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# Evaluation of automated sample preparation for detection of *Bacillus cereus* DNA in animal samples, animal feed and food

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Abstract

The automated purification of bacterial DNA from different samples using the ABI Prism 6700 Automated Nucleic Acid Workstation was often hindered by clogging of the purification membrane by particles in the samples. The introduction of a centrifugation step facilitated the possibility to purify *Bacillus cereus* DNA from 26 different animal-, animal feed- and food samples. The centrifugation can be done at high speed in order to make the pellet hard. When centrifugation was done at 9000 rpm for 5 min (Ct 30.2) as much DNA was recovered as when centrifugation was performed at 2100 rpm for 2 min. (Ct 30.8). It could be seen that free DNA in complex matrices was protected when proteinase K was used as lysis method (Ct 27.9). DNA was degraded in matrices treated by boiling (Ct 38.4), at room temperature (Ct 33.9) as well as by treatment with a commercial lysis buffer; powerlyse (Ct 30.1). The use of a centrifugation step has to be tested also with viable cells.



Keywords

*B. anthracis*, *B. cereus*, Automated sample preparation, ABI Prism 6700 Automated Nucleic Acid Workstation



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# Evaluation of automated sample preparation for detection of *Bacillus cereus* DNA in animal samples, animal feed and food

Sara Ehrs

## Sammanfattning

*Bacillus anthracis* är en mycket fruktad bakterie som orsakar den dödliga sjukdomen mjältbrand, även kallad antrax. Att bakterien utgör ett bioterrorhot visade sig när flera personer i USA 2001 dog efter att ha hanterat brev med antraxsporer.

I Sverige bedriver SVA (Statens veterinärmedicinska anstalt) tillsammans med SLV (Livsmedelsverket) på uppdrag av KBM (Krisberedskapsmyndigheten) ett projekt för att skapa ett resurslaboratorium som ska kunna ta emot och analysera stora mängder prover vid avsiktlig smittspridning av patogena mikroorganismer, däribland *B. anthracis.*

Mängden prover vid smittspridning blir ofta stor och för en snabb analys av proverna krävs automatiserade provarbetningsstrategier. Proverna kan skilja sig mycket åt i fysisk och kemisk karaktär och man förväntas kunna analysera allt ifrån livsmedels-, foder- och djurprover.

Den detektionsmetod som användes analyserar DNA ifrån mikroorganismerna. De olika proverna innehåller ofta ämnen som stör analysen, därför är det viktigt att bakterie DNA extraheras och renas ifrån själva provet. I det här examensarbetet utvärderades en robot för DNA extraktion för att se om den kan tillämpas på de olika provtyperna. För utvecklingsarbetet användes den mindre farliga bakterien *B. cereus* eftersom den inte utgör en lika stor säkerhetsrisk.

Resultatet visade att de ämnen som stör analysen helt kunde renas bort, men vissa provtyper som sväller mycket i vatten eller innehåller mycket partiklar var svåra att hantera för roboten.

Examensarbete 20p, Molekylär bioteknikprogrammet

Uppsala universitet juni 2006

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**ABBREVIATIONS**

| | |
|---|---|
| BHI | Brain heart infusion |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| BSL | Biosafety level |
| CFU | Colony forming units |
| Ct | Cycle of threshold |
| dsDNA | Double-stranded DNA |
| FSU | Former Soviet Union |
| r.t. | Room temperature |
| PCR | Polymerase chain reaction |
| 6100 prepstation | ABI Prism™ 6100 Nucleic Acid PrepStation |
| 6700 workstation | ABI Prism™ 6700 Automatic Nucleic Acid Workstation |

# 1 Introduction

## 1.1 Emergency planning

In 2001 the world was in shock when several people died in a bioterror attack in the USA. The reason for the deaths was spores of the feared and deadly bacteria *Bacillus anthracis* that had been sent in letters by mail. The intentional spreading of pathogens was unexpected and terrifying and therefore preparation for future possible threats is of major importance. For that reason the Swedish Emergency Management Agency has started a project, which is run by the Swedish National Veterinary Institute and the Swedish National Food Administration. The aim of the project is to build up a laboratory for rapid and large-scaled diagnostics of pathogens. The research is focused on developing methods for the increase of the capacity and the goal is to be able to analyse samples from complex matrices i. e. food-, animal- and water samples.

An important aspect is the safe handling of the samples. *B. anthracis* is classified as a Biosafety level-3 (BSL-3) organism. There are four levels of biological safety levels (BSL 1-4) and each level has different guidelines for laboratory facilities, safety equipment, laboratory practices and techniques. Infectious agents that may cause serious or potentially lethal diseases as a result of exposure by inhalation have to be handled in a BSL-3 laboratory. Therefore, to reduce the risks, the method development performed in this thesis project was done with *Bacillus cereus* in a BSL-2 laboratory. The idea is that methods based on the model microorganism *B. cereus* in a later stage can be transferred to a BSL-3 laboratory and applied on *B. anthracis*.

## 1.2 Bacillus anthracis

*Bacillus anthracis* is a gram-positive, sporeforming rodshaped bacterium. The spores are able to survive in soil for decades [1]. *B. anthracis* is a member of the *Bacillus* group, which also include many bacteria that are common in our surroundings. The closest relative is *B. cereus* and comparative studies of genome variation have concluded that they are one species [2]. *B. anthracis,* as opposed to *B. cereus,* is carrying the two plasmids ($pXO_1$ and $pXO_2$) that contain genes encoding two virulent toxins and a capsule that protects the bacteria from phagocytosis. [3]

*B. anthracis* can cause the very serious disease anthrax. Anthrax is a zoonose that mostly infect grazing animals, like cows, horses and sheep. The animals are infected from spores in the ground, which come from animals that have been infected earlier. Humans can become infected with anthrax when handling animals or animal products, e.g. fur, skin or bonemeal. Infection can also be the result of consumption of meat or milk from infected animals [1]. Anthrax is not known to spread between humans. There are three different types of anthrax affecting skin (cutaneous), lungs (pulmonary or inhalational) or digestive tract (gastrointestinal). Approximately 95% of the human cases of anthrax are cutaneous anthrax. This form of anthrax is associated with skin infections or sores in combination with the handling of products from infected animals. The first symptom is redness of the skin followed by a small sore that finally develops into a skin ulcer that enlarges, blackens, and become dry and tough. The mortality from untreated cutaneous anthrax is between 10-20%. Inhalation anthrax occurs after the inhalation of spores. Extrapolations of animal studies suggest that the human $LD_{50}$ (dose sufficient to kill 50% of infected persons) is 2500 to 55000 inhaled spores, and as few as one to three spores may be sufficient to cause infection [4]. Disease begins like a cold or flu and symptoms can include a sore throat, mild fever and muscle ache but will

suddenly develop into high fever, laboured breathing, excessive sweating and shock that can result in death in average three days [1, 4]. Almost as dangerous is the intestinal anthrax that follows from intake of food or water contaminated with *B. anthracis*. The first symptoms are nausea, loss of appetite followed by bloody diarrhoea, fever, bad stomach pain and sepsis. Inhalation and gastrointestinal anthrax has, if untreated, a very high mortality rate, close to 100% [5]. It is important with antibiotics treatment in an early phase of the disease, a delay will lessen the chances of survival.

## 1.3 Bacillus cereus

*B. cereus,* one of the bacteria closest related to *B. anthracis*, can cause food borne illness. Two toxins produced by *B. cereus* mediate the disease. One is a heat-stable enterotoxin that causes an emetic (causing vomiting) form of the disease and one heat-labile enterotoxin that causes a diarrhoeal form of the disease. The infectious dose of the diarrhoeal form is $10^5$-$10^7$ cells and the infectious dose of the emetic (causing vomiting) form is $10^5$-$10^8$ cells per g of food. The bacterium is found in the soil and from there it is spread to food, such as rice, milk or meat products. *B. cereus* spores survive pasteurisation and boiling and if the rice or milk is not refrigerated, the spores can germinate and the bacteria can multiply rapidly. The heat-stable enterotoxin is not destroyed when the food is reheated [6].

## 1.4 Bacillus anthracis as a biological weapon

*B. anthracis* is an appealing biological weapon due to its virulence and the spore's strong resistance to heat and drying. Several countries, there among USA, the former Soviet Union (FSU), England and Japan had anthrax based bioweapon development programs in the 1930- and 40s. After 1972 most biological weapon programs were terminated after most countries signed the Biological Weapon Convention. Despite prohibition of offensive bioweapon research and development, many countries are still believed to have biological weapons [3, 4].

In 2001, several people died in USA after anthrax spores had been mailed in envelopes. This incident demonstrated the powerful use of anthrax as a weapon of bioterrorism. The anthrax attacks of 2001 used one of many possible methods of attack. Another possible spreading is the aerosol release of *B. anthracis*. An aerosol attack would be of great danger to the civilian population. In 1970, the World Health Organization estimated that the release of 50 kg *B. anthracis* over an urban population of 5 million people would sicken 250 000 and kill 100 000[4]. In 1979, an accident in a microbiology facility plant in Sverdlovsk, FSU, resulted in an aerosol release of *B. anthracis*. An epidemic of anthrax occurred among the population that lived or worked in a narrow downwind zone from the plant. A large number of livestock also died in the same area, out to a distance of 50 km [3]. At first FSU denied that the epidemic was caused by accidental aerosol release of spores and attributed the disease to cutaneous and gastrointestinal anthrax caused by the consumption of contaminated meat. After the collapse of Soviet Union, the truth was revealed, and defected officials in the FSU military admitted that 42 people had died from inhalational anthrax [3].

In case of a big bioterrorism attack, the need of medical resources would be enormous. Naturally occurring anthrax is sensitive to many types of antibiotics. However there is a big concern that for bioweapon purposes, a modified strain resistant to many common antibiotics can be used. To be effective, prophylaxis has to be given in an early state. In practise, it means that prophylaxis is given as a preventive until analysis is finished. If analyses show that the result is negative, the prophylaxis is interrupted. However, if the results are positive the treatment goes on for 60 days. The treatment goes on for such a long time because at the

accident in Sverdlovsk people fell ill more than 40 days after exposure. The reason for this could be the late germination of inhaled latent anthrax spores. In Sweden, the few times treatment has been needed, antibiotics have been taken from the pharmacy. Should we have to use it in larger quantities, there are readiness supplies [7].

## *1.5 Detection of Bacillus anthracis*

The standard method for diagnosis of *B. anthracis* is by determining the growth of the organism on a selective medium. The major drawback with this method is that it is time consuming, taking 1 to 2 days to assure a negative result. A quicker test is using paper strips coated with antibodies against *B. anthracis.* However large numbers of spores (>10000) are required for a positive reaction. Many surface antigens of *B. anthracis* are shared by other *Bacillus* species. A problem with antibodies raised against vegetative cells or spores is the cross-reaction with other bacillus species, giving rise to false positives [8]. Molecular tools offer a rapid and sensitive detection of *B. anthracis*. With the use of real-time PCR a quick detection can be done in a couple of hours. Several genetic markers, both plasmid-borne and chromosomal, are available for *B. anthracis* [9].

### 1.5.1 Real-time PCR

Real-time PCR is a variant of PCR that allows the detection of the amplification during the run (Figure 1). The method is very time-saving since it is not necessary to analyse the samples afterwards with gel electrophoresis. The method is very sensitive and can detect DNA in the range of $10^{-15}$-$10^{-9}$ g in one sample [10].

There are different types of real-time PCR but in this assay SYBR Green Dye has been used. SYBR Green Dye binds to double stranded DNA (dsDNA). When bound to dsDNA, it starts to fluoresce. The more dsDNA produced, the more the signal will increase. When the signal has increased to a detectable level it can be displayed in an amplification plot. The cyclenumber where the signal reaches a certain level (threshold) is called the cycle of threshold (Ct). The number of cycles that is needed to reach the threshold is dependent on the initial concentration of DNA in the sample, i. e. the lower the Ct value, the higher the start concentration of DNA in the sample. An indication of the specificity of the PCR can be done with a melting curve analysis. This will reveal if the right DNA-sequence has been amplified or if there are primer dimers or other unspecific amplification products.

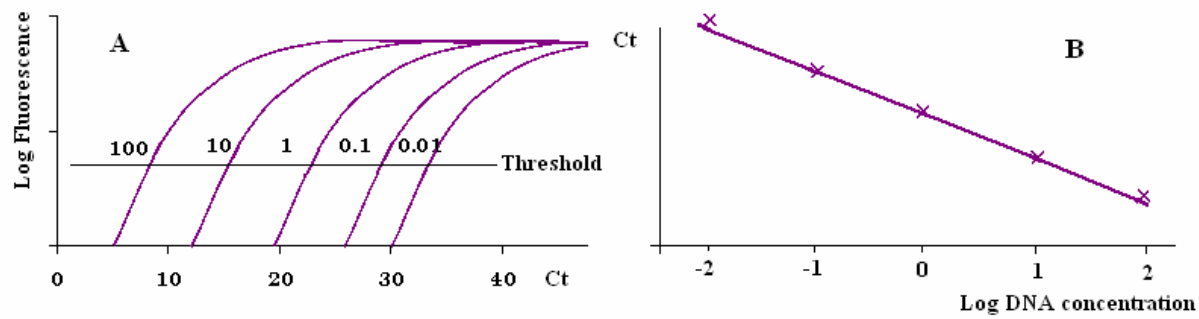



**Figure 1. Real-time PCR can be used for the construction of a standard curve**. (A) Plot of the log of florescence versus cycle. The threshold cycle (Ct) is calculated from the reference point (threshold). (B) The standard curve is generated by plotting Ct versus log of target DNA concentration. The amplification efficiency is determined from the slope of the standard curve. A slope of -3.32 represents a PCR efficiency of 100%. Or in other words, if the PCR efficiency is 100% the difference in Ct between a sample and a ten-time dilution of the sample is 3.32 Ct values. A one-time dilution is represented by approximately 1 Ct value.

## 1.6 Sample preparation

In an intentional spreading of *B. anthracis* a lot of situations have to be considered. The spreading can be through food to both humans and animals. This addresses the problem that samples can have a lot of different physical or chemical properties. For a good readiness, all sorts of samples should be able to be processed for the detection of *B. anthracis*. Important to take into account when designing a good process for detection of *B. anthracis* is the safety aspect. Handling of the samples should be safe for the personnel and environment. In a process it should therefore be as little hands-on as possible. The method must also be specific for the organism and the sensitivity high. When a lot of samples are handled, automation becomes more important, but also the application of the method on different samples. At last, the time of the process needs to be lowered as much as possible so that action can be taken as soon as possible.

It is sometimes preferred to extract and purify the bacterial DNA before detection with real-time PCR. This is done in order to remove substances that interfere with the PCR-reaction and make it less effective. There are several substances that are known to inhibit the PCR-reaction such as proteinases, heme compounds, lipids, salts, chelating agents, proteins and also DNA from organisms other than the target [11, 12]. Many sample preparation methods are being used to overcome these effects of PCR-inhibition such as dilution, centrifugation, adsorption and DNA extraction [11]. A typical sample preparation can be seen in Figure 2.

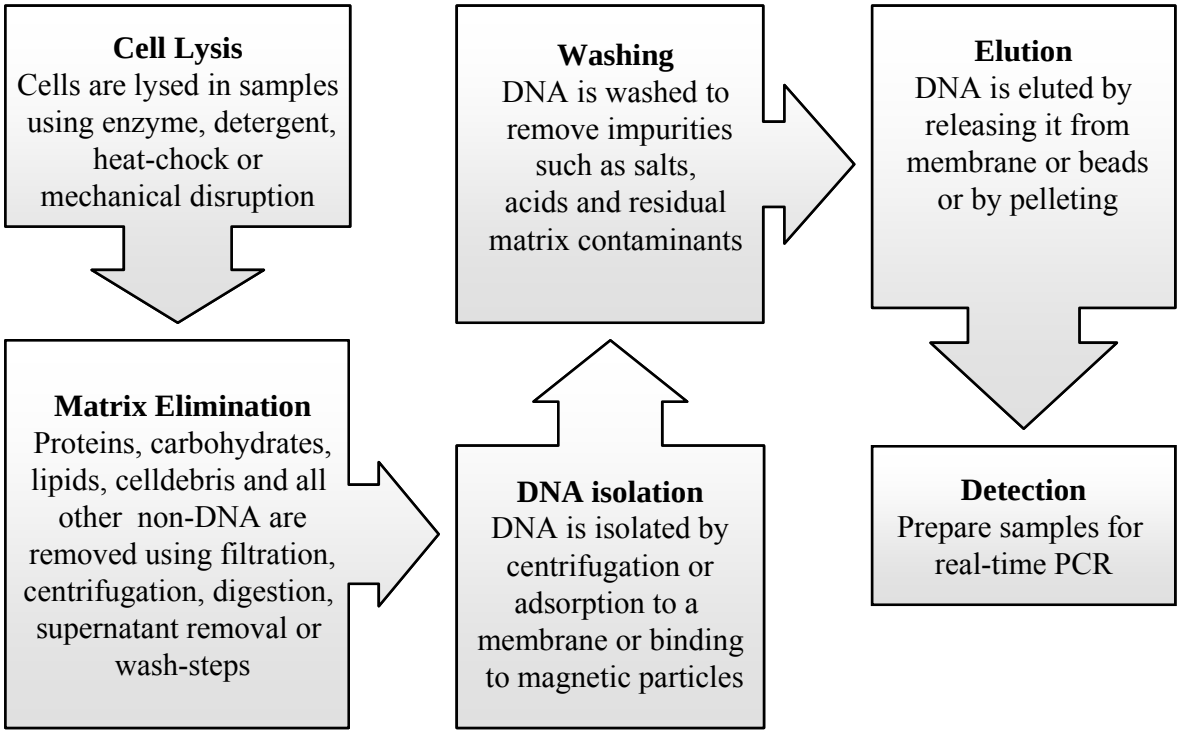



**Figure 2. Diagram of sample preparation**

### 1.6.1 Lysis of cells

For the detection of bacteria using real-time PCR, the DNA in the interior of the bacteria has to be released and become available for the PCR reaction. Therefore, viable or intact cells have to be lysed in order to release the DNA for detection. Gram-negative bacteria are fairly easy to lyse, osmotic chock or a mild detergent may be enough. The cell wall of gram-positive bacteria has a thick layer of peptidoglycan (Figure 3), which may be difficult to destroy, and therefore it might be necessary to add an enzymatic or mechanic step for the destruction of the cell wall.

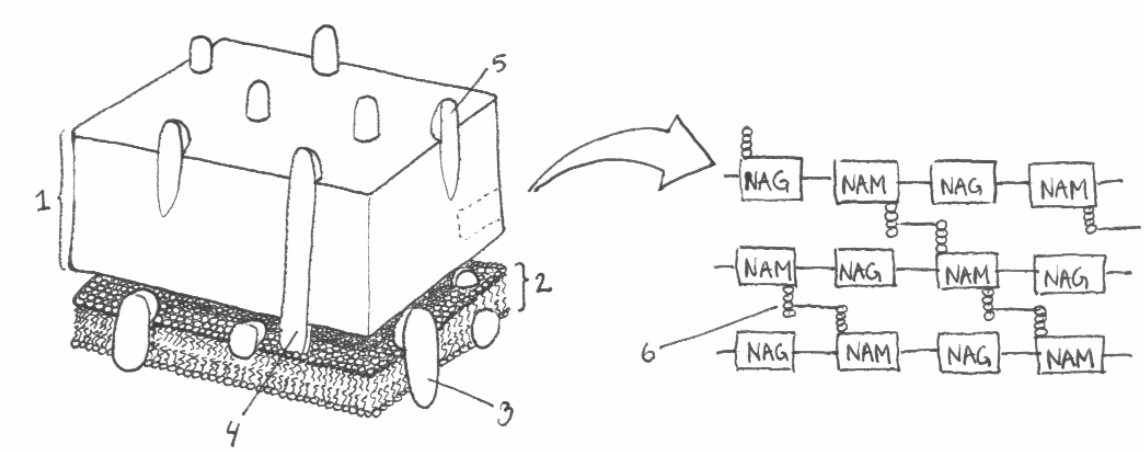



**Figure 3. The cell wall of gram-positive bacteria.** 1. Peptidoglycan layer (cell wall) 2. Cell membrane 3. Integral protein 4. Lipoteichoic acid 5. Teichoic acid. To the right: Schematic picture of one layer of peptidoglycan 6. Aminoacid crossbridge (tetrapeptide). The crossbridge differs among bacterial species. NAG: N-acetylglucosamine NAM: N-acetylmuramic acid. NAG and NAM are attached to each other via β-1,4-linkages.

Many different methods have been used for the lysis of cells and spores of *B. anthracis,* there among autoclaving, boiling, sonication and combinations of enzymes and detergents [13,14]. Other methods that are used for the disruption of the cell wall of gram-positive bacteria are bead beating and grinding. There are several different enzymes and detergents available for the destruction of the cell wall of gram-positive bacteria. In the literature all sorts of combinations of enzymes and detergents have been used. The properties of some enzymes and detergents have been listed in Table 1 and 2.

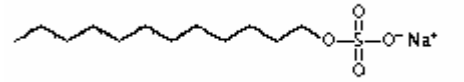
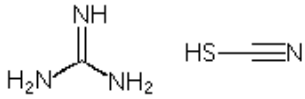
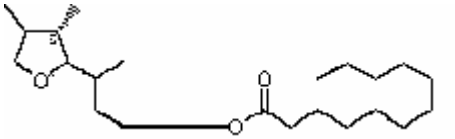
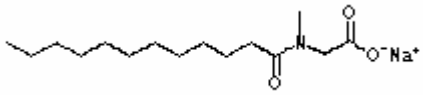
To detect as few numbers of cells as possible, a good lysis step should disrupt all cells and release the DNA but should not degrade DNA when it is unprotected. There are commercial lysis buffers for the lysis of gram-positive bacteria. Powerlyse (Genpoint, Oslo, Norway) and Ready-Lyse™ Lysozyme solution (Epicentre Biotechnologies, Madison, USA) are two of them.

**Table 1. Properties of enzymes.**

| Enzyme | Temperature[1)] | pH[2)] | Activity [3)] | References |
|---|---|---|---|---|
| Proteinase K | 60°C | 4-12.5 | Proteinase K is a serine protease that belongs to the subtilisin family. Hydrolyses peptide bonds. Hydrolyzes proteins and nucleases in DNA preparations.<br><br>It is active in the presence of 1% SDS, 4M Urea, 1% Triton X-100, 5% Tween-20 or 3M Guanidine HCl. PMSF, EGTA, DFP and EDTA has no effect on the enzyme.<br><br>Inactivation at 95°C for 10min.<br><br>Binds two $Ca^{2+}$ ions. | [15], [20], [21] |
| Mutanolysin | 37°C | 6.5-7 | Mutanolysin is a bacterial muramidase. It cleaves the β-N-acetylmuramyl-(1,4)-N-acetylglucosamine linkage of the polymer peptidoglycan-polysaccharide in the bacterial cell wall.<br><br>Mutanolysin lyses *Listeria* and other Gram-positive bacteria such as *Lactobacillus* and *Lactococcus*. | [11], [22], [23] |
| Labiase | 55°C | 4 | Labiase is an N-acetyl glukosaminidas with murimidase activity. Labiase contains β-N-acetyl-D-glucosaminidase and lysozyme activity. It is used for the lysis of many gram-positive bacteria such as *Lactobacillus*, *Aerococcus* and, *Streptococcus.* | [24], [25] |
| Lysozyme | 37°C | 6-9 | Lysozyme is a murimidase. It hydrolyzes the β-1,4 glycosidic bond between N-acetyl-D-glucosamine and N-acetylmuramic acid in the polysaccharide backbone of peptidoglycan in the bacterial cell wall.<br><br>Lysozyme is inhibited by indole derivatives (which bind to and distort the active site) and imidazole (formation of a charge-transfer complex). It is also inhibited by surface-active agents such as SDS, sodium dodecanate, and dodecyl alcohol. Other compounds of these types also inhibit lysozyme provided that the carbon chain present is at least 12 or more carbons in length.<br><br>At pH 6.2, maximal activity is observed over a wider range of ionic strengths (0.02 to 0.100 M) than at pH 9.2 (0.01 to 0.06 M). | [26], [27], [28] |
| Lysostaphin | 37°C | - | Lysostaphin is a glycyl-glycine endopeptidase. Hydrolysis of the Gly-\|-Gly bond in the pentaglycine inter-peptide bridges of the peptidoglycan. It is highly specific for *Staphylococcus* species.<br><br>Binds one $Zn^{2+}$ per subunit. | [11], [29], [30] |

1) Temperature optimum for activity, 2) pH for activity, 3) Reaction catalyzed, Co-factors, inhibitors, etc.

**Table 2. Properties of detergents**

| Detergent | Comments | References |
|---|---|---|
| Triton X-100 | Non-ionic<br>Inhibits PCR if the concentration is over 2%<br>  | [15], [16], [17] |
| SDS | Anionic<br>Inhibits PCR<br>  | [18], [19], [17] |
| Guanidine Thiocyanate | Chaotropic salt. Chaotropic salts increase the solubility of nonpolar substances in water. They denature proteins because they have the ability to disrupt hydrophobic interactions. They do not denaturate DNA or RNA.<br>  | [17] |
| Tween 20 | Non-ionic<br>PCR-facilitator<br>  | [17], [19] |
| N-lauryl sacrosine | Anionic<br>Inhibits PCR<br>Synonym: sarkosyl<br>  | [17], [19] |

## *1.7 Automated sample preparation*

The traditional DNA extraction technique is the phenol extraction but it is not very well suited for automation. For higher throughput systems the purification is often based on absorbents. The process and chemistry behind the different purification systems differ among the manufactures, but the principle behind the purification is often either of two kinds described below. The DNA is either absorbed onto a modified silica membrane in a 96-well flow-through plate or onto magnetic beads that can be separated from the sample using a magnet. When the DNA has been caught the inhibiting substances can be washed off in several steps before the purified DNA is released in a final step [31].

The fully automated system includes a unit for liquid handling that moves over the deckspace and moves liquid to and from microplates, tube racks and solvent reservoirs. Many of the manufactures have further developed the deckspace with other units such as heating blocks, shakers, sealing of plates and gripping arms that move microplates around the deckspace [31].

### 1.7.1 BioRobot EZ1

The BioRobot EZ1 (Qiagen, Hilden, Germany) is used to purify nucleic acids from clinical or forensic samples [32]. The purification principle is based on adsorption to magnetic particles. When cells are lysed the DNA are bound to the silica membrane of the magnetic particles. The magnetic particles with bound DNA are extracted from the sample using a magnet. Thereafter impurities are washed away and finally samples are eluted in 1.5 ml tubes. The reagents to the robot are supplied in pre-filled cartridges. The robot can process 1-6 samples at the same time within 15-20 minutes.

### 1.7.2 ABI Prism 6700 Automated Nucleic Acid Workstation

The ABI Prism™ 6700 Automated Nucleic Acid Workstation (Applied Biosystems, Foster City, USA) is used to purify nucleic acids from a variety of biological samples (Figure 4) [33]. The exact purification mechanism behind the 6700 workstation is still undisclosed by the company. But the extraction principle is based on a flow-through membrane with special physical properties that capture the DNA or RNA. When purifying nucleic acids, the samples are put on a 96-well tray with a membrane in each well. The tray with the membrane is different depending on if it is DNA or RNA that is purified. The samples are evacuated through the membranes using vacuum, but the nucleic acid is bound to the membrane. This is followed by a wash-step where compounds that are unspecifically bound to the filter are washed off. Finally the DNA is eluted using extreme pH.

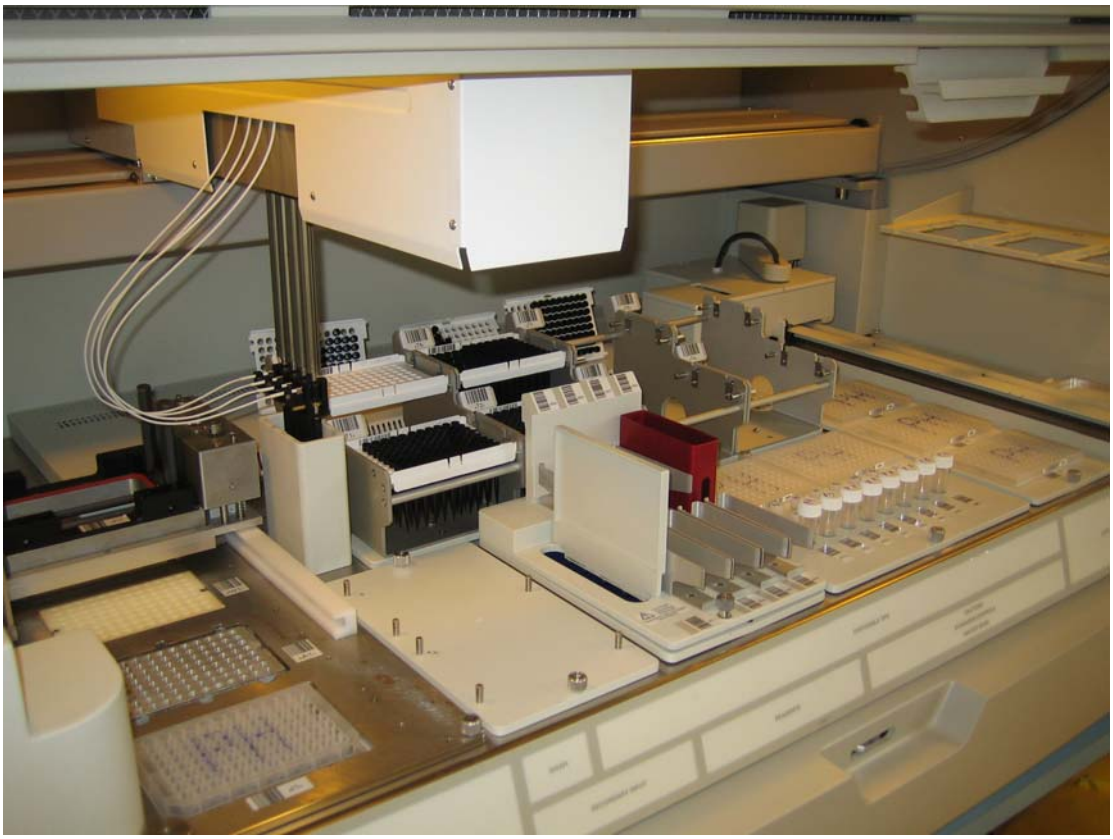


**Figure 4. ABI Prism 6700 Automated Nucleic Acid Workstation**

The 6700 workstation is fully automated and performs purification, reaction preparation to PCR tray set-up and sealing of real-time PCR trays. The liquid is handled using filter-plugged, conductive disposable tips. Since the pipette tips are conductive they sense when reaching the liquid and stays on the surface instead of going down in the liquid when filling up the tips with liquid. This reduces the risk for cross contamination. The robot is enclosed, making it a Class II biosafety cabinet.

The input is a 96-well microplate or a 96-deep-well plate with samples. Before the samples are put in the 6700 workstation they have to be subjected to a lysis step in order to release DNA from the cells. The lysed samples then have to be mixed with BloodPrep DNA Purification Solution (Applied Biosystems) to get the right binding properties of the DNA to the flow-through membrane. The BloodPrep DNA Purification Solution or a lysis buffer can be added to the sample by the robot but in this assay it was avoided since it would have disrupted the sample pellet. After the purification the robot can do two dilution series of the purified DNA before it sets up the PCR-plates. The output can be varied from run to run, but the maximum output is four 96-well PCR trays. An illustration of the different steps performed by the 6700 workstation can be seen in Figure 5.

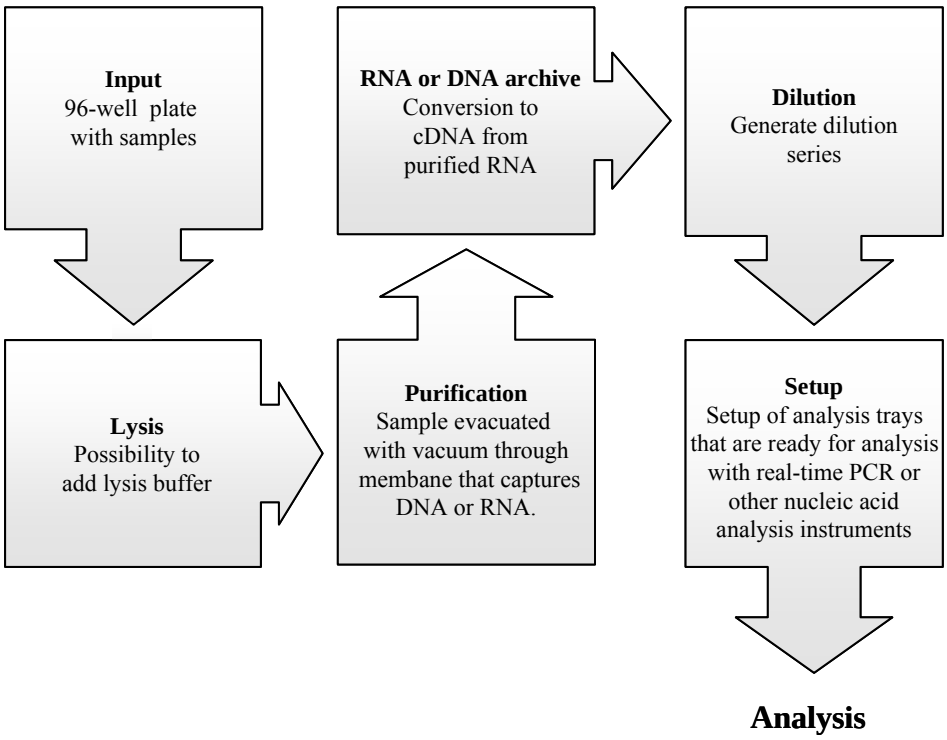



**Figure 5. Illustration of which steps can be performed by the ABI Prism 6700 Automated Nucleic Acid Workstation**

### 1.7.3 ABI Prism 6100 Nucleic Acid PrepStation

The ABI Prism™ 6100 Nucleic Acid PrepStation uses the same purification chemistry as the 6700 workstation. The difference is that the 6100 prepstation is manual and only comprise the purification step (Figure 6). Loading of the samples, wash buffers and elution buffers have to be done manually and then the real-time PCR plates also have to be set up manually.

**Figure 6. ABI Prism 6100 Nucleic Acid PrepStation**

The 6100 prepstation demands a lot of hands-on, which is not preferred in a BSL-3 laboratory. On the other hand the 6100 prepstation is superior to the 6700 workstation when it comes to supervision of the samples. The 6700 workstation does not make sure that the well in the filter tray has been evacuated before it loads the wash buffer, which might lead to overflow of the wells and contamination of samples. Therefore it is important to find a good protocol on the 6100 prepstation, where evacuation can be supervised, and then move it to the 6700 workstation.

The manufacturer provides protocols for the purification of DNA from blood, tissue and buccal swabs [34], but there are no available protocols for the DNA-extraction from other complex samples such as faeces, different food samples or environmental samples. The manufacturers' protocol for blood can be overviewed in Figure 7.

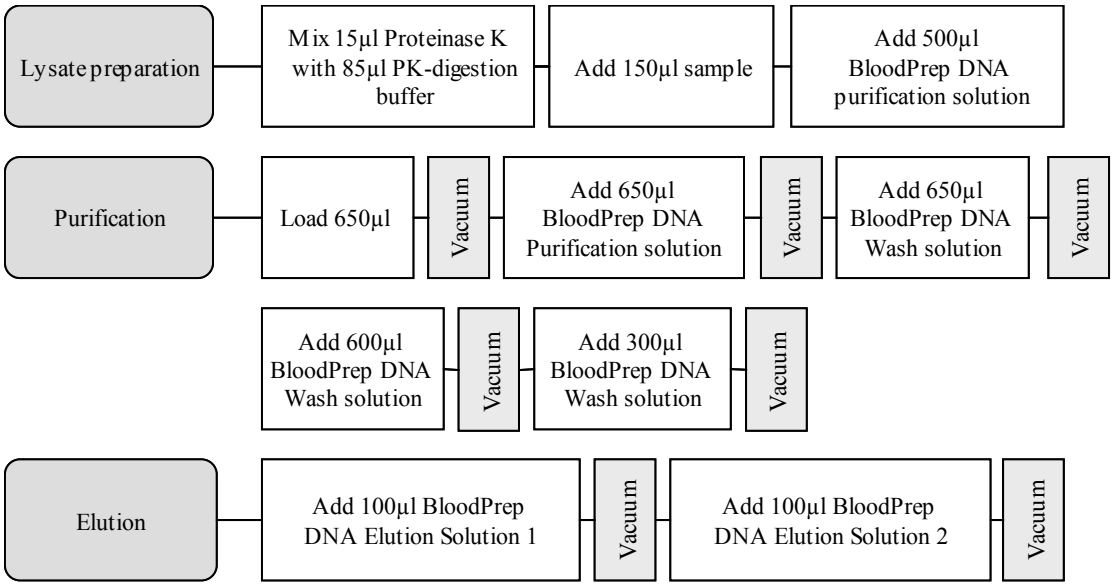



**Figure 7. BloodPrep protocol**

## *1.8 Objectives*

The aim of the study was to evaluate the ABI Prism 6700 Automated Nucleic Acid Workstation and the ABI Prism 6100 Nucleic Acid PrepStation for the isolation of DNA from *B. cereus*. *B. cereus* is a model organism for *B. anthracis* and the goal is to find a protocol that can be used to isolate DNA for the detection of *B. cereus* in animal samples, animal feed, food and environmental samples.

# 2 Material and Methods

## 2.1 Bacterial strains

*B. cereus* strain KBM1, stored at –70 °C, was inoculated in 10 ml BHI (Substrate department, SVA, Uppsala, Sweden), and incubated at 37 °C overnight. The concentration (cell forming units/ml, cfu/ml) was determined by serial dilutions in 0.86-0.9% NaCl (Substrate department, SVA) followed by plating and cell counting on horse blood agar (Substrate department, SVA).

## 2.2 Wash of B. cereus

500 µl of an overnight culture of *B. cereus* strain KBM1 was pelleted at different speeds ranging from 1000-10000 rcf for 5 min in an Eppendorf centrifuge 5417R (Eppendorf, Hamburg, Germany). The pellet was resuspended in 1 ml $ddH_2O$ (Substrate department, SVA) and the wash-step was repeated three times at the same speed of centrifugation. Samples for PCR were collected each time from the supernatant. 50 µl of Powerlyse (Genpoint, Oslo, Norway) was added. Lysis was interrupted after 4 min by addition of ethanol, and DNA was purified using the QIAmp DNA Mini Kit (Qiagen). The samples were eluted in 200 µl of AE-buffer (Qiagen). The DNA concentration was determined using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Eluates were diluted in $ddH_2O$ and DNA was amplified with the real-time PCR protocol described below with the exception that the 40 cycles were iterations of 95 °C, 15 s followed by 58 °C, 45 s and 72 °C, 35 s.

## 2.3 Screening of enzymes and detergents for lysis of B. cereus in pellets

Thirteen different lysis buffers were prepared according to Table 3. All detergents were prepared from stock solutions from Substrate department, SVA. The enzyme stocks used were 15.1 mg/ml Proteinase K (Roche Diagnostics GmbH, Mannheim, Germany), 5000 U/ml Mutanolysin (M 4782; Sigma, Saint Louis, USA), 10 mg/ml Lysozyme (L 3790; Sigma). 500 µl of a $1.5*10^7$ cfu/ml over night culture of *B. cereus* strain KBM1 was transferred into 15 1.5 ml tubes. The bacteria were pelleted at 8800 rpm for 5 min and resuspended in 1 ml of $ddH_2O$ and this was repeated three times. 200 µl of the lysis buffer was added to the pellet and the tubes were incubated according to the conditions given in Table 3. Lysis was interrupted by addition of 200 µl 99.5% ethanol, and DNA was purified using the QIAmp DNA Mini Kit. Samples were eluted in 200 µl of AE-buffer. The DNA concentration was determined using Nanodrop. Eluates were diluted in $ddH_2O$ and DNA was amplified with the real-time PCR protocol described below.

**Table 3. Lysis buffers.** All buffers were prepared in 50 mM Tris-HCl pH 8. Nr 14 and 15 are negative controls, consisting of 50 mM Tris-HCl pH 8.

| No. | Reagents | Incubation |
|---|---|---|
| 1 | 2% TritonX-100 | 37 °C 50 min + 95 °C 10 min |
| 2 | 1.13 mg/ml Proteinase K | 55 °C 60 min + 95 °C 10 min |
| 3 | 1 mg/ml Lysozyme | 37 °C 60 min |
| 4 | 500 U/ml Mutanolysin | 37 °C 60 min |
| 5 | 1.13 mg/ml Proteinase K,<br>1 mg/ml Lysozyme | 55 °C 60 min + 95 °C 10 min |
| 6 | 1.13 mg/ml Proteinase K, 500 U/ml Mutanolysin | 55 °C 60 min + 95 °C 10 min |

| | | |
|---|---|---|
| 7 | 1 mg/ml Lysozyme, 500 U/ml Mutanolysin | 37 °C 60 min |
| 8 | 1.13 mg/ml Proteinase K,<br>1 mg/ml Lysozyme, 500 U/ml Mutanolysin | 55 °C 60 min + 95 °C 10 min |
| 9 | 1.13 mg/ml Proteinase K, 1% SDS | 55 °C 60 min + 95 °C 10 min |
| 10 | 1.13 mg/ml Proteinase K, 0.05% Tween20 | 55 °C 60 min + 95 °C 10 min |
| 11 | 1.13 mg/ml Proteinase K, 1% SDS, 0.05% Tween20 | 55 °C 60 min + 95 °C 10 min |
| 12 | 1.) 1 mg/ml Lysozyme, 5 mM EDTA,<br>50 mM NaCl. 2.)1% SDS. 0.95 mg/ml proteinase K | 1.) 37 °C 30 min. 2.) added<br>55 °C 30 min + 95 °C 10 min |
| 13 | 1.13 mg/ml Proteinase K, 10 mM EDTA,<br>100 mM NaCl, 2% SDS, 2% Sarkosyl | 55 °C 60 min + 95 °C 10 min |
| 14 | 50 mM Tris-HCl pH8 | 55 °C 60 min + 95 °C 10 min |
| 15 | 50 mM Tris-HCl pH8 | 37 °C 60 min |

## *2.4 Preparation of DNA from B. cereus*

*B. cereus* strain KBM1, stored at –70 °C, was spread on horse blood agar. The plates were incubated at 37 °C overnight. A couple of colonies were resuspended in 200 µl G2-buffer (EZ1; Qiagen). Six samples were prepared and DNA extracted using the DNA tissue kit (Qiagen) and the EZ1 robot (Qiagen). The DNA was eluted in 100µl. The samples were pooled and the DNA concentration was determined to 163 ng/µl (260/280=1.81, 260/230=1.06) using Nanodrop ND-1000 spectrophotometer. Samples were divided into six tubes and kept at –20 °C.

## *2.5 Sample preparation*

All matrices, i. e. the food and animal samples used in this project, were taken from the -70 °C freezer (Appendix 1). They had been prepared by Stina Lindberg (Swedish National Veterinary Institute) [35]. In short; 10 g of matrix and 90 ml 0.86-0.9% NaCl were stomached at 230 rpm for 2 min using a bag containing a filter that remove the largest particles.

### 2.5.1 PCR-inhibition

150µl of each matrix was added to 500 µl BloodPrep DNA Purification Solution (Applied Biosystems). Samples were thoroughly mixed with vortex and then centrifuged at 2100 rpm for 2 min. 550 µl was added to the 6100 prepstation. The method for DNA isolation on the 6100 prepstation was followed. The DNA was eluted in 128 µl. Duplicates samples were prepared for each matrix. 45 µl of the eluate was mixed with 5 µl 0.16 ng/µl DNA from *B. cereus* strain KBM1. Samples were analyzed by real-time PCR.

### 2.5.2 DNA-recovery in all matrices

675 µl matrix was spiked with 75 µl 0.16 ng/µl DNA from *B. cereus* strain KBM1. Samples were thoroughly vortexed and 150 µl of sample was transferred into two 1.5 ml tubes containing 200 µl dd$H_2O$. 700 µl BloodPrep DNA Purification Solution was added and samples were thoroughly vortexed and centrifuged at 2100 rpm for 2 min. 950 µl of supernatant was loaded onto 6100 prepstation. The method for DNA isolation on the 6100 prepstation was followed. Samples were eluted in 136 µl and analyzed by real-time PCR.

### 2.5.3 Lysis methods

Selected matrices were spiked with 0.16 ng/µl or 0.32 ng/µl DNA from *B. cereus* strain KBM1. Samples were thoroughly vortexed and 150 µl of the spiked matrix was dispensed into 1.5 ml tubes. Lysis buffer or $ddH_2O$ was added and samples were incubated depending on lysis buffer according to the conditions in Table 4. 500 µl or 700 µl BloodPrep DNA Purification Solution was added and samples were thoroughly mixed. Samples that were to be purified on the 6700 workstation were moved to a deep-well plate for pre-centrifugation. The samples in 1.5 ml tubes were centrifuged at 2100 rpm for 2 min or 9000 rpm for 5 min in an Eppendorf centrifuge 5417R (Eppendorf). The samples in deep-well plates were centrifuged at 4000 rpm for 10 min in an Eppendorf centrifuge 5810R (Eppendorf). 950, 550, 475 or 375 µl of the supernatant was loaded manually to the 6100 prepstation or the deep-well plate was put in the 6700 workstation. The protocol for the workstations was followed; see below. Samples were eluted in 128, 136, 150 or 200 µl. The elution volume was adjusted so that the DNA concentration in the eluate theoretically would be the same as in the sample. The elution volume was calculated on the assumption that all DNA was eluted. Samples were analyzed by real-time PCR.

**Table 4. Lysis buffers and incubation times.**

| Lysis method | Reagenses | Volume added to sample (µl) | Incubation |
|---|---|---|---|
| Proteinase K | 1.12 mg/ml Proteinase K (Applied Biosystems), 50 mM Tris-HCl pH8 | 200 | 55 °C 60 min + 95 °C 10 min |
| Powerlyse | Powerlyse (Genpoint) | 100* | r.t. 4 min |
| Boiling | $ddH_2O$ | - | 99 °C 20 min** |
| Control | $ddH_2O$ | 200 | r.t. 60 min |
| Proteinase K | 1.2 mg/ml Proteinase K (Applied Biosystems) in PK-digestion buffer (Applied Biosystems) | 100 | 58 °C 10 min |
| Proteinase K | 1.2 mg/ml Proteinase K (Applied Biosystems) in PK-digestion buffer (Applied Biosystems) | 100 | 58 °C 60 min, 95 °C 10 min |
| Boiling | $ddH_2O$ | - | 99 °C 20 min* |
| Control | $ddH_2O$ | 100 | r.t. 60 min |

*After incubation 100 µl $ddH_2O$ was added so that the volume was the same in all samples.
** After incubation 200 µl $ddH_2O$ was added so that the volume was the same in all samples.
r.t. abbreviation for room temperature

## *2.6 DNA isolation on ABI Prism 6100 Nucleic Acid PrepStation*

DNA purifications were performed on an ABI Prism™ 6100 Nucleic Acid PrepStation (Applied Biosystems). Empty wells were sealed with adhesive tape to ensure even vacuum. In the following, if not other mentioned, vacuum was abrupt as soon as everything in the wells was evacuated. The samples were loaded into the wells and evacuated for 400 s at 80% vacuum. If the sample volume exceeded 650 µl, the rest was loaded in the well as soon as the first 650 µl had been evacuated. 650 µl BloodPrep DNA Purification Solution (Applied Biosystems) was added at 80% vacuum. This was followed by three wash-steps at 80% vacuum. The BloodPrep DNA wash-solution (Applied Biosystems) was added in the following amounts; 650 µl, 600 µl and 300 µl. A pre-elution vacuum at 100% for 120 s was

performed. Before the filter tray was moved to elution position a “touch off” was performed in order to release drips on the edge. 64-100 µl Elution Solution 1 (Applied Biosystems) was added and incubated with the DNA on the membrane for 180 s. Samples were eluted at 60% vacuum for 120 s. 64-100 µl BloodPrep DNA Elution Solution 2 (Appplied Biosystems) was added and vacuum was performed at 60% for 120 s. A touch off was performed and the plate was stored in the refrigerator or in the –20 °C freezer until analysis by real-time PCR.

## 2.7 DNA isolation on ABI Prism 6700 Automated Nucleic Acid Workstation

The software for the 6700 workstation was used to set up the protocol. The settings were programmed to do the following: 375 or 475 µl was taken from the samples in the deep-well and put on the filter tray. The samples were evacuated for 400 s at 80% vacuum. 650 µl BloodPrep DNA Purification Solution was added and 80% vacuum was applied for 400 s. This was followed by three wash-steps at 80% vacuum for 100 s. The BloodPrep DNA wash-solution was added in the following amounts; 650 µl, 600 µl and 300 µl. A pre-elution vacuum at 100% for 120 s was performed. 75 µl BloodPrep DNA Elution Solution 1 was added and incubated with the DNA on the membrane for 180 s. Samples were eluted at 60% vacuum for 120 s. 75 µl BloodPrep DNA Elution Solution 2 was added and vacuum was performed at 60% for 120 s.

## 2.8 Real-time PCR

The assay was performed on an ABI Prism 7500 (Applied Biosystems). The reverse primer was 5’-ATTGTTCCTTCTGCCGCTAAAA-3’ and the forward primer was 5’-TTGCTTGAAATTTATGAGCGTCTAC-3’ [39]. The reaction mixture contained 2.5 µl of the DNA template sample, 2 µl of each 10 pmol/µl primer (Thermo Electron Corp., Waltham, USA), 0.25 µl 20 mg/ml BSA (Sigma), 0.25 µl 1 U/µl AmpErase UNG (Applied Biosystems), 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems), 5.5 µl $ddH_2O$ (Sigma). The PCR-program was initiated with UNG activation at 50 °C for 2 min then a denaturation step at 95 °C for 10 min, followed by 40 cycles of repeated denaturation (95 °C, 15 s) and elongation (60 °C, 60 s). In the final cycle melting curves of the amplicons were determined by initially heating the mixture to 95 °C for 15 s, cooling it to 60 °C for 60 s, and controlled heating to 95 °C. All calculations of the Ct value were performed with the threshold set at 0.2. For simplicity, samples that were not detected were given Ct 40.00 (last cycle).

# 3 Results

## *3.1 Wash of B. cereus*

An overnight culture of bacteria contains a lot of dead cells, free DNA and celldebris. The DNA from dead cells will also be detected in the DNA assays; real-time PCR and at 260 nm absorbance. When these assays are used to study the effectiveness of a method for cell lysis it is important that as much free DNA as possible is removed so that only DNA from lysed cells will be seen in the assays. Removing free- or extra cellular DNA from the cell culture is a difficult task and here washing the cells in water were tested. Between the washes cells were pelleted using centrifugation. Since DNA is much lighter than cells, it stays in the supernatant when cells are pelleted and can be removed with the supernatant. A good speed of centrifugation is important. If the speed is too low, cells will not completely pellet, and cells are lost in the wash. If the speed is to high cells can be crushed from the force and release DNA, which can be incorporated with the cell-pellet. Also, if the centrifugational force is too high, free DNA in the supernatant might migrate to the cell-pellet.

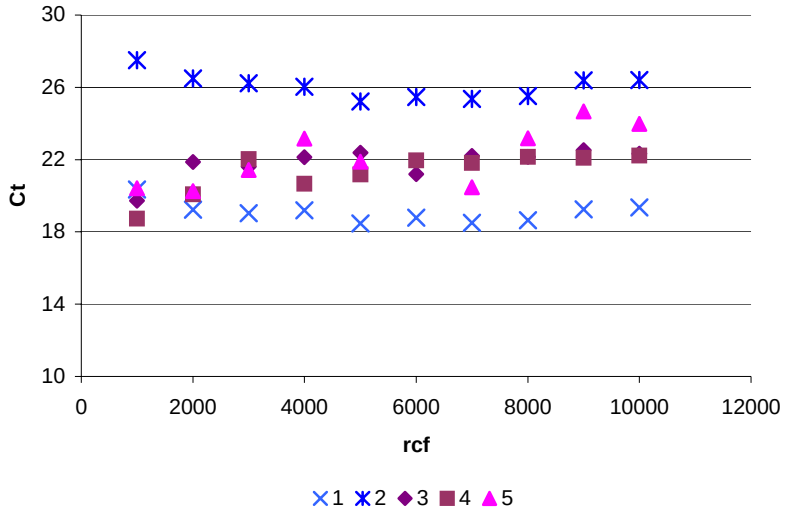



**Figure 8. Cell wash of *B. cereus* using different speed of centrifugation for pelleting.** Real-time PCR results from: 1. 10 x dilution of DNA purified from cell-lysate; 2. 100 x dilution of DNA purified from cell-lysate; 3. Supernatant after wash 1; 4. Supernatant after wash 2; 5. Supernatant after wash 3. For more information see material and methods. The values are based on one measurepoint.

Based on the results in Figure 8 we decided to use the speed of centrifugation of 8000 rcf. A lot of DNA was collected from the pellet (10 x dilution Ct 18.6, 100 x dilution Ct 25.5) and the concentration of DNA in the supernatant was among the lowest (wash 1; Ct 22.16, wash 2; Ct 22.2, wash 3; Ct 23.2). Even though cells were washed there was still a lot of DNA in the supernatant, though it was not enough to be measured with the nanodrop method, which means that the concentration was below 1.5 ng/µl. This DNA could come from the free DNA in the overnight culture or it could be from lysis of cells during the wash-step. Since there was no salt added to the water there was a possibility that cells were lysed due to osmotic shock.

## 3.2 Screening of enzymes and detergents for lysis of B. cereus in pellets

For the detection of bacteria using real-time PCR, the DNA has to be released and become available for the PCR reaction. Therefore, viable or intact cells have to be lysed in order to release the DNA for detection.

This study was focused on screening of a selected number of enzymes and detergents that could be used for the lysis of *B. cereus*. Different combinations of enzymes and detergents were also tested.

**Table 5. Determination of DNA-concentration in cell-lysates using nanodrop.** A washed pellet of *B. cereus* strain KBM1 containing $9*10^6$ cfu was lysed with different lysis buffers (for more details see material and methods). DNA from lysates was purified and the concentration of the DNA in the eluates was determined using the nanodrop method. The lysis of pellet with Powerlyse was done with another overnight culture. The eluate was also analysed using real-time PCR after a 100 x dilution in $ddH_2O$. The result was from one measurepoint.

| Sample ID | | ng/µl | 260/280 | 260/230 | Chromosome equivalent* | Ct |
|---|---|---|---|---|---|---|
| 8 | Proteinase K + Lysozyme + Mutanolysin | 64.9 | 2.0 | 2.3 | 11.4 | 20.4 |
| 6 | Proteinase K + Mutanolysin | 61.1 | 2.1 | 2.4 | 10.7 | 20.3 |
| 11 | Proteinase K + 1% SDS + 0.05% Tween20 | 57.7 | 2.0 | 3.9 | 10.1 | 20.4 |
| 12 | Lysozyme + EDTA + 1% SDS +proteinase K | 55.4 | 2.1 | 2.4 | 9.7 | 20.5 |
| 13 | Proteinase K + EDTA + 2% SDS + 2% Sarkosyl | 49.1 | 2.1 | 2.3 | 8.6 | 21.0 |
| 9 | Proteinase K + 1% SDS | 48.4 | 2.2 | 3.5 | 8.5 | 20.6 |
| 5 | Proteinase K + Lysozyme | 46.7 | 2.1 | 2.3 | 8.2 | 20.4 |
| 2 | Proteinase K | 37.6 | 2.0 | 2.4 | 6.6 | 20.3 |
| 10 | Proteinase K + 0.05% Tween20 | 35.2 | 2.0 | 2.2 | 6.2 | 20.4 |
| 4 | Mutanolysin | 29.4 | 2.1 | 2.3 | 5.2 | 21.3 |
| 3 | Lysozyme | 25.5 | 2.2 | 2.2 | 4.5 | 24.1 |
| NA | Powerlyse | 23.9 | 1.9 | 1.8 | 4.2 | - |
| 15 | Tris-HCl 55 °C +95 °C | 22.7 | 2.1 | 2.4 | 4.0 | 21.4 |
| 7 | Lysozyme + Mutanolysin | 13.5 | 2.1 | 2.2 | 2.4 | 24.6 |
| 14 | Tris-HCl 37 °C | 12.4 | 2.2 | 2.2 | 2.2 | 21.7 |
| 1 | TritonX-100 | 10.7 | 1.7 | 1.7 | 1.9 | 22.1 |

* $10^6$ chromosomes/µl

The calculations of chromosome equivalents are based on the assumption that the genome of *B. cereus* is $5.22*10^6$ bp [36]. One basepair weighs $1.01*10^{-21}$ g. According to this, the mass of one chromosome of *B. cereus* is $5.7*10^{-15}$ g.

If the purified DNA comes from colony-forming units only, the number of chromosome equivalents in the eluate should be $4.5*10^4$ chromosomes/µl since the cell pellet contained $9*10^6$ cfu and was eluted in 200 µl. According to the results in Table 5 there is approximately 40 to 250 times more DNA than expected in the eluate. This suggests that it is not enough to wash the cells with water to get rid of the majority of free DNA in an overnight culture. The extra DNA could also come from dead cells or viable cells that for some reason does not grow on the agar plate.

As long as there are more chromosome equivalents than viable cells in the samples, real-time PCR is not a good detection method when the effects of the different lysis buffers are to be studied.

It should be noted that most DNA was obtained with lysis buffers that included one or two enzymes that degrade the cell wall and proteinase K that degrades proteins. Less DNA was obtained when only the enzymes that degrade the cell wall, and no proteinase K, were included in the lysis buffer. For example, the difference in the amount of DNA obtained in sample 3 and 8 is approximately two times when measured with nanodrop. The difference between the two samples is 4 Ct values, which correspond to a good ten fold. The difference was rather big between the two detection methods when looking at these two samples. In most other cases the two methods yielded similar results. Another exception was in the samples where the lysis buffer contained lysozyme but lacked proteinase K.

## *3.3 Evaluation of ABI Prism 6100 Nucleic Acid PrepStation*

Real-time PCR offers a quick detection method of *B. anthracis*. Since most biological and environmental samples contain substances that inhibit the PCR it is relevant to perform a sample preparation step to purify and enrich the DNA. The challenge is to find a method that achieves good quality DNA from all different sample types. In this assay the 6100 prepstation will be evaluated as a method for DNA purification. For this evaluation 47 different samples (matrices) with different physical and chemical properties was used. The matrices were a selection of animal samples, animal feed, food and environmental samples (Appendix 1).

The 6100 prepstation purification principle is based on a flow-through filter and that fact caused a lot of problems at first. Many of the samples contained a lot of particles that clogged the filter. In the BloodPrep protocol [34] there is no centrifugation or filtration step to remove particles. Since almost all matrices were impossible to purify without removing particles first, a centrifugation step was introduced. At first centrifugation was done right after the lysis step, and then the supernatant was mixed with BloodPrep DNA Purification Solution. Later, we decided that centrifugation should be done after the addition of BloodPrep DNA Purification Solution since it contained detergents that kept the DNA in solution. In the first of the following experiments, the samples were centrifuged in 1.5 ml tubes at 2100 rpm for 2 min, but the pellet was still loose and sometimes caused problems. Therefore, a faster centrifugation step of 9000 rpm for 5 min was used (see 3.3.3) and this was used for the following experiments. In some experiments deep-well plates were used and they were centrifuged at 4000 rpm for 10 min.

After centrifugation the supernatant was put on the membrane of the 6100 prepstation. In the first of the following experiments 950/1050 µl of the centrifuged samples were put on the membranes. In the later experiments 475/950 µl or 375/850 µl of the centrifuged samples were put on the membranes. The partial volume that was put on the membrane was changed because this minimized the accidental disruption of the pellet.

### 3.3.1 PCR-inhibition and DNA-recovery in all matrices

To study if the 6100 prepstation was capable of removing the PCR-interfering substances from the different matrices, all matrices were prepared on the 6100 prepstation. The eluates from the samples were spiked with the same amount of DNA and analysed with real-time PCR. If PCR-inhibitors were present in the samples after the purification, the Ct value should

be raised compared to $ddH_2O$, which do not contain PCR-inhibitors. None of the matrices inhibited the real-time PCR (Figure 9). The mean value for all the samples was Ct 25.5. When samples were spiked with DNA before purification some matrices gave a higher Ct than the control ($ddH_2O$, Ct 26.0) (Figure 9). Mean value for all matrices was Ct 29.0. At this stage it was difficult to say if it was PCR-inhibitors that co-extracted with DNA or if the DNA was degraded in the matrix or if DNA precipitated with the pellet. Another possibility was that the membrane was saturated with background DNA leading to the loss of *Bacillus* DNA. 15 of the samples were not included in Figure 9 since they were problematic to purify because they sometimes clogged the membrane (Table 6).

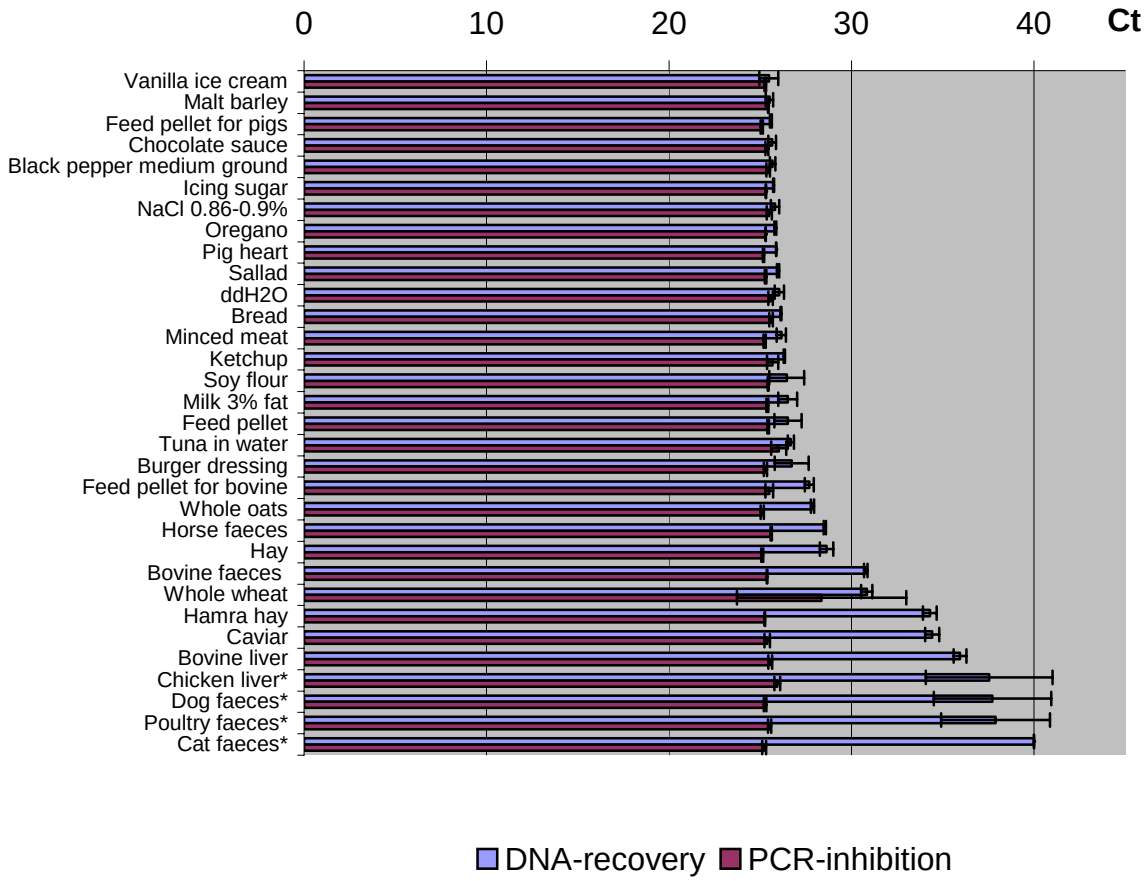



**Figure 9. PCR-inhibition and DNA-recovery in matrices purified with ABI Prism 6100 Nucleic Acid PrepStation**. Purple bars: PCR-inhibition; 150 µl matrix was mixed with 500 µl DNA purification solution, samples were centrifuged at 2100 rpm for 2 min. 550 µl was added to the membrane of the 6100 prepstation and elution was done in 128 µl. Eluates were spiked with 0.016 ng/µl DNA from *B. cereus* strain KBM1. Blue bars: DNA-recovery; 150 µl of matrix spiked with 0.016 ng/µl DNA from *B. cereus* strain KBM1 was added to 200 µl $ddH_2O$ (instead of lysis buffer). 700 µl DNA purification solution was added. Samples were centrifuged at 2100 rpm for 2 min and 950 µl of supernatant was added to the wells. Samples were eluted in 136 µl. Samples were run in duplicates and the reported Ct value is the mean of the two samples. *Undetected samples were given a Ct value of 40.0.

Common for the problematic matrices was that they did not form a sufficiently hard, small and compact pellet when centrifuged at 2100 rpm for 2 min. Many of the problematic matrices absorbed a lot of water or contained a lot of particles. The problem arose when particles clogged the membrane.

**Table 6. Ct values of problematic matrices spiked with 0.016ng/µl DNA from *B. cereus* strain KBM1.** The matrices in the table were difficult to purify on the 6100 prepstation. The matrices sometimes clogged the membrane in the 6100 prepstation. Here samples were run on the 6100 prepstation but samples that were not able to pass the filter were removed using a pipette and then the purification protocol for the 6100 prepstation was followed as much as possible, but when a solution did not go through the membrane it was removed. 150 µl of matrix spiked with 0.016 ng/µl DNA from *B. cereus* strain KBM1 was added to 200 µl $ddH_2O$ (instead of lysis buffer). 700 µl BloodPrep DNA Purification Solution was added. Samples were centrifuged at 2100 rpm for 2 min and 950 µl of supernatant was added to the wells in the 6100 prepstation. Samples were run in duplicates and the reported Ct value is the mean of the two samples.

| Matrix | Ct | Stdv |
|---|---|---|
| $ddH_2O$ | 25.0 | 0.3 |
| NaCl 0.86-0.9% | 25.6 | 0.1 |
| Wholemeal porridge | 25.7 | 0.1 |
| Curry | 26.1 | 0.2 |
| Feed pellet for dog | 26.3 | 0.7 |
| Corn flour | 27.2 | 0.8 |
| Growth feed pellet for pigs | 28.1 | 0.6 |
| Feed pellet for horse | 28.2 | 1.4 |
| Feed pellet for poultry | 28.7 | 1.3 |
| Yeast feed | 29.2 | 1.4 |
| Whole corn feed | 30.7 | 0.2 |
| Wholemeal gruel | 30.9 | 0.1 |
| Orange juice | 31.1 | 1.1 |
| Pig faeces | 33.6 | 0.1 |
| Egg* | 36.5 | - |
| Whipping cream* | 37.6 | - |
| Muesli* | - | - |

*No signal obtained for one or both of the samples.

### 3.3.2 The influence of lysis on DNA-recovery

It is important that the choice of lysis method actually lyses viable cells and spores but the degradation of free DNA by the method should also be considered. Here, three protocols for cell lysis were evaluated for the recovery of free DNA in different matrices using the 6100 prepstation. The chosen methods were boiling, a rapid and safe method; proteinase K treatment, an enzyme-based method and Powerlyse, a commercial lysis buffer with 4 M Guanidine thiocyanate (detergent) as one of the components. SDS was also studied as a lysis buffer but when it was mixed with BloodPrep DNA Purification Solution it reacted and formed a jelly-like substance.

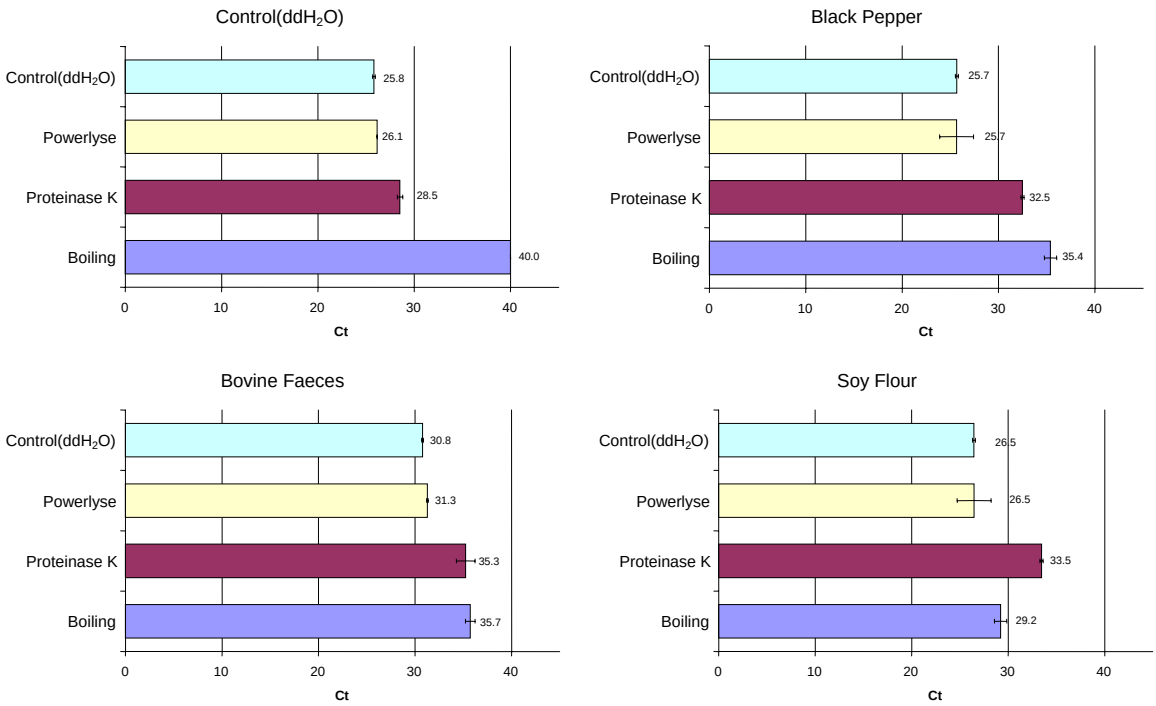



**Figure 10. Influence of the choice of lysis method on DNA-recovery in different matrices.** 150 µl of black pepper, soy flour, bovine faeces or $ddH_2O$ was spiked with 0.016 ng/µl of DNA from *B. cereus* strain KBM1. The matrices and the added DNA were treated with different lysis methods; 200 µl 1.12 mg/ml proteinase K in 50 mM Tris-HCl pH 8 56 °C 60 min + 95 °C 10 min, 100 µl Powerlyse r.t. 4 min, boiling 99 °C 20 min or 200 µl $ddH_2O$ (control) r.t. 60 min. After incubation 100 µl $ddH_2O$ was added to the sample with Powerlyse. After incubation the samples were mixed with 700 µl BloodPrep DNA Purification Solution (500 µl was added to the boiled samples). The samples were centrifuged at 2100 rpm for 2 min. 950 µl (550 µl for boiled samples) of the supernatant was loaded onto the 6100 prepstation. Samples were eluted in 136 µl (128 µl for boiled samples). PCR-results are from eluates after purification on the 6100 prepstation. All samples were done in duplicates. Mean values for the matrices were; Black pepper Ct 29.8 (4.6), soy flour Ct 28.9 (3.1), bovine faeces Ct 33.3 (2.4) and control ($ddH_2O$) Ct 31.0 (6.1). Samples that were not detected with the real-time PCR method were given Ct 40.0.

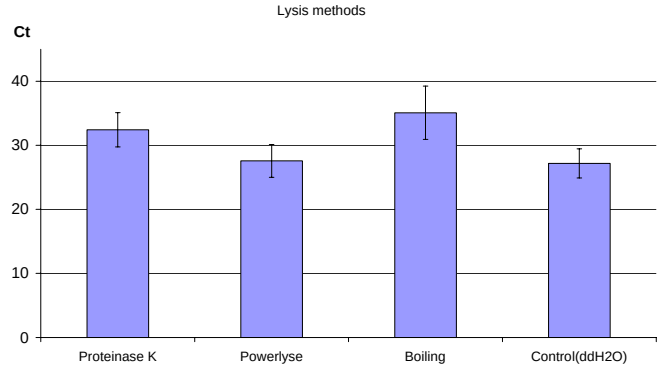



**Figure 11. Mean value of eight measurepoints of the different lysis methods in the tested matrices, i. e. black pepper, soy flour, bovine faeces and $ddH_2O$**. Proteinase K, Ct 32.4 (2.7); Powerlyse, Ct 27.6 (2.6); Boiling, Ct 35.1 (4.2); $ddH_2O$, Ct 27.2 (2.3).

The different lysis methods turned out to give different results of DNA recovery. Powerlyse was equal to the control but boiling and treatment with proteinase K seemed to degrade the DNA (Figure 11). In Figure 10 it can be seen that when Powerlyse was used on black pepper the same amount of DNA was recovered as when Powerlyse was used on water. Powerlyse on soy flour gave 1 Ct higher than Powerlyse on water, which in general can be said to represent

half of all DNA. The bovine faeces was the least successful matrix when it came to DNA-recovery. Somehow DNA was lost in bovine faeces; compared to water the results were approximately 5 Ct values higher in bovine faeces when treated with Powerlyse and proteinase K. 5 Ct values can in general be said to represent a loss of more than 90% of all DNA.

### 3.3.3 The influence of centrifugation on DNA-recovery

The largest problem with the 6100 prepstation purification system was clogging of the membrane. The problem arose from particles in the samples. The vacuum could then not handle to suck the sample through the membrane, which led to spoiling of the samples. To overcome the problem a centrifugation step was incorporated into the preparation step and only the supernatant was put on the membrane. This assumed that cells were lysed, because cells will pellet together with the particles in the matrix, while the DNA from lysed cells stays in the supernatant. But here only free DNA was studied, excluding that problem. In earlier experiments, centrifugation was done at 2100 rpm for 2 min, but then the pellet was still loose and incautious handling led to that particles clogged the membrane. The results in Figure 12 show that a faster centrifugation could be used, without loosing DNA. The mean values for the methods were Ct 30.2 (5.6) for 9000 rpm and Ct 30.8 (4.9) for 2100 rpm. For this purpose both previously problematic and non-problematic matrices were chosen.

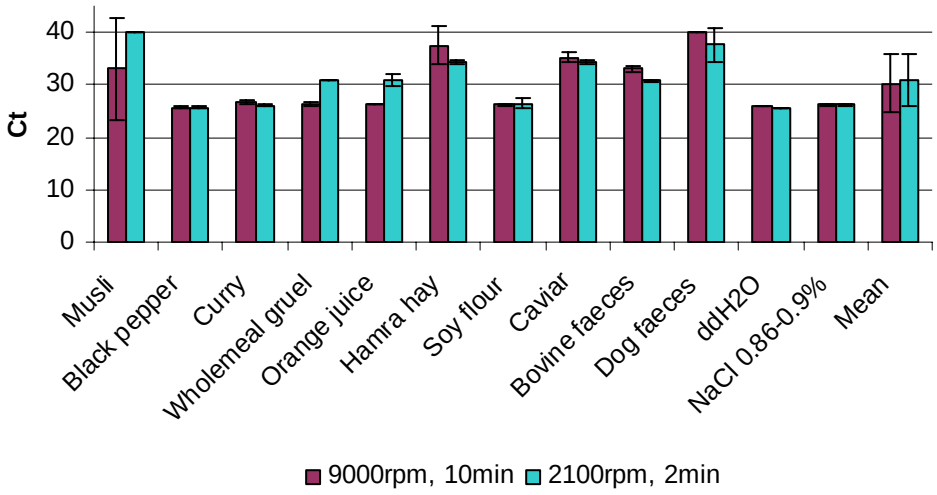



**Figure 12. Ct values of matrices centrifuged with different speed before purification on the 6100 prepstation.** 150 µl of matrix spiked with 0.016 ng/µl DNA from *B. cereus* strain KBM1 was added to 200 µl $ddH_2O$ (instead of lysis buffer). 700 µl BloodPrep DNA Purification Solution was added to give the right binding properties to the purification filter in the 6100 prepstation. Samples were centrifuged at either 2100 rpm for 2 min or 9000 rpm for 5 min. 950 µl of supernatant was added to the wells of the 6100 prepstation. Samples were eluted in 136 µl. Samples were run in duplicates and the reported Ct value is the mean of the two samples. Undetected samples were given the Ct value of 40.0.

### 3.3.4 Verification of ABI Prism 6700 Automated Nucleic Acid Workstation

The 6700 workstation is based on the same purification system as the 6100 prepstation. As the 6100 prepstation is superior when it comes to supervision of the samples it is advantageous to find a good protocol on the 6100 prepstation, where evacuation can be supervised, and then move it to the 6700 workstation.

We next tried DNA purification from some of the "easy" matrices using the 6700 workstation. For extra precaution, only 425/850 µl of the supernatant was loaded into the wells. This was done to make sure that the pipette did not disrupt the pellet. The results of the DNA recovery from the different matrices were compared to a real-time PCR standard curve that was prepared from the same dilution series of DNA that was used to spike the matrix. Theoretically, the concentration of DNA in the eluate should be half as much as the concentration of the spiked matrices. Only as a simple comparison, the Ct value this corresponds to was calculated from the standard curve. The theoretical value is only to be taken as a guideline. The standard curve was done in $ddH_2O$ but for a better comparison it could have been diluted in Elution Solution 1 and 2, since there is a chance that the elution buffer affects the real-time PCR in comparison to $ddH_2O$. The recovery was better in some of the matrices than in water. The Ct for the different matrices ranged from 0.3 Ct-values lower than the theoretical value to almost two Ct-values above the theoretical value (Table 7).

**Table 7. DNA-recovery in matrices purified with ABI Prism 6700 Automated Nucleic Acid Workstation.** 150 µl of matrix spiked with 0.032 ng/µl DNA from *B. cereus* KBM1 was mixed with 700 µl BloodPrep DNA Purification Solution. Samples were centrifuged in a 96-deep-well plate at 4000 rpm for 10 min. The deep-well plate was placed in the 6700 Workstation, which was programmed to put 425 µl onto the membrane. Samples were eluted in 150 µl. Samples were run in duplicates. Real-time PCR was set up manually from the elution plate.

| Matrix | Ct | Stdv |
|---|---|---|
| Salad | 26.3 | 0.2 |
| Black pepper medium ground | 26.4 | 0.1 |
| Oregano | 26.5 | 0.2 |
| Malt barley | 26.5 | 0.5 |
| Icing sugar | 26.9 | 0.1 |
| Bread | 26.9 | 0.4 |
| Pig heart | 27.1 | 0.1 |
| Soy flour | 27.3 | 0.0 |
| Chocolate sauce | 27.8 | 0.0 |
| $ddH_2O$ | 27.8 | 0.3 |
| NaCl 0.86-0.9% | 27.8 | 1.4 |
| Feed pellet for pigs | 28.3 | 0.2 |
| Theoretical value | 26.6 | |

To verify that both the 6100 prepstation and the 6700 workstation gave the same results, three matrices and water were spiked with DNA. Samples were treated either with water or with Powerlyse and then purified with both the 6100 prepstation and the 6700 workstation. The result shows (Table 8) that the 6100 prepstation and the 6700 workstation give more or less the same result.

**Table 8. Comparison between ABI Prism 6100 Nucleic Acid PrepStation and ABI Prism 6700 Automated Nucleic Acid Workstation**. 150 µl of matrix spiked with 0.032 ng/µl DNA from *B. cereus* KBM1 was mixed with either 100 µl ddH2O and incubated at r.t. for 60 min or 100 µl Powerlyse and incubated at r.t. for 4 min. 500 µl BloodPrep DNA Purification Solution was added and samples were centrifuged in deep-well plates at 4000 rpm for 10 min. 37 5µl was added to the wells. Samples were purified either on the 6100 prepstation or the 6700 workstation. Samples were eluted in 150 µl. Samples were done in triplicates.

| Treatment | Matrix | 6100 | Stdv | 6700 | Stdv |
|---|---|---|---|---|---|
| $ddH_20$, r. t. 60 min | Black pepper | 26.8 | 0.5 | 27.9 | 0.2 |
| | Hamra hay | 39.7 | ** | - | *** |
| | Bovine faeces | 39.2 | ** | 34.7 | ** |
| | $ddH_2O$ | 27.2 | 0.1 | 29.6 | 2.6 |
| Mean $ddH_2O$ | | 30.10 | 5.76 | 29.56 | 2.84 |
| Powerlyse, r.t. 4 min | Black pepper | 27.1 | 0.6 | 27.7 | 0.7 |
| | Hamra hay | 32.1 | 0.9 | 31.3 | 0.3 |
| | Bovine faeces | 34.7 | 0.8 | 34.1 | 1.5 |
| | $ddH_2O$ | 27.2 | 0.8 | 27.3 | 0.3 |
| Mean Powerlyse | | 30.3 | 3.5 | 30.1 | 3.0 |
| Mean both methods | | 30.2 | 4.39 | 29.9 | 2.9 |

*No detection of DNA in one of the samples
**No detection of DNA in two of the samples
***No detection of DNA in any of the three samples

### 3.3.5 The effect of boiling and PK-digestion buffer on DNA recovery

In 3.3.2 it was seen that the DNA could not be detected in the samples prepared with certain lysis methods. It was surprising that DNA added to water and then boiled could not be detected with the real-time PCR method, so we decided to re-do that experiment using new water. It was also tested if proteinase K did not have to be inactivated. In the protocol for BloodPrep [34] proteinase K is not inactivated. The proteinase K treatment was this time done in PK-digestion buffer instead of 50 mM Tris-HCl pH 8, because that was the recommendation in the BloodPrep protocol. This was not used in the earlier experiments because it was desirable to compare the results with previous experiments where proteinase K from Roche Diagnostics had been used.

In Figure 13 it can be seen that the DNA could not be detected after it had been boiled in water. Mixed in with the different matrix types, the DNA was only barely detected after boiling. It could also be seen that an inactivation of proteinase K was not necessary. In Figure 14 it can be seen that the proteinase K treatment was superior the control and Powerlyse in all the matrices. First this was thought to be the result of a background flora of *B. cereus* but this theory was later rejected (see 3.3.6).

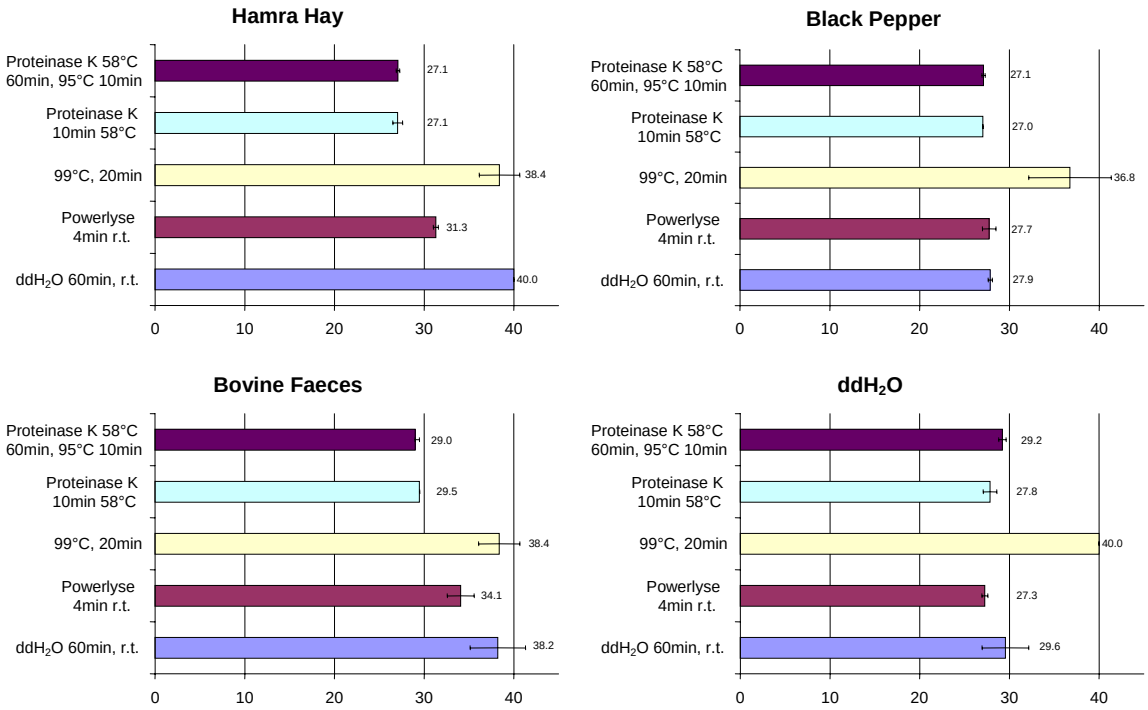



**Figure 13. Influence of lysis method on DNA-recovery in different matrices.** Black pepper, hamra hay, bovine faeces or $ddH_2O$ was spiked with 0.032 ng/µl of DNA from *B. cereus* strain KBM1. The matrices were treated with different lysis methods; 1.2 mg/ml proteinase K in PK-digestion buffer 58 °C 60 min + 95 °C 10 min, 1.2 mg/ml proteinase K in PK-digestion buffer 58 °C 10 min, Powerlyse r.t. 4 min, boiling 99 °C 20 min or $ddH_2O$ (control) r.t. 60 min. 150 µl of the matrix was mixed with 100 µl of the lysis buffer and incubated. 100 µl $ddH_2O$ was added to the boiled samples. 500 µl BloodPrep DNA Purification Solution was added and the samples were moved to a deep-well plate and centrifuged at 4000 rpm for 10 min. 375 µl of supernatant was purified using the 6700 workstation. Samples were eluted in 150 µl. All samples were done in duplicates except samples treated with Powerlyse or water, which were done in triplicates. Mean values and standard deviation for the matrices were: Black pepper; Ct 29.4 (4.2), hamra hay; Ct 34.5 (5.5), Bovine faeces; Ct 35.3 (4.0) and $ddH_2O$; Ct 30.6 (5.2). Samples that were not detected in the real-time PCR were given the Ct value of 40.0.

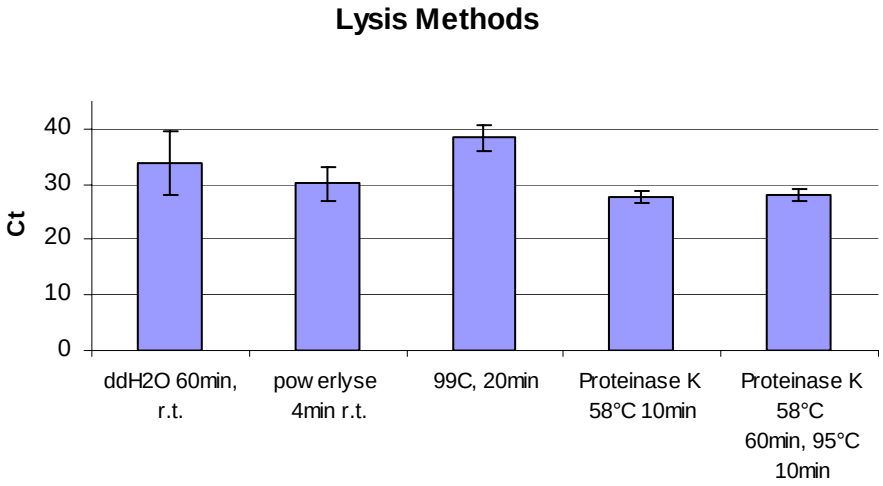



**Figure 14. Mean value of eight measurepoints of the different lysis methods in all matrices, i. e. black pepper, hamra hay, bovine faeces and $ddH_2O$**. $ddH_2O$, Ct 33.2 (5.8); Powerlyse, Ct 30.1 (3.0); boiling, Ct 38.4 (2.5); proteinase K 10 min, Ct 27.9 (1.1); proteinase K 60 min, Ct 28.1 (1.1).

### 3.3.6 Negative control of matrices

Since bovine faeces and hamra hay gave so much better results with the proteinase K treatment it was suspected that there was a background flora of *B. cereus*. Therefore the matrices hamra hay, bovine faeces, soy flour and black pepper were treated with proteinase K in PK-digestion buffer without spiking of DNA and purified with the 6100 prepstation. None of the samples yielded a signal in the real-time PCR, showing that there was no *B. cereus* DNA in the matrices (background) as previously suspected.

### 3.3.7 The volume of BloodPrep DNA Purification Solution needed in a pure system (dd$H_2O$)

When comparing all the performed experiments it seemed like the added volume of BloodPrep DNA Purification Solution influenced the DNA-recovery. Therefore this was tested on water. In Table 9 it can be seen that the amount of DNA purification solution does not have an influence on DNA-recovery in water. However it might have an influence in matrices, but this has to be investigated. It can also be seen that the recovery is not a 100% when compared to Ct values obtained from the standard curve (same calculations as in 3.3.4).

**Table 9. Influence of the volume of DNA purification solution on DNA-recovery in water.** 150 µl of dd$H_2O$ spiked with 0.032 ng/µl DNA from *B. cereus* KBM1 was added to 100 µl dd$H_2O$, 100 µl Powerlyse or 100 µl proteinase K solution (15 µl proteinase K + 85 µl PK-digestion buffer). 500 µl or 700µl BloodPrep DNA Purification Solution was added. Samples were centrifuged at 9000 rpm for 5 min. 375 µl or 475 µl was purified with the 6100 prepstation. Samples were eluted in 150 µl. Samples were done in duplicates.

| Lysis method | DNA pur sol | Ct | Stdv |
|---|---|---|---|
| dd$H_2O$ | 500 | 26.2 | 0.0 |
| dd$H_2O$ | 700 | 27.0 | 0.2 |
| ProtK + PK-buffer | 500 | 26.2 | 0.1 |
| ProtK + PK-buffer | 700 | 26.8 | 0.1 |
| Powerlyse | 500 | 26.2 | 0.1 |
| Powerlyse | 700 | 26.5 | 0.2 |
| Theoretical | | 25.6 | |

# 4 Discussion

ABI Prism 6700 Automated Nucleic Acid Workstation and the ABI Prism 6100 Nucleic Acid PrepStation were evaluated for the purification of *B. cereus* DNA from animal samples, animal feed, food and environmental samples.

It was here shown that the 6700 workstation and the 6100 prepstation could be used for the purification of DNA from 26 different sample types. This was possible after the introduction of a centrifugation step into the existing standard protocol for DNA purification from blood [34]. (Figure 15)

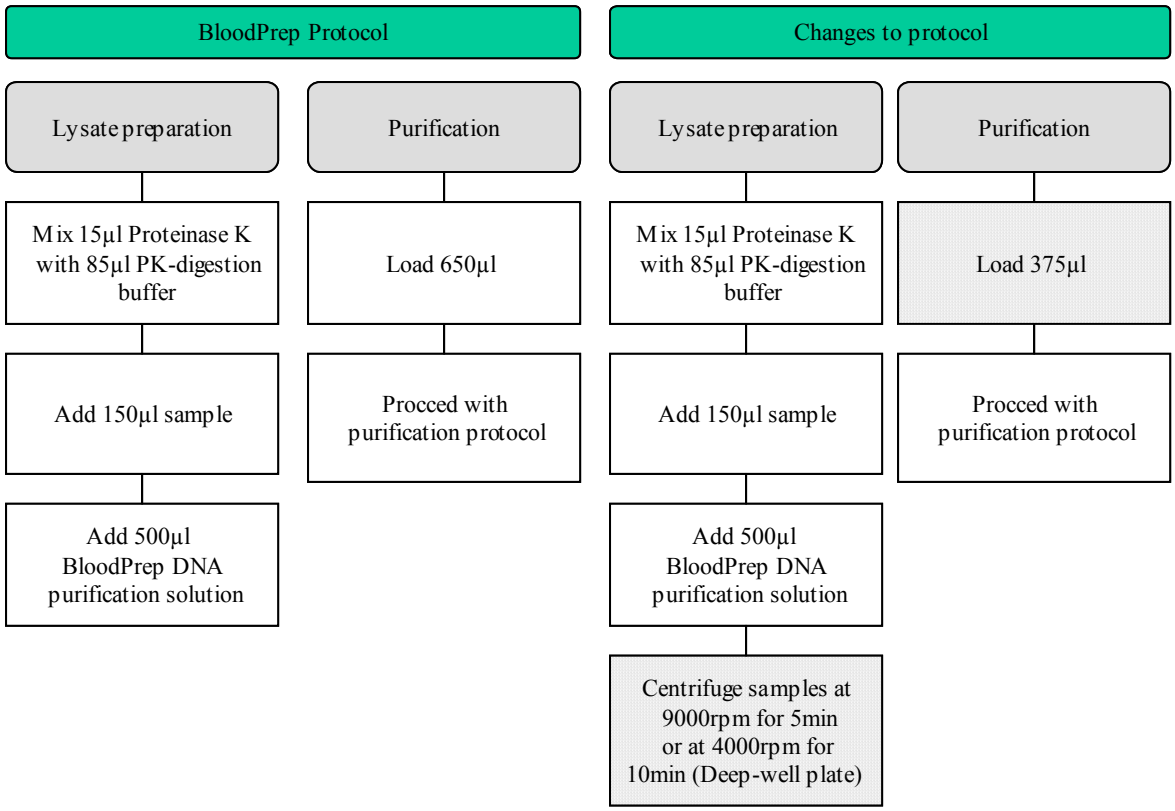



**Figure 15. Changes to protocol.** The original protocol was for the DNA extraction from blood by ABI Prism 6100 Nucleic Acid PrepStation from Applied Biosystems

Some samples were difficult to purify on the 6100 prepstation (Table 6). These samples include matrices that absorb a lot of water (muesli, porridge), contain a lot of particles (curry) or contain a non-liquid phase that cannot be centrifuged to the bottom (egg, whipping cream). With the new suggested protocol in Figure 15 even some of the difficult samples can probably be purified using the 6100 prepstation.

The greatest advantage of the 6100 prepstation is that none of the samples showed any signs of PCR inhibition after purification (Figure 9). Lindberg noted that some of these samples still contained inhibiting substances after the purification on Biorobot EZ1 (Qiagen) [35]. Especially inhibiting in those experiments were bovine faeces, dog faeces and black pepper but here they show no signs of being inhibiting after purification using the 6100 prepstation.

These results demonstrate that the choice of sample preparation station has to be considered for each sample type.

It is important to find a method that is reliable for the recovery of DNA. An aspect that should be regarded is the degradation of DNA when using different lysis methods. A good lysis method should lyse all cells and release the DNA but not degrade the DNA when it is in solution. Here proteinase K, Powerlyse and boiling were studied to see how the methods affect the recovery of DNA from different matrices. From a biosafety aspect, the boiling method should be preferred. It is a quick method and hands-on steps as addition of enzymes or detergents are avoided. However, here it was though shown that the boiling method reduced the possibility to detect the DNA with real-time PCR, which most likely is the effect of DNA degradation. It is difficult to speculate which method is the best since the lysis of cells were not studied; only how much free DNA that can be recovered. The best method for DNA recovery was the lysis with proteinase K in PK-digestion buffer. This is especially seen in bovine faeces and hamra hay (see 3.3.5). These matrices may contain DNases that degrade the free DNA but somehow the proteinase K in combination with PK-digestion buffer protects the DNA from degradation. It is possible that proteinase K digests DNases and thus protects the DNA. One possibility why this was not seen in 3.3.2 is that proteinase K from Applied Biosystems only is active in PK-digestion buffer also supplied by the manufacturer. The other explanation is that there could be some protective substance in PK-digestion buffer. Black pepper is a processed food, and has probably been heated or radiated, which inactivates the DNases. Therefore DNA is probably not degraded in this matrix and no difference is seen to the control (dd$H_2O$ in r.t.). Another explanation could be that DNA is pelleted together with the matrix, when no detergents are added. The content of PK-digestion buffer is unknown but Powerlyse is known to contain guanidine thiocyanate that can act protective of DNA and liberate the DNA from interactions with the matrix. Maybe that is why Powerlyse is a better method than water only.

For the simulation of infected samples, it would have been preferred to spike the samples with viable cells of *B. cereus*. It would then be possible to study the whole chain of sample preparation from lysis of cells to purification and detection (Figure 2). The spiking of samples with overnight culture was though biased by free DNA or DNA from dead cells (see 3.2). An attempt was done to wash the cells but there was still a lot of free DNA (see 3.1). Cells were washed in dd$H_2O$ but perhaps a different wash buffer should have been used to make sure that cells were not lysed because of osmosis. Instead an isotonic solution of NaCl was considered for that purpose, but in an experiment it was shown that 0.86-0.9% NaCl inhibit the PCR-reaction (results not shown). The amount of DNA in the supernatant can then not be measured with reliable result without purification. *Fyske et al*., 2003, used ice-cold 10 mM Tris-HCl pH 8 to wash *B. cereus* but that was not tested here [37]. There are several other methods that can be considered for the minimisation of extracellular or free DNA. *Kuske et al*., 1998, used cells in mid-log phase to minimise the amount of free DNA and they also propose that previous reports of lysis of *Bacillus* spores are biased by extracellular DNA [38]. Another method could be to remove the free DNA using magnetic beads that adsorb DNA. Before viable cells can be used a good protocol for the removal and verification of removed free DNA has to be found, which was out of the scope for this thesis. Real-time PCR detects viable cells, dead cells and free DNA. Here we used cfu (colony forming units) to count the number of cells, but it would have been better to count the cells in a microscope or with flow cytometry to obtain the relationship between dead and viable cells and then more conclusions could have been drawn about the amount of free DNA. The signal might not arise from free DNA, but rather from DNA from dead and non-growing cells.

The new protocol for DNA purification using the Applied Biosystems purification system seems promising, but it has to be tested on more samples. For example, the faster centrifugation step can be the determinant that enables the purification of the difficult matrices. The recovery of free DNA in the faeces, chicken liver, caviar and hay samples (Figure 9) can probably be enhanced by using proteinase K in PK-digestion buffer, but this has to be tested. In order to study the whole chain of sample preparation the next self-evident step is to find a good lysis step for viable cells, but first the problem with free DNA in cell-cultures has to be overcome. A promising method for cell lysis is the use of an enzyme for the destruction of the cell wall in combination with proteinase K for the protection against DNases. When a good lysis step has been found, the use of a centrifugation step in the purification protocol has to be evaluated for viable cells.

The use of an automated process can reduce the time required for the analysis. More samples can be run at the same time, and less man-time is needed. This reduces both the costs and the workload for the work force. The work is also safer as less hands-on is needed.

There are several aspects to be considered when chosing sample preparation station. It has been shown that the 6700 workstation is a suitable platform for DNA purification preceding the real-time PCR. The advantage with the 6700 workstation is the purity of the extracted DNA and the fact that it performs the setup of real-time PCR plates automatically. The draw-back with the 6700 workstation is the risk for overflow of wells leading to cross-contamination of the samples. Since the 6700 workstation is only a BSL-2 cabinet it also has to be build into a BSL-3 cabinet before it can be used for *B. anthracis.* The 6100 prepstation on the other hand, is small enough to be put into an already existing BSL-3 cabinet but its disadvantage is that it needs a lot of hands-on handling. The EZ1 is also a small robot that could fit into a small cabinet but it has the disadvantage that it takes only six samples at the time and PCR-inhibition is also a bigger problem compared to the Applied Biosystems purification system.

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First of all I wish to thank my supervisor Rickard Knutsson for the opportunity to perform my degree project at SVA. I would also like to thank you for answering all questions that arose during the run of the project. I also wish to thank Sara Johansson for all help and guidance both in the laboratory and with questions that arose during the work. I also wish to thank my scientific reviewer Björn Herrmann and also Karin Holmström, Sara Ahlgren and Pia Damm for revision of the report and helpful suggestions to improvements.

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# Appendix 1

| Sample Name | Matris | Matrix |
|---|---|---|
| M1 | Standard mjölk | Milk 3% fat |
| M2 | Vispgrädde | Whipping cream |
| M3 | Vaniljglass | Vanilla ice cream |
| M4 | Vetemjöl | Wheat flour |
| M5 | Majsmjöl | Corn flour |
| M6 | Müsli | Muesli |
| M7 | Florsocker | Icing sugar |
| M8 | Hamburgedressing | Burger dressing |
| M9 | Tonfisk i vatten | Tuna in water |
| M10 | Chokladsås | Chocolate sauce |
| M11 | Svartpeppar mellan | Black pepper medium ground |
| M12 | Oregano | Oregano |
| M13 | Curry | Curry |
| M14 | Fullkornsvälling | Wholemeal gruel |
| M15 | Fullkornsgröt | Wholemeal porridge |
| M16 | Ketchup | Ketchup |
| M17 | Apelsinjuice | Orange juice |
| M18 | Hel fodermajs | Whole corn fodder |
| M19 | Helt vete | Whole wheat |
| M20 | Jästfoder | Yeast feed |
| M21 | Foderpellets | Feed pellet |
| M22 | Foderpellets för höns | Feed pellet for poultry |
| M23 | Maltkorn | Malt barley |
| M24 | Foderpellets för nöt | Feed pellet for bovine |
| M25 | Hö | Hay |
| M26 | Fodermjöl för svin | Feed pellet for pigs |
| M27 | Hel havre | Whole oats |
| M28 | Miljödamm | Environmental dust |
| M29 | Tillväxtfoder för svin | Growth feed pellets for pigs |
| M30 | Hamrahö | Hamra hay |
| M31 | Hundtorrfoder | Feed pellet for dog |
| M32 | Grovt hästkraftfoder | Feed pellet for horse |
| M33 | Sojamjöl | Soy flour |
| M34 | Kaviar | Caviar |
| M35 | Nötfärs | Minced meat |
| M36 | Ägg | Egg |
| M37 | Bröd | Bread |
| M38 | Sallad | Sallad |
| M39 | Ungnötslever | Bovine liver |
| M40 | Grishjärta | Pig heart |
| M41 | Kycklinglever | Chicken liver |
| M42 | Hästträck | Horse faeces |
| M43 | Griströck | Pig faeces |
| M44 | Nötträck | Bovine faeces |
| M45 | Hundträck | Dog faeces |
| M46 | Katt-träck | Cat faeces |
| M47 | Kycklingträck | Poultry faeces |