many clinical laboratory personnel handle potentially pathogenic samples daily for example blood and stool samples. These samples may contain pathogenic bacteria, viruses and parasites. This poses potential health risk to person handling the sample. Therefore, inactivation of the possible pathogens is important. Bacterial pathogens can be inactivated by using disinfect reagents in sample storing (for example guanidium thiocyanate), using unfavourable pH or using harmful reagent for pathogens. If sample is

collected correctly and sample tube is handled in the appropriate manner, the sample should not pose health risk to personnel after the collection of the sample [4].

3.2 DNA Stability

Stability of DNA is dependent of hydrogen bonds between DNA strands and base stacking interactions. Water molecules surrounding the DNA effects the balance and stability of the DNA. This makes the buffer where DNA is stored extremely important so that hydrogen bonds hold. Also, very important factor of DNA stability is temperature. In too high temperatures DNA strands can denaturate, separating from each other. Also spontaneous hydrolysis can damage DNA and high temperature can speed up the hydrolysis. Hydrolysis can be blocked by chelation of divalent cations, like Mg^{2+}, for example with EDTA [5; 26].

4 Preanalytics and Challenges of Stool Sample Tube

4.1 Sampling and Storing of Stool Sample

Sampling of the stool sample can vary depending on the further analysis, analysable bacteria, virus or parasite. Before some of the sampling, patient must follow certain diet, avoid specific medicine or alcohol. Patient is given equipment and sampling instructions to follow and collect the sample according to the instructions. It is important that patient does not contaminate the sample or the outside of the sample tube [6].

There are different kind of sample tubes and methods of collecting the sample, usually stool samples are taken with swab or scooping some of the stool to the given sterile tube, as shown in figure 1. Swabs are taken by swabbing the cotton swab in the stool and transferring the swab to either liquid or gel sample tube. After collecting the sample, patient must label the sample with name, social security number, time and date of the sampling. Sample should be delivered to the laboratory as soon as possible. If sample tube can't be delivered immediately, sample must be stored in +4 °C. Some of the samples must be taken to laboratory within same day. Appendix 1 has HUSLAB laboratory's instructions for taking sample for parasite detection for nucleic acid assays.

Figure 1. Example of swab sample tube for nucleic acid assays. Sample is collected with swab. (Photo copied from from http://products.copangroup.com/index.php/products/genetics/enat [28])

When storing stool sample, the cold chain must be uninterrupted and the time between collecting the sample and analysis must be cut to the minimum. Stool samples can be stored in +4 °C for short term and for longer storing time -20 °C freezer is required. DNA stability suffers with time, if the sample is not stored correctly.

4.2 Sample Preparation

Stool sample is challenging sample for molecular diagnostic, because it is not homogenous, has lot of solid matter and the sample is complex and rich in macromolecules. Stool sample can be prepared before analysing to minimize these problems. Some of the commonly used methods are to filter, centrifuge, dilute or homogenise the sample before moving to analysing the sample. These methods are usually time consuming, so user benefits not doing these steps [7].

Stool matter is usually stored in –20 °C for longer periods of time. To prepare the stool sample analysis; frozen fecal matter was weight and diluted into PBS solution (15 %

stool in PBS). Stool suspension was then vortexed continuously for 10 minutes and before pipetting the suspension was left to set to prevent the solid matter getting to samples or clogging the pipette. Also, good method of avoiding the effects of inhibitors is to dilute the sample before the PCR. Samples were diluted in 1:10 ratio and analysed in parallel to the undiluted samples to get idea of inhibition in the samples.

4.3 PCR Inhibition

PCR is very precise method to use, but PCR inhibitors have always been problem for the analysis. Most PCR inhibitors interfere with DNA polymerase and inhibit the PCR reaction that way or inhibitors can directly bind to the DNA obstructing the copying of the DNA. This affects the amplification of DNA sequence, which leads to weaker signal or false negative results. Most problematic issue concerning the PCR inhibitors is the presence of inhibitors in the sample. PCR inhibitors can also come from processing containers or contaminated reagents. Most common samples that contain known inhibitors are blood, soil, stool and fabrics. Also, the materials and reagents that come to contact with the sample include inhibitors, such as many salts, ionic detergents, ethanol, isopropanol and phenol. These reagents are often used during sample processing [8; 9].

To prevent the PCR inhibitors, the sample should be processed with as little inhibitors as possible. This is problem if the sample itself contains a lot of inhibitors. In case of samples that are on other material, sample can be collected with swab-transfer method, which eliminates some of the inhibitors [9].

Stool is one of the most complex samples when PCR is used, because feces contain lot of bacteria and inhibitors that come from food or other biological material [7]. Handling of stool sample is also problematic, since stool sample is not homogeneous and solid matter in feces can interfere with used systems. Stool samples contain hard to remove inhibitors such as: polysaccharides, fecal fats, haemoglobin, bile salts and uric acids [10]. These PCR inhibitors might weaken the PCR results or give wrong negative results.

5 DNA extraction

DNA extraction is commonly used method in laboratories. DNA was first extracted by Friedrich Miescher in 1869 from leucocyte cells. Miescher intended to study proteins of the cell, when he discovered DNA, which he first called nuclei. After the discovery he shifted his studies to DNA. He developed method to first separating cells' nuclei and isolation the DNA [11].

There is number of ways to extract nucleic acids (NA) from samples. The most used methods are phenol-chloroform method, salting out, ion exchange and silica binding methods. Nowadays many extractions of NA in laboratories are made by commercial kits or automated equipment, which usually are based on silica binding method. When choosing the method of nucleic acid extraction, one must consider the chemistry and materials used in particular method, sample, cost of the method, contamination risks and the further analysis of DNA [12].

Typically, the first step of DNA extraction is creating lysate, breaking cellular structure and releasing DNA from cells. DNA is then separated from cellular debris and other unwanted material by centrifuging the sample. After that, target DNA is purified from unwanted nucleic acids and proteins for further analysis of sample [13].

In this study purification of DNA was made by silica DNA binding method. This method was used, because it was automated in used laboratory (easyMAG equipment). Silica binding method is based on the silica's nucleic acid binding attribute when there is high concentration of chaotropic salts present, such as guanidium thiocyanate. These salts facilitate binding the nucleic acids to silica particles, nucleic acids are then washed, and DNA can then be eluted with for example water or TE buffer. Absence of chaotropic reagent releases the nucleic acid from silica [13].

5.1 DNA extraction with Zymo Quick-DNA Fecal/Soil Microbe Miniprep Kit

In this study DNA was first extracted with Zymo Research Corporation's Quick-DNA Fecal/Soil Microbe Miniprep Kit, modifying the kits instructions by replacing the kits washing buffers with 70 % ethanol and DNA elution buffer with TE pH 8.0. This was made to ensure

the compositions abilities to bind the NA to column and release of the NA to elution buffer in known circumstances. In this extraction kit DNA is extracted by using spin columns with silica membrane. Appendix 2 has Quick-DNA Fecal/Soil Microbe Miniprep kit's protocol.

5.2 DNA extraction with NucliSENS easyMAG instrument

DNA and RNA from the samples were also extracted with NucliSENS easyMAG instrument, which extraction method is based on silica particles nucleic acid binding properties when guanidinium thiocyanate or other chaotropic reagent is present, as illustrated in figure 2. Silica is separated with magnets, NA is washed and eluted to a desired volume. Most of the extractions made in this study was made with EasyMAG instrument. In one extraction 24 samples can be extracted and one run takes about 1 hour [14].

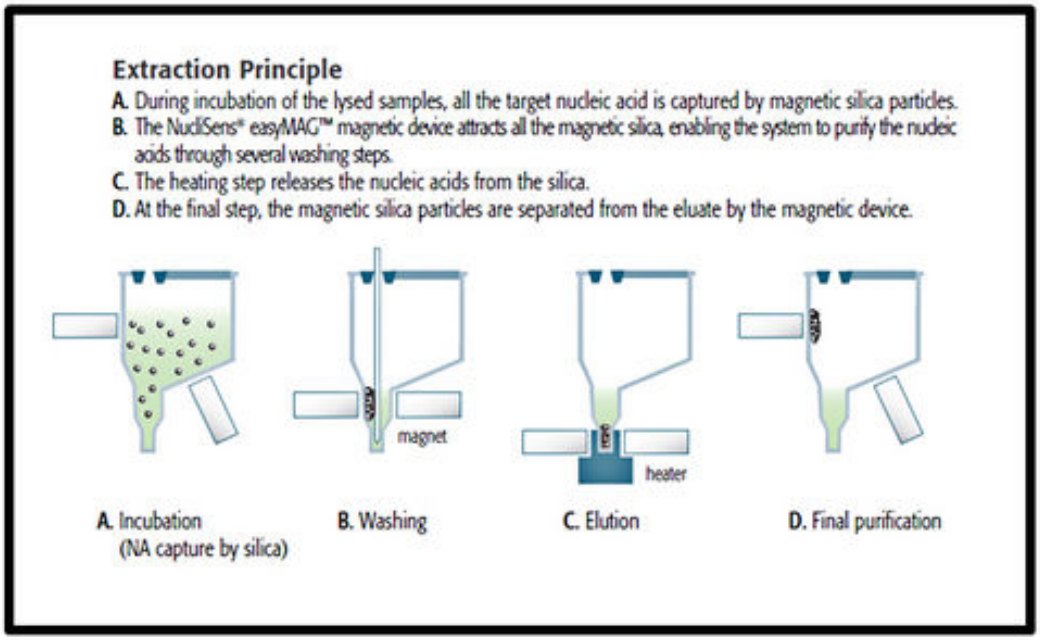

Figure 2. Extraction princicple of EasyMAG. [14]

6 Materials and methods

First background research was made to determine some of compositions that could be suitable for sample tube. Figure 3 illustrates used methods and flow of the analysis.

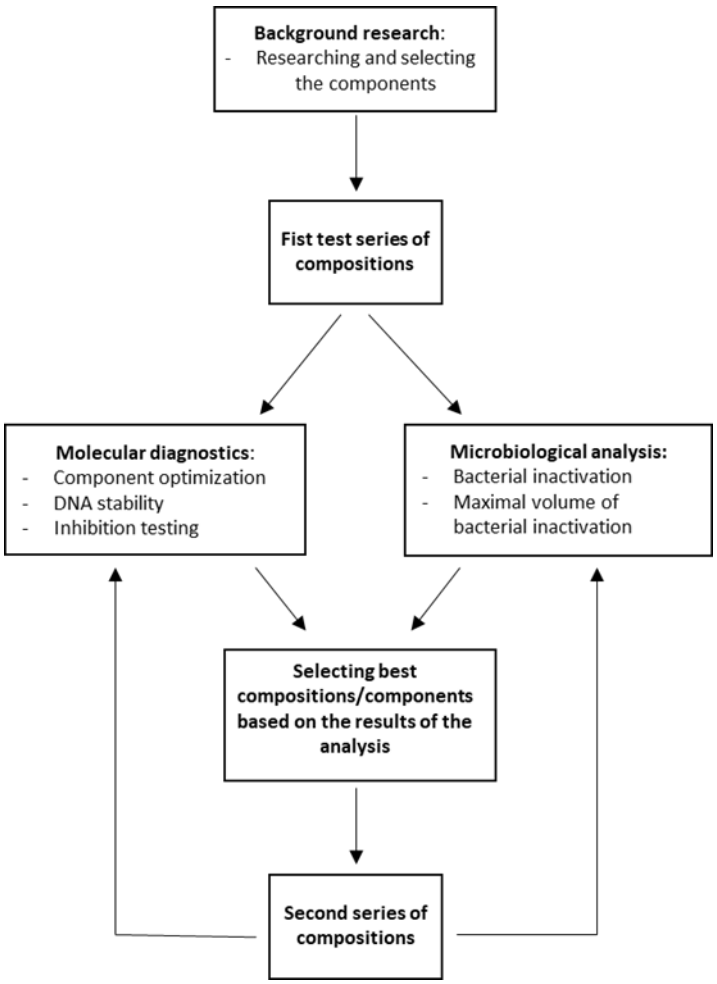

Figure 3. Flow chart of the analysis of sample tube compositions.

Range of concentrations were selected for each component and different kind of compositions were made with MODDE Pro –software. MODDE Pro –software is Sartorius Stedim Biotechs software used to create experimental setups [15]. Software gave total of 24 different compositions, which were taken to analysis. These 24 compositions had some parallel compositions to verify the results. The first series of compositions is called N-series (shown in table 1.).

Table 1. Compositions of N-series. 0 means no component/detergent was added to the composition. Concentrations stated are relative. Component 1 and component 6 was added in every composition, just with different concentrations.

	Com-ponent 1	Com-ponent 2	Com-ponent 3	Com-ponent 4	Com-ponent 5	Com-ponent 6	Com-ponent 7	Deter-gent 1	Deter-gent 2	Deter-gent 3
N1	1	0	0	0	0	2	0	5	0	0
N2	1	1	0	1	0	1	1	0	1	0
N3	2	0	0	0	1	1	1	5	5	0
N4	2	0	1	1	0	1	1	1	0	0
N5	2	0	1	0	1	2	0	0	1	0
N6	1	1	1	1	1	2	0	1	5	0
N7	2	1	1	1	1	1	0	5	0	1
N8	2	0	0	1	1	2	1	1	1	1
N9	1	0	0	0	0	1	0	0	5	1
N10	2	1	1	0	0	2	1	0	0	1
N11	1	1	1	0	1	1	1	5	1	1
N12	2	1	0	0	0	1	0	1	5	1
N13	1	1	0	0	1	2	1	1	0	5
N14	1	0	1	0	0	1	0	1	1	5
N15	1	0	1	1	0	2	1	0	5	5
N16	1	0	0	1	1	1	0	0	0	5
N17	2	1	0	1	0	2	0	5	1	5
N18	1	0	0	0	0	1	0	0	5	1
N19	1	1	0	0	1	2	1	1	0	5
N20	2	1	1	1	1	1	0	5	0	1
N21	2	1	1	0	1	1	0	1	0	0
N22	1	1	0	2	1	2	0	5	1	0
N23	1	0	0	2	0	1	0	1	5	0
N24	2	0	1	2	0	2	0	5	0	0

These 24 compositions where tested with different molecular- and microbiological analysis. The second series of compositions is called S-series. S-series was made the same way as N-series with MODDE-software, some of the components were eliminated from compositions based on results of N-series analysis and some concentrations of components were fixed. Compositions of S-series are illustrated in table 2.

Table 2. Compositions of S-series. 0 means no component/detergent was added to the composition. Concentrations stated are relative. Components 1, 5 and 6 were fixed according to the analysis done of N-series compositions. Component 7 was determined not to be important for composition. Component 3 and detergent 3 was also taken away from compositions.

	Component 1	Component 2	Component 4	Component 5	Component 6	Detergent 1	Detergent 2
S1	2	1	4	1	1	0	0
S2	2	3,3	2,6	1	1	0	0
S3	2	2,5	0	1	1	0	0
S4	2	1	1,3	1	1	1	0
S5	2	1,7	0	1	1	1	0
S6	2	3,3	0	1	1	10	0
S7	2	3,3	4	1	1	10	0
S8	2	1	2,6	1	1	10	0
S9	2	1	2,6	1	1	0	1
S10	2	3,3	1,3	1	1	0	1
S11	2	2,5	4	1	1	1	1
S12	2	1	0	1	1	10	1
S13	2	1,7	4	1	1	10	1
S14	2	1	0	1	1	0	10
S15	2	3,3	4	1	1	0	10
S16	2	2,1	2	1	1	0	10
S17	2	3,3	0	1	1	1	10
S18	2	1	4	1	1	1	10
S19	2	1	0	1	1	10	10
S20	2	1	4	1	1	10	10
S21	2	3,3	4	1	1	10	10
S22	2	2,5	0	1	1	10	10
S23	2	2,1	2	1	1	1	1
S24	2	2,1	2	1	1	1	1

6.1 Nucleic Acid Binding

NA binding test was first started by preparing two sets of N-series samples, one with stool background (15 % stool in PBS) and one without stool background. In this analysis compositions ability to bind the NA was tested. Targets for PCR amplification added were invE gBlock plasmids and process control (PRC) for Mobidiags own extraction equipment. Table 3 illustrates the preparing of samples. 1:10 of dilution was prepared of the

samples and analysed parallel to undiluted samples to examine, if PCR inhibitors are left to the sample after NA extraction.

Table 3. Prepared samples of N-series compositions.

	Sample material	**Amout added to 1 ml of composition**
Set 1	Stool suspension	175 µl
	invE gBlock 10^7	5 µl
	PRC	20 µl
Set 2	PBS	175 µl
	invE gBlock 10^7	5 µl
	PRC	20 µl

PCR was prepared from samples with Mobidiags bacterial gastro enteritis detection kit. Appendix 3 is Mobidiags bacterial gastro enteritis detection kit PCR quick guide. This detection kit is multiplex real-time PCR kit for screening of pathogenic bacteria from stool samples [27]. Bio-Rads CFX96 Real-Time System, C1000 Thermal Cycler (figure 4) was used to analyse the PCR plate.

Figure 4. Picture of Bio-Rad CFX96 C1000 thermal cycler.

Protocol used for the pathogen detection with thermal cycler is illustrated in table 4. Results of the thermal cycler were analysed with Bio-Rad CFX manager –software. Cq results and yield were analysed from samples.

Table 4. Protocol used for the thermal cycler.

Temperature	Time	Amount of cycles	
95 °C	10 min	1x	
95 °C	15 s	45x	Fluorescence readout at the end of each 60 °C cycle
60 °C	1 min		

6.2 Salmonella Lysis Test

Salmonella lysis test was made to test the compositions ability to release the NA from cells to the composition. Samples were prepared by adding 200 µl of $5x10^8$ CFU/ml *Salmonella bongori* suspension and 20 µl PRC to 1 ml of each of the test composition. Controls of eNAT (COPAN diagnostics) and PBS were prepared the same way. Suspension was incubated in the compositions and controls for 30 min in room temperature and then centrifuged in 13 000 rpm for 1 minute to pellet intact bacterial cells. 200 µl of the supernatant was taken to easyMAG extraction. After extraction PCR was prepared from the samples and 1:10 dilution was analysed parallel to the undiluted samples.

6.3 Nucleic Acid Stability

NA stability was tested with S-series compositions. First, 175 µl stool-PBS solution and 16 µl of process control (PRC) were added to 1 ml of composition and controls of PBS, eNAT and DNA/RNA shield. For targets, 9 µl of different kind of nucleic acids and plasmids were added to the samples (approximately 1 million copies of target gene per sample), to have various selection of targets. These targets were: plasmids ipaH and stx 2 and invA -gene (extracted *Salmonella bongori* DNA). Samples were put to different temperatures and extractions was made in different time points to examine the PCR signal to determine in which time point and temperature compositions fail in terms of NA stability. Table 5 shows time points extractions were made and temperatures where samples were stored. Samples and controls were extracted with easyMAG equipment. First extraction was made 30 minutes of incubation in room temperature. PCR were prepared

from the samples in each timepoint and temperature using Mobidiags bacterial pathogen detection kit and PCR plates were then analysed with Bio-Rad CFX96 Real-Time System, Thermal Cycler.

Table 5. Temperatures where samples were stored, and time points were extraction of the samples were taken.

	Temperatures			
Time points	**-20 °C**	**+4 °C**	**RT**	**+37 °C**
30 min			x	
3 days		x	x	x
2 weeks		x	x	x
4 weeks	x	x	x	x

6.4 Microbial Inactivation

In this test it was determined, if different organisms are efficiently inactivated in the different test compositions. This was done by choosing four test organisms so that they would be as different as possible and have different attributes and morphology. The organisms that were chosen based on these conditions were: *Escherichia coli* (Sa1558), *Staphylococcus aureus* (F704), *Rhodococcus equi* (Sa1635) and *Candida albicans* (Fa232). *E. coli* was selected because of it is widely used in laboratories and is very common intestine bacteria. *S. aureus* was selected to represent the common gram-positive bacteria. *Rhodococcus equi* is acid-fast bacteria and *Candida albicans* is representing yeasts.

6.4.1 *Escherichia coli*

Escherichia coli is rod shaped, about 2 µm long, gram-negative bacteria that live in digestive tracts of humans and animals. *E. coli* is commonly used bacteria in laboratories because the genetics of *E. coli* are fairly known, and they are easy to grow and manipulate. Some of *E. coli* types are harmless and are not pathogens. However, some may

cause diarrhea or urinary tract infections. One of the bacteria that is cause of food poisoning is *E. coli* O157:H7, which is part of EHEC –serogroup. Other pathogenic *E. coli* serogroups are ETEC, EAEC, STEAEC, DAEC, AIEC, EPEC and EIEC [16].

In this analysis *E. coli* was incubated in CLED (cysteine lactose electrolyte deficient) agar for 48 h in +37 °C. *E. coli* forms yellow colonies in CLED agar. Bacteria that grow yellow colonies in CLED agar indicate lactose positive and blue colonies indicate lactose negative bacteria [17].

6.4.2 *Straphylococcus aureus*

Straphylococcus aureus is gram-positive cocci shaped bacteria that is about 1 µm in diameter. In blood agar *S. aureus* forms big golden-yellow to white colonies. *S. aureus* is common bacteria found on the skin, nose and pharynx of humans. It is potential pathogen that may cause food poisoning and skin infections like boils and sties. Some *S. aureus* strains can be resistant to multiple antibiotics and are known as MRSA. MRSA can spread in hospitals, where poor hygiene and weak immune system can infect patients. Even though *S. aureus* in some cases cause infections in humans, approximately 30 % of population carry the bacteria in their normal flora [18].

S. aureus was incubated in sheep blood agar for 48 h in +37 °C. Sheep blood agar contains different nutrients and 5 to 10 % sheep blood. This agar detects hemolysis around the colonies. Hemolysis is destruction of red blood cells in the agar and it turns the agar colourless, green or brown around colonies. There are 3 types of hemolysis (alpha-, beta- and gamma hemolysis) that can be detected from the agar, observing the type and the radius of hemolysis around the colonies can help to detect the bacteria in agar. *S. aureus* causes beta-hemolysis in sheep blood agar (figure 5), which lyses the red cell in agar and turns agar around the colonies colourless [19; 20].

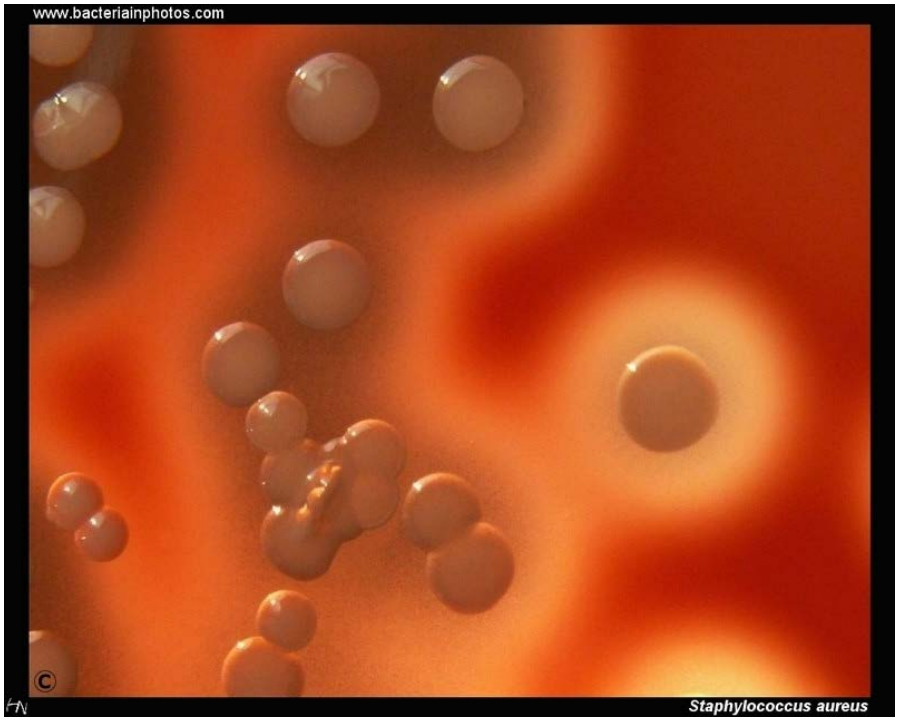

Figure 5. *S. aureus* colonies in sheep blood agar with beta-hemolysis. (Photo copied from http://www.bacteriainphotos.com/Staphylococcus%20aureus.html [29])

6.4.3 *Rhodococcus equi*

Rhodococcus equi is gram-positive, intracellular, coccobacillus bacteria that can be found in soil and water. *R. equi* does not form spores and is nonmotile. It has strong cell wall that has mycolic acids, so it can resist some acid and can also grow in anaerobic conditions. *R. equi* is known to infect horses and particularly young foals, but it is also known to infect humans with weak immune system. *R. equi* infections lead to pneumonia, which causes swelling of tissue in lungs. In case of *R. equi* infections antibiotics are used as treatment [21].

Rhodococcus equi was incubated in chocolate agar for 48 h in +37 °C. Chocolate agar is very rich medium for more fastidious organisms. Chocolate agar is made by heating blood agar until blood cells are lysed, turning the agar brown, which gives the agar its name. When blood cells are boiled they release nutrients such as haemoglobin and hemin into the agar [22].

6.4.4 *Candida albicans*

C. albicans is a yeast that can be found in human intestinal tract, mouth and colon. *Candida albicans* can grow in ether yeast or filamentous cell form depending on the environmental factors for example temperature, pH and CO_2 concentration effect the form. *C. albicans* can also form biofilm from cellulose to protect itself, which makes it resistant to environmental factors. *C. albicans* is usually harmless, but might infect those with low immunoprotection. If *C. albicans* gets overgrown in human body, it causes infections for mouth, throat, genitals or bloodstream. *C. albicans* was cultured to chocolate agar and incubated for 48 h in +37 °C [23].

6.5 Microbial Inactivation Testing

In inactivation testing, selected test organisms were incubated in compositions and growth was inspected from agar plates. In parallel of each composition 1:10 dilution was also prepared and analysed. First dilutions series was made of the bacteria so that suitable number of bacteria could be added to compositions. Right concentration was determined with DensiCHEK densitometer. *E. coli*, *S. aureus*, *R. equi* and *C. albicans* suspensions were adjusted to 0.5 McFarland standard ($1{,}5x10^8$ CFU/ml). In each composition 200 µl of $1{,}5x10^7$ CFU/ml bacteria solution was added in compositions and controls so that each composition had 2,5 million CFU. Compositions were then incubated in room temperature for 30 min. 100 µl of solutions was cultured in suitable agar plate for the organism. Agar plates where incubated in +37 °C for 48 h and colonies were counted.

6.6 Maximal Volume of Inactivation

After inactivation testing compositions maximal volume of microbial inactivation was further tested spiking compositions with bacterial suspension ($1{,}5x10^7$ CFU/ml) in different volumes. Spiked volumes were from 0.25 ml to 3 ml bacterial suspension to either 0.25 or 0.5 ml of test composition. These solutions were incubated in room temperature for 30 minutes and then cultured on agar plate and incubated for 48 h in +37 °C. Colonies were examined and counted from the composition- and control agar plates.

The microbial inactivation abilities of different composition, components were also tested to see performance of components individually. Components 1 and 2 and detergents 1, 2 and 3 were selected to this analysis. These components were tested in different concentrations. Table 6 shows the relative concentrations of components tested. In this analysis suspension was made by mixing $1.5x10^8$ CFU/ml *E. coli* and *S. aureus* suspension and making dilution series of the mix. 200 µl of $1.5x10^7$ CFU/ml suspension was then added to 1 ml of each component. Components were then incubated for 30 min and 100 µl was cultured to agar plates. Organisms were incubated for 48 h in +37 °C.

Table 6. Components and the relative concentrations tested for microbial inactivation.

	Component 1	**Component 2**	**Detergent 1**	**Detergent 2**	**Detergent 3**
Concentration 1	1	1	1	1	1
Concentration 2	2	1.5	2	2	2
Concentration 3	3	2	2.5	2.5	2.5

6.7 Detergent Optimization

The use of detergent 2 will be highly regulated due to environmental concerns, so detergent in composition 3 should be preferably replaced. In this method one new detergent (detergent 4) was taken into analysis, trying to find suitable detergent for composition. First, composition N3 was prepared without any detergents and added different concentrations of detergent 1, detergent 3 and detergent 4. Table 7 shows the prepared compositions and relative concentrations added. 200 µl of $5x10^8$ CFU/ml *Salmonella bongori* suspension was added to 1 ml of composition and left to incubate for 30 minutes. These samples were then extracted with EasyMAG and Zymo DNA kit, and then PCR was prepared from the samples.

Table 7. Relative concentrations of added detergents. Detergents were added to N3 composition without detergents.

	Detergent 1	**Detergent 3**	**Detergent 4**
Concentration 1	0	0	0
Concentration 2	1	1	1
Concentration 3	5	5	5
Concentration 4	10	10	10
Concentration 5	20	20	20

6.8 Inhibition Testing

In this analysis different components and the compositions of those components inhibitory effects were analyzed. Components examined in this analysis were component 1, 2 and 4 as well as detergents 1 and 2. Each component was tested in five different concentration to determine in which concentration component inhibits PCR. Table 8 demonstrates the components prepared. Calibration standard for bacterial pathogen detection was used as a template.

Table 8. Components prepared for inhibition testing. Concentrations are relative.

Component 1	10	5	2.5	1.25	0.625
Component 2	10	5	2.5	1.25	0.625
Detergent 1	10	2.5	0.63	0.16	0.04
Detergent 2	10	2.5	0.63	0.16	0.04
Component 4	10	5	2.5	1.25	0.625

Figure 6 shows the detection panel of bacterial pathogen detection kit; calibration DNA standard 3 includes all the target genes shown on the figure.

Species	Target gene	Assay mix
EHEC	*stx1, stx2*	1
EPEC	*eae*	1
ETEC	*est, elt*	2
Yersinia pseudotuberculosis / pestis	*virF, rumB*	2
Yersinia enterocolitica / pseudotuberculosis / pestis	*virF*	2
Campylobacter jejuni	*rimM*	2
Campylobacter coli	*gyrB*	2
Shigella spp. / EIEC	*ipaH, invE*	3
EAEC	*aggR*	3
Salmonella spp.	*invA*	3

Figure 6. Mobidiags bacterial pathogen detection kit target panel. (Appendix 3)

7 Results

7.1 Results of Nucleic Acid Binding and *Salmonella* Lysis Test

From Cq results of the PCR, number of copies of the target gene were calculated and compared to the undiluted samples to get yield of the samples. Cq results of the samples were also compared to the control eNAT. eNAT is sample tube for clinical samples to be analysed with molecular diagnostic methods [24]. Assembled results of PCR (table 9) helped to narrow the compositions down to four of the best compositions, which were taken to further analysis.

Table 9. Collected results of N-series compositions. Compositions bolded were deemed to perform the best overall from the series and further tested with microbial inactivation.

	Nucleic acid binding			**Salmonella lysis test**		
Sample	**Undiluted samples (invE)**	**Diluted samples 1:10 (invE)**	**Yield %**	**Undiluted samples (invA)**	**Diluted samples 1:10 (invA)**	**Yield %**
N1	-	-	-	13775	15605	88
N2	3050	2498	122	44566	43574	102
N3	**107910**	**125277**	**86**	**48866**	**58376**	**84**
N4	128169	181984	70	34221	72350	47
N5	48672	48592	100	46946	50249	93
N6	**514224**	**532059**	**97**	**50292**	**57727**	**87**
N7	-	-	-	55343	103038	54
N8	228	89	-	30787	21012	147
N9	41672	49440	84	4445	8093	55
N10	152947	175002	87	44961	46963	96
N11	-	-	-	33750	43701	77
N12	252522	255619	99	61064	41860	146
N13	**23360**	**22598**	**103**	**54725**	**56568**	**97**
N14	36573	43728	84	58659	45785	128
N15	60588	60404	100	51910	59014	88
N16	3094	10132	31	49669	75165	66
N17	-	-	-	59507	44063	135
N18	23834	23212	103	53225	55459	96
N19	17011	19636	87	59545	63378	94
N20	-	-	-	54245	49917	109
N21	-	-		28304	22806	124
N22	-	-	-	56254	68469	82
N23	5465	5754	95	56238	52737	107
N24	**129965**	**160344**	**81**	**53388**	**64302**	**83**
eNAT	95441	103170	93	50418	51780	97

Taking into consideration of copies of the target gene, yield of the diluted and the undiluted samples, foaming and solubility of the composition (results not presented here) four of the best compositions were selected from this series. Yield should preferably be close to 100 % in both analysis.

PCR results of the NA binding show that samples with no stool background (figure 7) have very little inhibition and most of the compositions show good results of the amplification of target gene. Some inhibition can be seen in undiluted samples, which is not present in diluted samples, which was expected. Overall most of the compositions performed as well as eNAT control in terms of amplification of target gene.

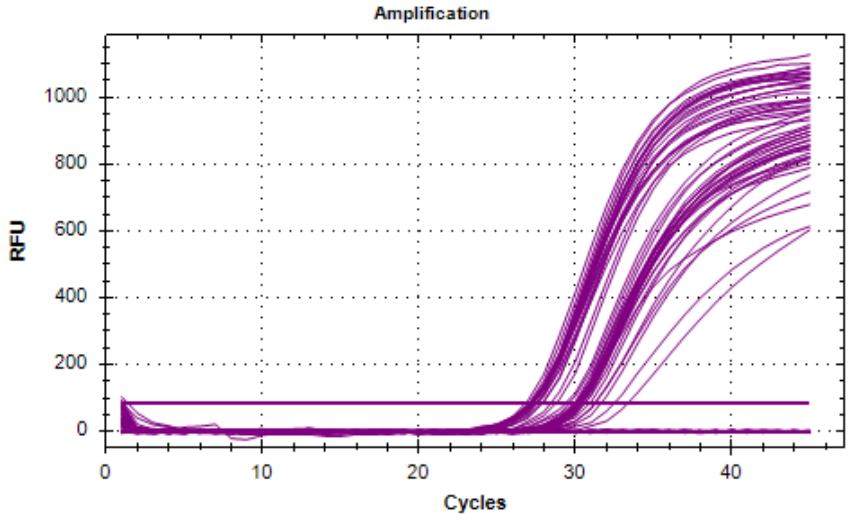

Figure 7. PCR of compositions N1-N24 and 1:10 dilutions with no stool background. Amplification of invE plasmid.

Four of the best components determined had no noticeable inhibition in the PCR (figure 8).

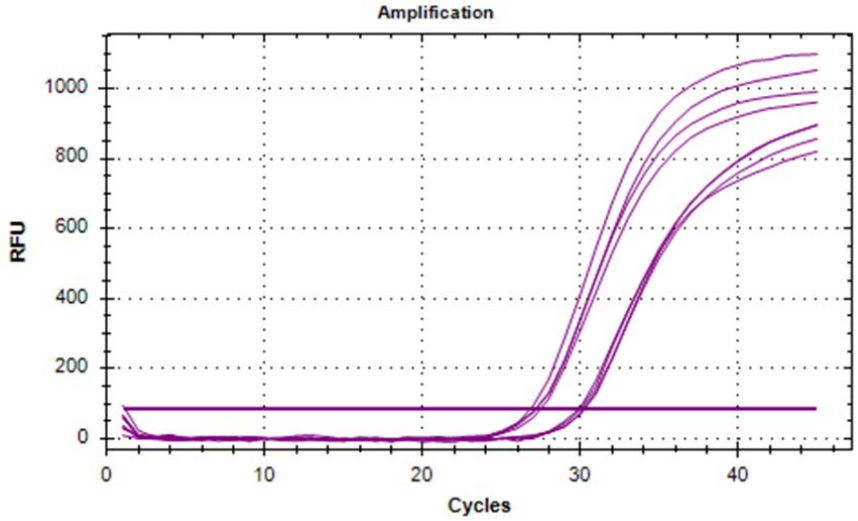

Figure 8. PCR of compositions N3, N6, N13 and N24 and 1:10 dilutions with no stool background. Amplification of invE plasmid.

PCR results with stool background have noticeable inhibition in samples (figure 9). In conclusion stool background inhibits PCR in most of the compositions in some extend.

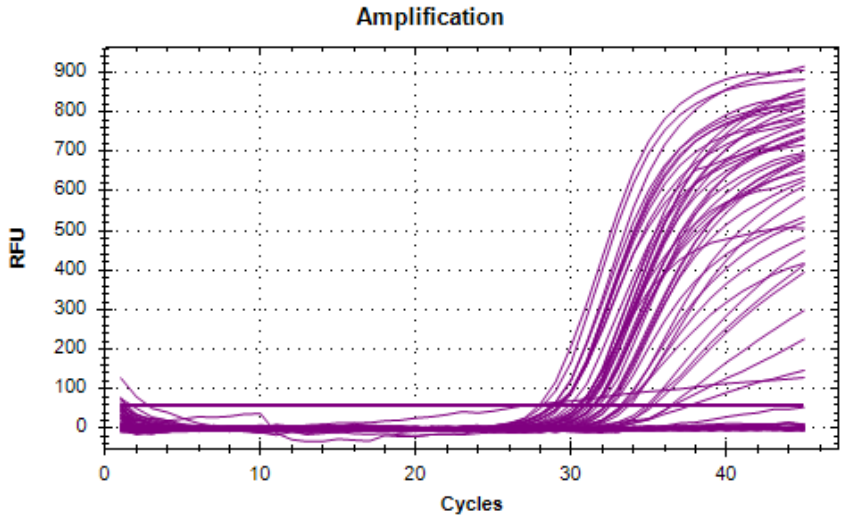

Figure 9. PCR of compositions N1-N24 and 1:10 dilutions with stool background. Amplification of invE plasmid.

Figure 10 shows PCR results for 4 of the best N-series compositions with stool background. The best performing compositions had almost no inhibition compared to the samples with no stool background.

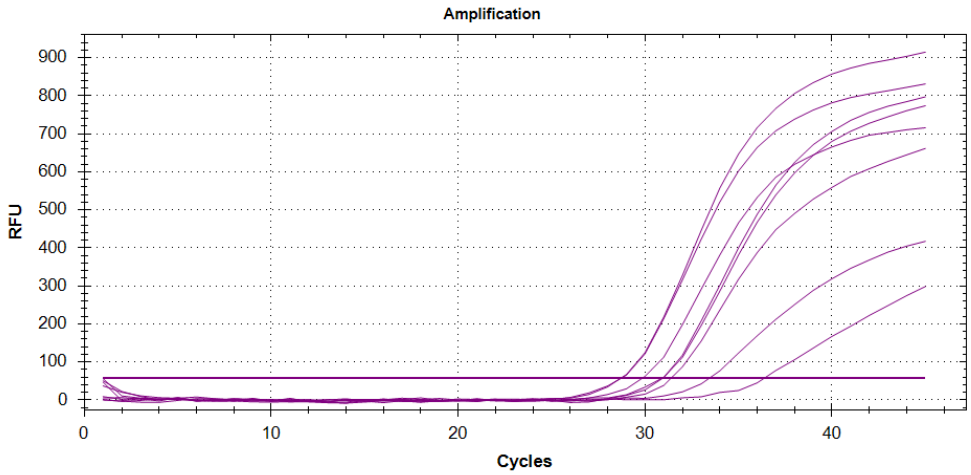

Figure 10. PCR of compositions N3, N6, N13 and N24 and 1:10 dilutions with stool background. Amplification of invE plasmid.

7.2 Results of Nucleic Acid Stability

Most of the compositions should keep the NA stabile in -20 ˚C at least the 4-week period that was last tested timepoint in this study. -20 ˚C results are shown in table 12 and all the compositions and controls are shown to stabilize DNA. Results for the RT samples (shown in table 10) show that some of the compositions stabilises the NA even in RT. Compared to the controls: Zymo Research Corp. DNA/RNA shield [25] and eNAT. DNA/RNA shield is attended for NA stability and transport for clinical samples. PBS control shows that NA is not stabile after 4-week period and has no amplification of target gene. Samples S12 and S13 failed to stabilize the NA. Tables 11 and table 12 show results of +37 ˚C and -20 ˚C stability testing.

Table 10. Results of room temperature NA stability testing comparing extraction results of 30 min to 4-week timepoint.

Room temperature						
Compositions	**30 min**			**4 weeks**		
	stx 2 (number of copies)	ipaH (number of copies)	invA (number of copies)	stx 2 (number of copies)	ipaH (number of copies)	invA (number of copies)
S1	7766	5681	8097	7895	9810	9786
S2	11961	8793	12232	7315	10702	11671
S3	11765	7658	11888	12826	9864	14448
S4	16125	11114	12777	13183	13332	14057
S5	11286	10593	13553	10848	12924	14639
S6	13211	10120	13160	6920	6237	10315
S7	8172	6092	8823	4929	4873	10908
S8	8507	6495	8519	2009	3199	3207
S9	10254	8233	11436	6861	9148	9316
S10	12591	9057	13801	9958	9469	15437
S11	11226	9058	11996	8334	10030	11675
S12	13521	14435	13757	0	0	0
S13	10973	8476	10793	23	25	42
S14	12666	13691	12913	18639	22326	23099
S15	12884	10277	13652	6809	5957	12632
S16	6807	5767	8081	11109	10804	12027
S17	10943	5719	9780	9096	4134	9724
S18	10614	15034	11435	11253	11896	15275
S19	7856	6084	9711	6694	6833	10119
S20	9698	7924	9227	7851	9721	10766
S21	22929	11666	10311	13441	8732	9173
S22	9100	7153	11027	9122	5929	11174
S23	8612	6804	10313	6832	7757	10421
S24	6973	5502	7104	6816	6980	7502
eNAT	6141	5578	6212	5621	6595	8167
PBS	15043	6158	6392	0	0	0
DNA/RNA Shield	5395	5217	5470	4343	5502	5439

Table 11. Results of +37℃ NA stability testing comparing extraction results of 3 day to 4-week timepoint.

+37℃						
Compositions	**3 days**			**4 weeks**		
	stx 2 (number of copies)	ipaH (number of copies)	invA (number of copies)	stx 2 (number of copies)	ipaH (number of copies)	invA (number of copies)
S1	2761	2589	4713	5387	3638	6824
S2	887	1112	1668	6201	2504	7358
S3	5312	4061	8079	3170	1985	6025
S4	8719	8183	14972	8706	6636	13970
S5	4727	6382	6905	5163	3720	7604
S6	381	840	1596	3937	2134	5544
S7	618	474	1117	5087	2139	4112
S8	6748	5013	6313	5258	7672	5702
S9	2990	2849	5484	4492	3334	6411
S10	1240	1308	2104	3236	2000	4046
S11	2565	2897	3840	5432	3565	7045
S12	4365	3308	6133	5796	4249	7705
S13	0	0	0	4939	3398	6277
S14	8174	7130	10203	2556	1726	3581
S15	5773	5526	8880	4453	2330	5232
S16	6236	4714	5083	3716	2454	4625
S17	6434	3943	7204	6425	2369	6703
S18	8801	7270	10543	7523	5268	8859
S19	5852	4912	8267	15721	10740	17324
S20	9984	6636	6064	6368	3872	6491
S21	831	490	1002	6520	3151	7783
S22	11666	7312	13187	5593	2853	7232
S23	8689	7144	7610	4650	3396	5796
S24	9485	7489	6202	3817	2889	5947
eNAT	1473	1274	2192	6677	3832	7625
PBS	0	0	0	0	0	0
DNA/RNA Shield	1616	1220	2240	10288	5447	7934

Table 12. Results of -20℃ NA stability testing in 4-week timepoint.

-20 ˚C			
Compositions	**4 weeks**		
	stx 2 (number of copies)	ipaH (number of copies)	invA (number of copies)
S1	7766	5681	8097
S2	11961	8793	12232
S3	11765	7658	11888
S4	16125	11114	12777
S5	11286	10593	13553
S6	13211	10120	13160
S7	8172	6092	8823
S8	8507	6495	8519
S9	10254	8233	11436
S10	12591	9057	13801
S11	11226	9058	11996
S12	13521	14435	13757
S13	10973	8476	10793
S14	12666	13691	12913
S15	12884	10277	13652
S16	6807	5767	8081
S17	10943	5719	9780
S18	10614	15034	11435
S19	7856	6084	9711
S20	9698	7924	9227
S21	22929	11666	10311
S22	9100	7153	11027
S23	8612	6804	10313
S24	6973	5502	7104
eNAT	6141	5578	6212
PBS	15043	6158	6392
DNA/RNA Shield	5395	5217	5470

More timepoints are needed to be tested to get more reliable data to determine the NA stability capabilities of the compositions. NA stability testing is still ongoing.

7.3 Results of Microbial Inactivation

Every composition of N-series and 1:10 dilution of composition-bacterial mixes were cultured. Controls used where eNAT and PBS. Table 13 shows results of four best N-series compositions and controls. All the best compositions inactivated all organisms in this analysis. Compositions N3, N6, N13 and N24 were tested further with maximal inactivation volume. Same method and controls were used for S-series compositions. For S-series only best 5 compositions were tested, based on the molecular analysis. Table 14 illustrates results five of the best compositions of S-series. All the best compositions of S-series inactivated organisms.

Table 13. Microbial inactivation of the compositions of N-series. PBS and eNAT acted as controls for analysis.

Sample	***E. coli* (CFU)**	***S. aureus* (CFU)**	***R. equi* (CFU)**	***C. albicans* (CFU)**
PBS	>1000	>1000	>1000	>1000
PBS 1:10	>1000	>1000	>1000	>1000
eNAT	0	0	0	0
N3	0	0	0	0
N6	0	0	0	0
N13	0	0	0	0
N24	0	0	0	0

Table 14. Microbial inactivation of the compositions of S-series. PBS and eNAT acted as controls for analysis.

Sample	***E. coli* (CFU)**	***S. aureus* (CFU)**	***R. equi* (CFU)**	***C. albicans* (CFU)**
PBS	>1000	>1000	>1000	>1000
PBS 1:10	>1000	>1000	>1000	>1000
eNAT	0	0	0	0
S4	0	0	0	0
S14	0	0	0	0
S18	0	0	0	0
S21	0	0	0	0
S22	0	0	0	0

Maximal volume of inactivation was tested to see the volume, were compositions fail to inactivate the organisms. Volumes were altered according to the attributes of the bacteria, for example *R. equi* was the most resistant organism, because of its cell wall and ability to resist many environmental stress factors. Controls used in this analysis were eNAT and DNA/RNA shield. Many of the compositions tested performed as well as or better than eNAT and DNA/RNA shield control. N6 composition killed the bacteria the worst and N24 killed bacteria the best. N6 and N13 where the worst out of the 4 compositions. Concentration of component 1 seems to be important factor in inactivation of the bacteria. N6 and N13 have half of the concentration of N3 and N24, which performed better for inactivation analysis. Results are shown in tables 15-18.

Table 15. 0.25 ml of composition or control was spiked with 1.5 to 3 ml of $1.5x10^7$ *E. coli* suspension.

Volume of bacterial suspension of *E. coli* (ml)	**eNAT (CFU)**	**DNA/RNA Shield (CFU)**	**N3 (CFU)**	**N6 (CFU)**	**N13 (CFU)**	**N24 (CFU)**
1.5	0	0	0	0	0	0
1.75	0	0	0	0	0	0
2	0	0	0	0	0	0
2.5	ca. 190	45	48	10	21	38

Table 16. 0.25 ml of composition or control was spiked with 0.75 to 2 ml of $1.5x10^7$ *S. aureus* suspension.

Volume of bacterial suspension of *S. aureus* (ml)	**eNAT (CFU)**	**DNA/RNA Shield (CFU)**	**N3 (CFU)**	**N6 (CFU)**	**N13 (CFU)**	**N24 (CFU)**
0.75	0	0	0	0	0	0
1	0	0	0	ca. 200	12	0
1.5	>1000	>1000	25	>1000	>1000	0
1.75	>1000	>1000	ca. 300	>1000	>1000	32
2	>1000	>1000	>1000	>1000	>1000	>1000

Table 17. 0.5 ml of composition or control was spiked with 0.25 to 3 ml of $1.5x10^7$ *R. equi* suspension.

Volume of bacterial suspension of *R. equi* (ml)	**eNAT (CFU)**	**DNA/RNA Shield (CFU)**	**N3 (CFU)**	**N6 (CFU)**	**N13 (CFU)**	**N24 (CFU)**
0.25	0	0	0	0	0	0
0.5	0	0	0	0	0	0
1	0	0	0	1	0	0
2	>1000	>1000	>1000	>1000	>1000	ca. 300
3	>1000	>1000	>1000	>1000	>1000	>1000

Table 18. 0.5 ml composition or control was spiked with 0.25 to 3 ml of $1.5x10^7$ *C.albicans* suspension.

Volume of bacterial suspension of *C. albicans* (ml)	**eNAT (CFU)**	**DNA/RNA Shield (CFU)**	**N3 (CFU)**	**N6 (CFU)**	**N13 (CFU)**	**N24 (CFU)**
0.25	0	0	0	0	0	0
0.5	0	0	0	0	0	0
1	0	0	0	74	0	0
2	15	59	0	>1000	0	3
3	88	>1000	ca. 200	>1000	>1000	ca. 150

Results of microbial analysis of different components show that component 1 inactivates the bacteria in all concentrations. Also, detergent 1 inactivates organisms very effectively, except for *R. equi,* which had some growth in all the concentrations. Components 2 and detergents 2 and 3 do not inactivate any of the organisms in any of the concentrations. Controls used were PBS and eNAT. All the results of the components are shown in table 19.

Table 19. Microbial inactivation of different components.

Suspension of *E. coli* and *S. aureus* ($1{,}5x10^7$ CFU/ml)		**Suspension of *R. equi* and *C. albicans* ($1{,}5x10^7$ CFU/ml)**	
Component	**CFU**	**Component**	**CFU**
Component 1 (concentration 1)	0	Component 1 (concentration 1)	0
Component 1 (concentration 2)	0	Component 1 (concentration 2)	0
Component 1 (concentration 3)	0	Component 1 (concentration 3)	0
Component 2 (concentration 1)	>1000	Component 2 (concentration 1)	>1000
Component 2 (concentration 1,5)	>1000	Component 2 (concentration 1,5)	>1000
Component 2 (concentration 2)	>1000	Component 2 (concentration 2)	>1000
Detergent 1 (concentration 1)	0	Detergent 1 (concentration 1)	29*
Detergent 1 (concentration 2)	0	Detergent 1 (concentration 2)	18*
Detergent 1 (concentration 2,5)	0	Detergent 1 (concentration 2,5)	12*
Detergent 2 (concentration 1)	>1000	Detergent 2 (concentration 1)	>1000
Detergent 2 (concentration 2)	>1000	Detergent 2 (concentration 2)	>1000
Detergent 2 (concentration 2,5)	>1000	Detergent 2 (concentration 2,5)	>1000
Detergent 3 (concentration 1)	>1000	Detergent 3 (concentration 1)	>1000
Detergent 3 (concentration 2)	>1000	Detergent 3 (concentration 2)	>1000
Detergent 3 (concentration 2,5)	>1000	Detergent 3 (concentration 2,5)	>1000
PBS	>1000	PBS	>1000
eNAT	0	eNAT	0

*Colonies were only *R. equi* bacteria. No *C. albicans* colonies found in these agar plates.

7.4 Results of Detergent Optimization

Detergent 3 was found to be the best option out of the detergents tested in this analysis. Detergent 3 does not inhibit the reaction and has the best results of amplification of sample out of the detergents tested. Detergent 4 performed fairly well in the analysis, no particular inhibition was found in samples. Detergent 1 performed the worst out of the detergents tested, because inhibition was found in reactions.

In easyMAG extraction PCR (shown in figure 11) detergent 1 shows some inhibition, whereas zymo extraction kit PCR (shown in figure 12) detergent 1 shows significant inhibition. This may indicate that detergent binds to the NA. The difference between the methods might occur because easyMAG is automated and zymo extraction kit is manually made extraction. In zymo extraction kit 70 % ethanol was also used to wash the

samples, instead of the kits own wash buffers. There were found to be no significant difference between the extraction methods with detergent 3 and 4.

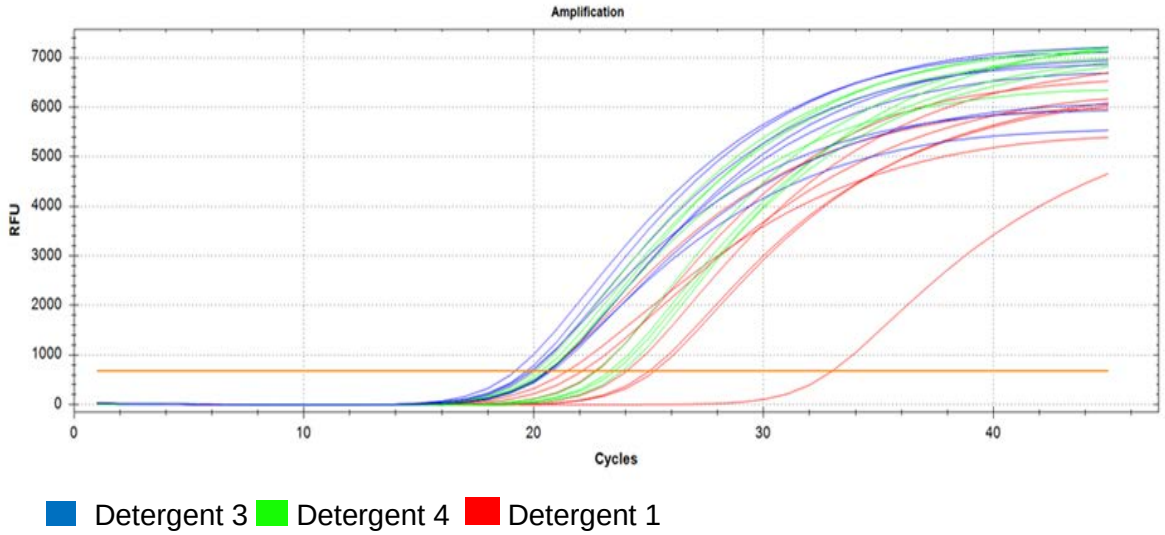

Figure 11. PCR analysis of the different detergents in different concentrations. Samples where extracted with EasyMAG equipment. Amplification of invA gene.

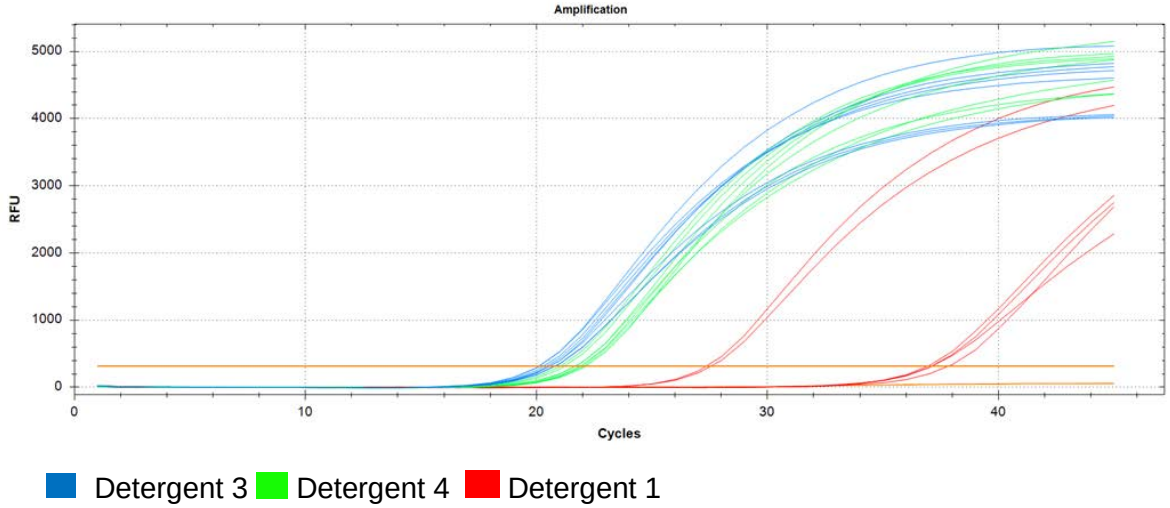

Figure 12. PCR analysis of the different detergents in different concentrations. Samples where extracted with Zymo Quick-DNA Fecal/Soil Microbe Miniprep Kit. Amplification of invA gene.

7.5 Results of Inhibition Testing

Some of the components and detergents were found to inhibit PCR reaction. Table 20 illustrates the concentrations of components that inhibited the reaction. All components show inhibition in high concentrations. Inhibition was found to be worst with detergent 1, which inhibits PCR reaction still in low concentrations. Figure 13 shows the amplification of the sxt1 plasmid of the samples.

Table 20. Concentrations that show inhibition in PCR reaction are highlighted in yellow. Concentrations shown are relative.

Component 1	10	5	2.5	1.25	0.625
Component 2	10	5	2.5	1.25	0.625
Detergent 1	10	2.5	0.63	0.16	0.04
Detergent 2	10	2.5	0.63	0.16	0.04
Component 4	10	5	2.5	1.25	0.625

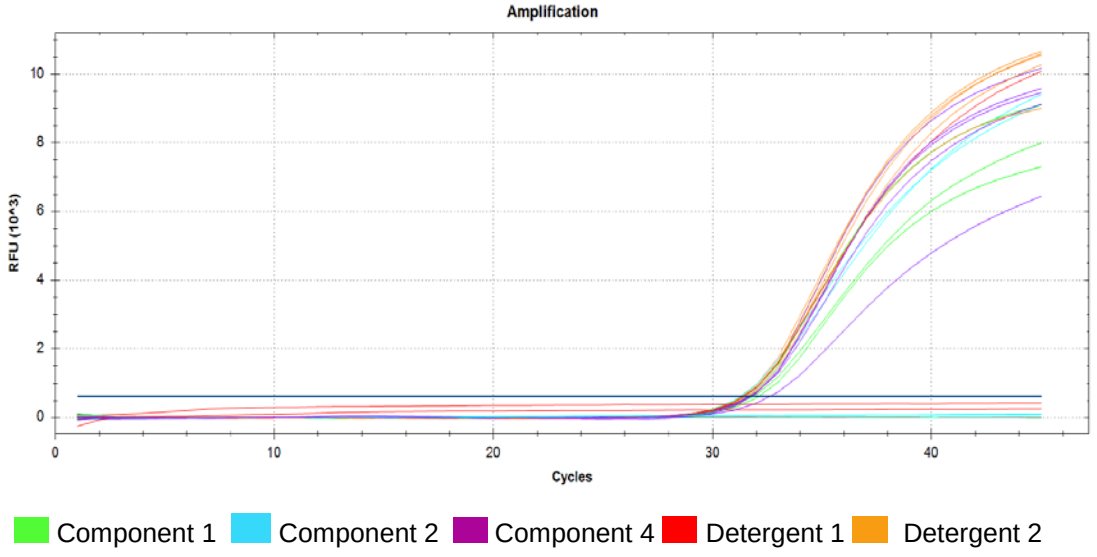

Figure 13. Target gene viewed in this figure is stx1 gene. Different components and detergents are shown in different colours.

8 Discussion

With the above methods, this study was done to ensure that requirements of the sample tube were met. Some of the method attributes and chemistry are not known, and this has to be taken in the consideration when interpreting the results. Also, some of the methods should be repeated to ensure the gained results. Microbial inactivation testing could be developed more by first culturing only the composition to agar plate and then culturing bacterial suspension on top to ensure that compositions do not just inhibit the growth of the bacteria.

Development of the sample tube in question continues with more stability testing and component testing. Follow-up research should include analysis of NA stability further, inactivation of viruses and testing of possible tube. Composition consists of components that might cause corrosion of the tube, so testing endurance of the tube is needed. Possible tube candidate has screw top, which might be vulnerable to strong reagents.

Some of the component concentrations were concluded in these analysis, like concentration of component 1 and detergent to use in future sample tube (detergent 3). Compositions tested in these analyses were also found to inactivate organisms and the inactivation capabilities of single components were also determined. Figure 14 illustrates possible sample tube.

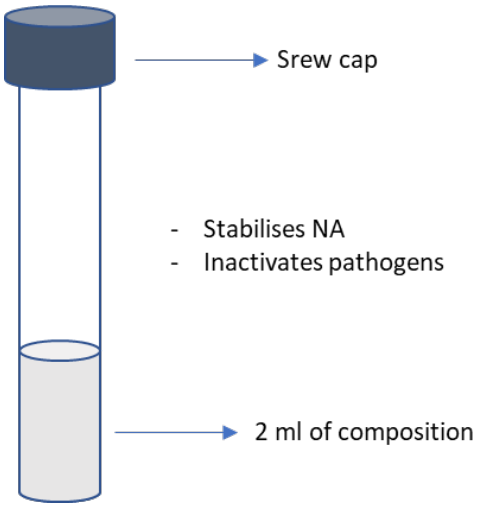

Figure 14. Illustration of stool sample tube.

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HUSLAB instructions for sampling parasite sample for nucleic acid assay (F-ParaNhO)

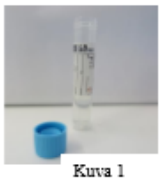
husLab
HELSINGIN JA UUDENMAAN SAIRAANHOITOPIIRI

Ohje laboratorioon tulevalle

ULOSTEEN PARASIITTINÄYTE (F-ParaNhO)

Saat näytteenottotarvikkeet HUSLABin lähimmästä toimipisteestä

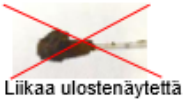
Kaarimalja tai vastaava astia (esim. kertakäyttölautanen)
Näytteenottotikku
Näyteputki (eNAT™, sininen korkki)
Suljettava muovipussi ja nimitarra


Kuva 1

Ulostenäytteen ottaminen

1) Ota ulostenäyte kaarimaljaan tai vastaavaan astiaan esim. kertakäyttölautaselle.
2) Avaa näyteputken vaaleansininen korkki ja aseta putki pystyasentoon tasaiselle alustalle (kuva1).
3) Ota näytteenottotikku pois suojapakkauksesta.
4) Työnnä näytteenottotikun pää ulosteeseen. Älä laita ulostetta tikkuun liikaa, vain ohut kerros tikun päähän.


Sopiva määrä ulostenäytettä


Liikaa ulostenäytettä

5) Työnnä näytteenottotikku näyteputken nesteeseen ja sekoita hyvin.
6) Ota näytteenottotikku pois nesteestä ja hävitä se esim. sekajätteen mukana.
7) Sulje näyteputki huolellisesti putken omalla, sinisellä korkilla.
8) Kirjoita tarraan nimesi, henkilötunnuksesi ja näytteenottopäivämäärä ja aika.
9) Laita sinikorkkinen näyteputki ja tarra muovipussiin. Sulje pussi huolellisesti.
10) Tuo näyteputki mielellään samana päivänä laboratorioon. Jos et voi tuoda näytettä samana päivänä, säilytä näyte jääkaapissa seuraavaan päivään asti.

Lisätiedustelut

Jos sinulla on kysyttävää voit soittaa HUSLABin palvelunumeroon p. 09 471 86800 ma - pe klo 7.30-15.30. Palvelunumerosta ei valitettavasti voida antaa laboratoriovastauksia.

KL 6375 F –ParaNhO

Instruction manual, Quick-DNA Fecal/Soil Microbe Miniprep Kit (Page 5)

Protocol

For optimal performance, add beta-mercaptoethanol (user supplied) to the **Genomic Lysis Buffer** to a final dilution of 0.5%(v/v) *i.e.*, 500 µl per 100 ml.

1. Add ≤ 150 mg of fecal sample or ≤ 250 mg of soil sample to a **ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm)**. Add 750 µl **Lysis Solution** to the tube[1].

   Note: Alternatively, add water sample[2] or 50-100 mg (wet weight) fungal/bacterial cells[3] that have been resuspended in up to 200 µl of water or isotonic buffer (e.g., PBS) to a ZR BashingBead™ Lysis Tube.

2. Secure in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for ≥ 5 minutes.

   Note: Required processing time will vary depending on the device and application and therefore should be evaluated on a case by case basis.

   For example, processing times may be as little as 3 minutes when using high-speed cell disrupters (e.g., the portable TerraLyzer™ Sample Processor, FastPrep® -24, or similar) or as long as 20 minutes when using lower speeds (e.g., Disruptor Genie™, or standard benchtop vortexes). See manufacturer's literature for operating information.

3. Centrifuge the ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm) in a microcentrifuge at ≥ 10,000 x *g* for 1 minute.

4. Transfer up to 400 µl supernatant to a **Zymo-Spin™ IV Spin Filter (Orange Top)** in a **Collection Tube** and centrifuge at 8,000 x *g* for 1 minute.

   Note: Snap off the base of the Zymo-Spin™ IV Spin Filter (Orange Top) prior to use

5. Add 1,200 µl of **Genomic Lysis Buffer** to the filtrate in the Collection Tube from Step 4.

6. Transfer 800 µl of the mixture from Step 5 to a **Zymo-Spin™ IIC Column**[4] in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute.

7. Discard the flow through from the Collection Tube and repeat Step 6.

8. Add 200 µl **DNA Pre-Wash Buffer** to the Zymo-Spin™ IIC Column in a <u>new</u> Collection Tube and centrifuge at 10,000 x *g* for 1 minute.

9. Add 500 µl **g-DNA Wash Buffer** to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x *g* for 1 minute.

10. Transfer the Zymo-Spin™ IIC Column to a clean 1.5 ml microcentrifuge tube and add 100 µl (50 µl minimum) **DNA Elution Buffer** directly to the column matrix. Centrifuge at 10,000 x *g* for 30 seconds to elute the DNA[5, 6].

11. Snap off the base of the **Zymo-Spin™ IV-HRC Spin Filter (Green Top)** and place into a clean Collection Tube. Centrifuge at 8,000 x *g* for 3 mins.

    Note: If the HRC matrix is dry, add 400-600 µl water prior to prepping the filter.

12. Transfer the eluted DNA to a prepared Zymo-Spin™ IV-HRC Spin Filter (Green Top) in a clean 1.5 ml microcentrifuge tube and centrifuge at exactly 8,000 x *g* for 1 minute.

    The filtered DNA is now suitable for PCR and other downstream applications.

[1] Cap tube tightly to prevent leakage.

[2] For water samples, filter using desired non-silica based filter (not provided). Cut the filter into small pieces before adding to the lysis tube.

[3] This equates to approximately 10^9 bacterial cells, 10^8 yeast cells and 10^7 mammalian cells.

[4] The Zymo-Spin™ IIC Column has a maximum capacity of 800 µl.

[5] In some cases a brown-colored pellet may form at the bottom of the tube after centrifugation. Avoid this pellet when collecting the eluted DNA.

[6] If fungi or bacterial cultures were sampled, the DNA is now suitable for PCR as well as other downstream applications.

Amplidiag Bacterial GE, PCR Quick Guide

MOBIDIAG

Amplidiag® Bacterial GE
PCR Quick Guide

CE IVD

Version 1.4 • May 2017 • English

1 Take PCR reagents to room temperature. Once thawed, store the reagents at +4 °C for up to one month.

2 Vortex and spin down all reagents.

3 Prepare Amplidiag Bacterial GE Reaction Mixes to clean laboratory tubes. Please see volumes needed for each Reaction Mix in tables below. Make about 10 % more of the Reaction Mixes to ensure sufficient volumes needed for pipetting.

Reaction Mix 1	Volume (µl)/reaction
2x PCR Master Mix	10
Assay Mix 1	5
Total volume	15

Reaction Mix 2	Volume (µl)/reaction
2x PCR Master Mix	10
Assay Mix 2	5
Total volume	15

Reaction Mix 3	Volume (µl)/reaction
2x PCR Master Mix	10
Assay Mix 3	5
Total volume	15

4 Mix thoroughly and spin down Reaction Mixes. Avoid bubble formation. Dispense 15 µl of the Reaction Mix to the desired reaction wells of the analysis plate according to the plate setup illustrated below.

5 Add 5 µl of DNA sample to the reaction wells according to the plate setup illustrated below. Remember to add all three controls to the dedicated wells on the plate.

6 Seal the plate and spin down the liquids.

7 Place the plate to the real-time PCR device and start the device software. Use the Amplidiag Bacterial GE run template provided by Mobidiag.

PCR plate setup

Symbol legend:

1-30 Samples 1-30

NC Use Negative Control Water as sample (clear cap)

PC Use Control DNA Mix as sample (red cap)

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