Optimization of a sampling and analysis process to study the effects of skin care products on the microbial skin flora.

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1 Abstract.

Our skin is home to a vast community of microorganisms, the skin micro flora, which affect our health and well-being. Yet, there are many aspects of this topic that are still unexplored. This project aimed to optimize a method for analysis of the skin micro flora and the effect of skin care products on the skin micro flora, from sampling and sample preparation to analysis with quantitative polymerase chain reaction (qPCR). Different testing parameters, such as sampling methods, extraction kits and master mixes, were evaluated in a lab setting using *Staphylococcus aureus*. By adding bacterial solution to a surface of Vitro-skin, collecting the bacteria through sampling, extracting the sample, and analyzing the sample using qPCR, a method description was formulated. The *S. aureus* solution was used in two different concentrations, $5*10^6$ and $5*10^8$ colony forming units (CFU)/ml and 20 µL per test, totalling an inoculation of either 10^5 or 10^7 CFU to each test site. Some chosen skin care products were investigated for inhibitory effects on the qPCR analysis.

Five sampling methods, two extraction kits and two master mixes were tested in total. The two extraction kits, Qiagen DNA microbiome kit and ThermoFisher PureLink microbiome kit, proved to have very different strengths with the DNA microbiome kit being very reliable and applicable to many different sampling methods and the PureLink microbiome kit being more specialized and producing both higher and lower DNA yields depending on the sampling method used. Out of the two master mixes tested, the Genesig Oasig master mix and Quantabio Toughmix master mix, the Toughmix proved to be more resilient while the Oasig master mix's performance was affected by the addition of skin care products to the analysis. Quality control work was performed in the form of extraction controls included in the PCR analysis and total DNA concentrations made on samples in addition to the qPCR. Results were confirmed by statistical analysis, Anova and bonferroni-corrected t-tests.

In the end the Copan ESwab 480C flocked swab, ThermoFisher PureLink Microbiome DNA purification kit and Quantabio Toughmix 2x master mix were shown to be the best combination. Furthermore there were inhibitory effects from some skin care products on the qPCR analysis, where the addition of a moist toilet paper solution resulted in a 15-cycle increase and a Demakup solution a four-cycle increase in Cq values compared to samples without any added additives. Because of the COVID-19 pandemic there was a shortage of lab material, some planned tests could not be carried out, and work will be continued beyond this particular project, including further work in verifying the quality of the proposed method.

1.1 Sammanfattning.

Vår hud är hem för en stor grupp av mikroorganismer, hudens mikroflora, som påverkar vår hälsa och vårt välbefinnande. Ändå finns det många aspekter av detta ämne som ännu är outforskade. Detta projekt syftade till att optimera en metod för analys av hudens mikroflora och effekten av hudvårdsprodukter på hudens mikroflora, från provtagning och provberedning till analys med kvantitativ polymeraskedjereaktion (qPCR). Olika testparametrar, såsom provtagningsmetoder, extraktionskitt och PCR mastermixar, utvärderades i en laboratoriemiljö med *Staphylococcus aureus*. Genom att

tillsätta bakteriell lösning till en yta av Vitro-skin, samla bakterierna genom provtagning, extrahera provet och analysera med qPCR formulerades en metodbeskrivning. *S. aureus* -lösning användes i två olika koncentrationer, $5*10^6$ och $5*10^8$ CFU/ml och 20 µL per test, totalt en ympning av antingen 10^5 eller 10^7 celler till varje testyta. Några utvalda hudvårdsprodukter undersöktes med avseende på hämmande effekter på qPCR-analysen.

Fem provtagningsmetoder, två extraktionskitt och två master mixar testades totalt. De två extraktionskitten, Qiagen DNA-mikrobiomkitt och ThermoFisher PureLink-mikrobiomkitt, visade sig ha olika styrkor där DNA-mikrobiomkittet var mycket tillförlitlig och användbart för olika provtagningsmetoder och PureLink-mikrobiomkittet som var mer specialiserat producerade både högre och lägre DNA-mängd beroende på vilken provtagningsmetod som användes. Av de två testade mastermixarna, Genesig Oasig master mix och Quantabio Toughmix master mix, visade sig Toughmix vara mer motståndskraftig medan Oasig master mixens prestanda påverkades av tillsatsen av hudvårdsprodukter till analysen. Kvalitetskontroll utfördes i form av extraktionskontroller som ingick i PCR-analysen och total DNA-koncentrationsmätningar gjorda på prover utöver qPCR. Resultaten bekräftades med statistisk analys, Anova och bonferroni-justerade t-test.

I slutändan bedömdes Copan ESwab 480C topsen, Thermofisher PureLink Microbiome DNA-extraktionskitt och Quantabio Toughmix 2x master mix vara den bästa kombinationen. Vidare fanns hämmande effekter från vissa hudvårdsprodukter på qPCR-analysen, där tillsats av lösnigen från en intimvåtservett resulterade i en ökning på 15 cykler och lösningen från en sminkborttagningsservett en ökning på fyra cykler av Cq-värdet jämfört med prover utan tillsatts. På grund av COVID-19-pandemin var det brist på laboratoriematerial, och det fanns några planerade tester som inte kunde genomföras. Arbetet kommer att fortsätta efter detta specifika projekt, inklusive ytterligare arbete för att säkerställa kvaliteten på den föreslagna metoden.

2 Preface.

This report was written as the finishing part of my Master thesis project and as a sub-project of a bigger research project within RISE Research Institutes of Sweden AB (RISE). Upon completion this will finish my degree of Master of science in engineering, focusing on biotechnology (In Swedish: Civilingenjör inom bioteknik), at the Faculty of Engineering (LTH) at Lunds university, Sweden. It is my own personal opinion that there is a growing need for a broad perspective within research, and there is much to gain from combining multiple disciplines and competencies. Because of this I have tried to keep a broad focus in my education, in choice of courses and projects, while building a solid basis of knowledge and strong ability to tackle new projects with integrity. This led me to a project in forensic science at the division of Applied Microbiology (TMB) at LTH in the autumn of 2019, which aimed to investigate sampling of human DNA from crime scenes. When it was time to choose a topic for my masters thesis in 2020, and I found a project about developing a method for sampling bacterial DNA, it felt like a natural progression from my earlier work.

This is a Masters thesis, and hopefully a document that will prove useful to RISE in their future work. It is primarily aimed at the scientific community, personnel within hygiene and health related research and companies within the same sector. But, as I am passionate about spreading science in the general population and I myself come from a very different background as a musician, I also hope to write this in a way that would be comprehensible also to someone with a shallow understanding of microbiology and bacteria, whilst not sacrificing the scientific integrity of the text.

I have been alone in executing this project as a student, but along the way I have had a tremendous amount of help from my fantastic supervisors as well as the fantastic lab staff at RISE lab facilities in Gothenburg, without whom I probably still would be running around like a headless chicken. I also would like to thank RISE for this opportunity and LTH for the support. The Covid-19 pandemic did certainly present its fair share of difficulties for this project, from almost all supervisor contact being digital and public transport commuting to the sudden shortage of pipette tips due to Covid-testing and research work all over the world. But in the end, there was work done, and there is a report, and I wish whoever reads it good reading and hopefully some new insights into the fantastic world of swabs, extractions and bacterial DNA.

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3 Introduction.

This Master thesis is part of a larger project with the aim to establish and validate a good and reliable method for high-throughput analysis of skin samples using quantitative polymerase chain reaction (qPCR), with a particular interest in the effect of skin care products on the analysis. It has been proven that the bacteria that inhabit the human skin play a big part in the health and function of the skin as well as some vital functions of the immune system. Despite this much is still unknown about the symbiosis between the skin micro flora and its host, and there is a need for more research into this area. By broadening our understanding of the role the skin micro flora play in skin conditions, immune system priming and the transfer of pathogens via the skin we may find solutions to chronic skin conditions, find ways to strengthen our immune systems and help improve the everyday health of a vast amount of people.

As skin care products have become a fundamental part of many peoples' daily routine today, and as there is a need to perform large-scale mapping of the skin microflora in individuals when skin care products are and aren't present, it is crucial to understand how these products impact the analysis process. To do this a dependable and accurate analysis method is necessary. Figure 1 shows a description of what a general PCR process looks like today. First there is a sample, which can come from a myriad of sources and contains or is suspected to contain some kind of genetic material. The sample must then be prepared for analysis, which can mean many different things depending on what analysis it is being prepared for, before it is analysed using a specific type of PCR chosen for that purpose. In the case of this project qPCR will be used.

PCR has been described as having done to microbiology what the internet did to communication since it's introduction (Bartlett and Stirling 2003). However accurate, it is undeniable that PCR has revolutionized microbiological lab work and it is thanks to this that many microbiologists and research facilities now seeks to replace older and more time- or resource-consuming methods with PCR based ones. PCR have been used for the analysis of microbes for over forty years, and so there is already a vast quantity of established protocols for a number of different applications. To perform testing and ensure high-qualitative results a complete testing method, where a qPCR protocol is paired with a suitable sampling method and sample preparation method, is needed.

The finished method would ideally to be versatile, cheap and easy to use, suitable for large volumes of samples and be able to withstand any or most inhibitory effects from skin care products. When optimizing sampling and analysis protocols it is important to consider the specific research question it is going to be used for and adjust the method. All of these steps needs to be adapted to the desired purpose in different ways, this have been described in more detail under section 4, and then validated for the intended purpose.

3.1 The aim of the project

The aim of this project was to first establish a sampling and DNA extraction protocol, and then optimize the qPCR and investigate the effects from skin care products, using the sampling protocol as a starting point for the subsequent tests. The setup was to sample surfaces of artificial skin inoculated with known concentrations of *S. aureus*, using different sampling techniques, extract DNA from these samples, using different DNA

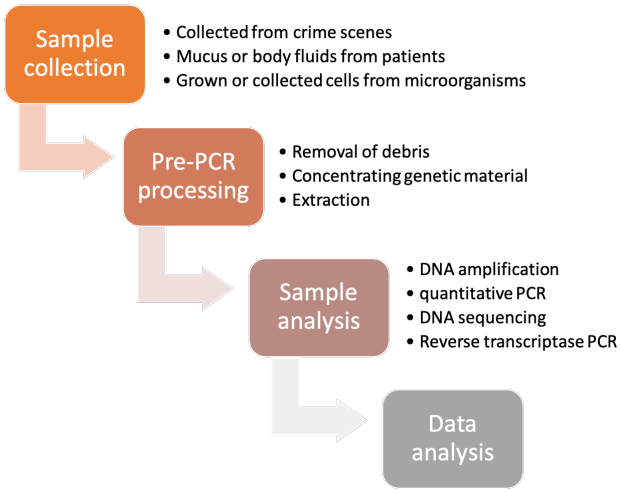


Figure 1: The outline of a general PCR analysis workflow, not specific to this project. There needs to be a sample of genetic material, or suspected to contain genetic material. The sample is then prepared, in what manner is dependent on what kind of sample and how impure it is.

extraction kits, and evaluate the recovery of bacterial DNA from these samples using qPCR. After a method was determined the inhibitory effect of skin care products on the qPCR analysis was established by incorporating chosen skin care products into sample analysis, and tested against different PCR master mixes to optimize the process.

The objectives of this project was to answer these three questions:

- What is the best sampling method/DNA extraction method combination?
- What master mix is the most optimal for this analysis?
- Does skin care products have an inhibitory effect on the qPCR analysis?

4 Theory.

4.1 The skin micro flora.

4.1.1 The host/micro flora symbiosis.

The human skin is home to a myriad of microbes (studies have estimated bacterial cell density on human skin to be, on average, 10^7 cells/cm^2 (Sender, Fuchs, and Milo 2016)), amongst the most common are bacteria from the genera *Staphylococcus* and *Corynebacterium* (Grice and Segre 2011; Chiller, Selkin, and Murakawa 2001), who affect their hosts health and well-being in many ways. *Staphylococcus epidermidis*, for example, have been shown to help inhibit growth of its pathogenic close relative *Staphylococcus aureus*, as well as other pathogens (Grice and Segre 2011). *Cutibacterium acnes*, formerly *Propinibacterium acnes*, similarly protects the host by acidifying its surroundings, making it inhospitable for other microorganisms, pathogens and non-pathogens (Chiller, Selkin, and Murakawa 2001; Fournière et al. 2020). There are also indications that benign bacteria living on the skin can stimulate the immune system and prime the immune response of its host (Chiller, Selkin, and Murakawa 2001; Fournière et al. 2020).

The skin microflora has been proven crucial for the quick development of a ready and able immune system in neonatal babies, the introduction and colonization of microbes in and on a baby just emerged from a sterile environment prepares the burgeoning immune system for life outside of the womb (Capone et al. 2011). Throughout a person's life there is extensive communication between the immune system and the microorganisms colonizing the skin, through the use of metabolites and other signal molecules (Swaney and Kalan 2021). The immune system can, for example, differentiate between *S. aureus* and *S. epidermis* through cytokine signalling, which in turn limit regulatory T-cells, who helps regulate antibody responses in the body (Fournière et al. 2020).

An imbalanced or unhealthy skin flora can likewise have a negative impact on the host. The presence of pathogens on the skin, antibiotic resistant strains in particular, increases the risk of serious infection in the event of skin break, and many skin conditions such as psoriasis, eczema and acne have been linked to the presence of certain types of bacteria on the skin (Grice and Segre 2011; Blanchet-Réthoré et al. 2017). A balanced and healthy skin microflora could be the deciding factor between a serious or chronic, debilitating condition and maintained well being.

There are many factors that can affect the composition of the skin flora. Intrinsic factors such as age, sex, diet and body chemistry matter, as well as extrinsic factors such as everyday environment (C. Wallen-Russel and S. Wallen-Russel 2017; Leong et al. 2019) and hygiene routine (Benhadou et al. 2018; Lee et al. 2017; Blanchet-Réthoré et al. 2017). In their review Wallen-Russel and Wallen-Russel (2017) compares the compositions of skin micro floras in people from developed areas (primarily western culture populations) to people from less developed areas, such as indigenous groups. While many of the improvements in general health and well being, as well as medical successes, for the past 200 years can be contributed to an increased awareness of cleanliness and hygiene, this study show that a higher living standard, or a higher standard for hygiene and cleanliness primarily in the home, have contributed to a decrease of diversity in skin micro flora composition and a rise in microbe related skin conditions. Compared to each other, the

groups with a lower living standard had a much higher diversity in microbes inhabiting their skin, and much lower levels of chronic skin conditions (C. Wallen-Russel and S. Wallen-Russel 2017).

It has been established that the skin micro flora affect the skin, but the specific mechanisms of how and why are still in the dark. There is much work left to be done in the field to fully understand the skin flora and its role in human life.

4.1.2 The skin flora and skin care products.

It is problematic to make any generalized statements about the effects of skin care products on the skin micro flora, because both those groups encompass a vast selection of diverse elements. If by "skin care products" one means products intended for use in any hygiene, cosmetics and personal care routines that category contains everything from deodorants, soaps and body powders to moisturizers and skin creams. With such a wide range of products and functions it is natural that they will have different effects on any microbes that they come into contact with. In addition, there is a lot of inter-personnel variation when it comes to skin health, skin texture/quality and how the skin reacts to different products. As mentioned before, research have shown a decrease in microbial diversity in regions with a higher hygiene and health standard, which correlates with an increase in skin problems and chronic conditions (C. Wallen-Russel and S. Wallen-Russel 2017). There are strong indications that this is because of hygiene and skin care routines that upsets the natural balance between the host and the bacterial community.

Many skin creams and deodorants, amongst others, are marketed as "anti-bacterial", and pushes an image of bacteria on the skin as something bad that must be remedied. Even though many of the claims put forward might be unsubstantiated, such as essential oils being anti-bacterial or anti-bacterial products protecting against COVID-19 (Kim n.d.), it points toward a trend of wanting to remove or kill microbes on the skin, and skin care products aimed toward doing just that. On the other end of the spectrum are efforts to "re-balance" damaged skin with probiotic products to remedy skin conditions believed to be caused by or exasperated by microorganisms (Blanchet-Réthoré et al. 2017). Atopic dermatitis (AD) severity have been correlated with *S. aureus* dominating the affected skin, and by introducing probiotic *Lactobacillus johnsonii* NCC 533, using a skin cream as application vector, researcher could show a decrease of *S. aureus* presence and lessening of AD symptoms (Blanchet-Réthoré et al. 2017).

Skin care products can affect the skin flora directly by adding or removing bacteria. It can also have indirect effects such as alterations of the skin environment, making the skin more or less favorable for certain types of bacteria. The effects of a product on the skin can be quite persisting, with some ingredients being detectable on the skin up to two weeks after application stop (Bouslimani et al. 2019). This means that they can have an effect for a long time on the skin flora as well. An experiment first intending to investigate the skin hydration levels' effect on skin flora diversity found that rather than the increased hydration levels caused by face creams the skin flora was affected by the cream itself. Certain typical skin bacterial groups such as *Propionibacterium, Staphylococcus,* and *Corynebacterium* decreased, and *Ralstonia*-members increased. *Propionibacterium* is typically lipophilic, and predominantly resides in sebaceous (oily) parts of the skin, while *Ralstonia* is a less common skin bacteria. However, it was theorized that, while lipophilic,

Propionibacterium could not utilize the oil components of the skin care products as nutrition, but *Ralstonia* could. So even though it is a less common group of bacteria in the skin it could compete opportunistically when introduced to certain components (Lee et al. 2017).

In short: it is difficult to say what effect a skin care product will have on the skin micro flora. Some products will strip a community, and leave room for opportunistic monocultures to form. Others will affect things indirectly by acting as nutrients or co-factors, or by altering the skin environment altogether, favoring certain species.

4.2 The sampling and analysis process.

It is evident that there are many things that factor into the skin micro flora-host symbiosis. This means that there is a lot of factors to consider when developing a testing method for skin micro flora research. In this section the theory behind the different steps, that have been considered in this project, is described, divided into a section about sampling and sample preparation and a section about PCR and qPCR specifically. This section serves as a spotlight on the methods used in this project, and there would be much more to be said on the subject if this was a comprehensive literature study, which it is not. Suffice it to say that pre-PCR processing of samples is merely the process of turning a raw sample into a substance that is amplifiable via PCR, and that means very different things depending on the sample, the type of PCR used and the purpose of the analysis. For this project the pre-PCR processing is comprised of sampling and DNA extraction, but this is not necessarily the case for all PCR analyzes.

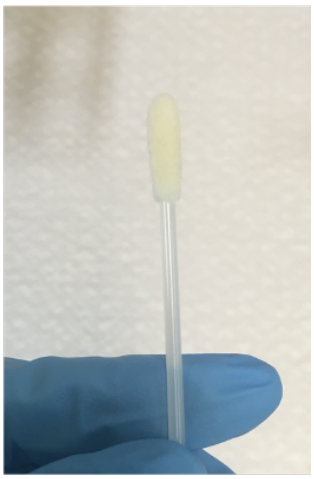
4.2.1 Sampling.

Sampling of microorganisms is not new, but new knowledge and technique is still continuously developed and implemented. Microorganisms were discovered by Robert Hooke and Antoni van Leeuwenhoek somewhere between 1665-83 (Gest 2004) but biotechnology, and the use of microorganisms had existed long before that. Many food related processes such as fermenting and souring utilizes microorganisms, and have been used by mankind for hundreds of years (Keller 1979; Chambers and Pretorius 2010). When "preferable" cultures have formed, they could be indirectly sampled and transferred through inoculation with some of the product itself, as is done with sourdough starters for example. As the field of microbiology have grown and developed the finesse of the sampling methods have increased, and today there are multiple methods for sampling microorganisms directly (Ismail et al. 2013).

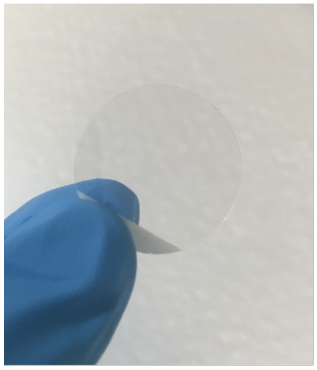
The choice of sampling method is dependent on the purpose and target surface/medium to be sampled, a method for collecting samples from wooden surfaces might be poorly suited for collecting samples from soft tissue and mucous membranes (Ismail et al. 2013).

When sampling microorganisms from the skin there are a lot of options available for consideration. Tape strips are a popular option within forensics (Fierer et al. 2010) and research in skin diseases (Taslimi et al. 2017; Clausen et al. 2018), swabs are one of the most common method used for sampling directly from the skin (Digel et al. 2018), and scraping is commonly used when high yields are important and in food safety (Digel et al. 2018). These are the basis for the methods that have been chosen for this project, but

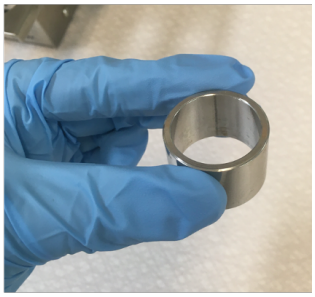
there are more methods like wiping, sonication and bulk sampling, which are also used (Digel et al. 2018; Ismail et al. 2013).



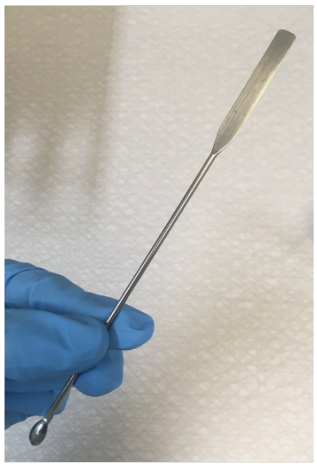
(a) A flocked nylon sampling swab. Swab is rubbed against target, and picks up cells.



(b) A round sampling tape. Tape is dabbed at target, cells are caught on the sticky bit.



(c) Metal ring used for sampling skin through scraping. Ring is put to skin and a buffer solution is added to the inside.



(d) Metal spatula used together with the metal ring to scrape skin when sampling. After scraping the solution is retrieved via pipette.

Figure 2: Example images of some common sampling methods. These images, together with some others also figure in section 5, as they are the methods actually used in this project.

Each method have its strengths and weaknesses, and they should be carefully considered

when choosing a method. Swab sampling is non-invasive and versatile, but can have inconsistency issues due to variations in pressure, number of strokes, etc. between personnel. Swabs come in many different materials and sizes, such as wool, foam and nylon for example, the choice of which can also have a huge impact on the results (Bruijns, Tiggelaar, and Gardeniers 2018). Tape sampling is very gentle, easy to keep consistent between different people using the method, and generally yield high degrees of materials collected. However, with tape there is a greater need for after-sampling processing to yield a sample due to its sticky nature. (Taslimi et al. 2017; Forsberg et al. 2016). Scraping can be performed using different techniques. In this project a method was used where a metal ring with a diameter of 2.8 cm was placed on the area to be sampled. Inside the ring 1 ml of physiological saline solution was applied and the area scraped with a metal spatula. The sample was then retrieved by collecting the solution with a pipette. Scraping can be perceived as unpleasant for subjects, it has a high degree of uncertainty due to variability in approach similarly to swabs, but it also have a high yield (Digel et al. 2018). When choosing a sampling method it is important to consider many factors, and chose a method that is suitable for a specific set of needs.

4.2.2 Sample preparation.

When a sample has been collected it needs to be prepared before it can be analysed. This section will focus on describing the sample preparation process called DNA extraction, because that is what has been used in the project. However, that is far from the only method for preparing samples for PCR analysis. All the steps in a PCR analysis prior to the detection of PCR products falls under the category "pre-PCR processing" (together with master mix optimization and addition of facilitators, see section 4.3), which aims at any step taken to convert a complex sample into an amplifiable one. DNA extraction is one such step that is available. There are also enrichment methods such as sample growth cultures, which aims to bring samples up to detectable concentrations of target organisms by growing them (Löfström et al. 2004), physical methods, that utilizes the physical properties of molecules to separate them, or simply mitigate disturbances from non-target molecules, such as centrifugation, dilution and filtration. DNA extraction is an extensively used method, but it is not the only one, and it is not always strictly necessary either (Rådström, Knutsson, Wolffs, Lövenklev, et al. 2004; Rådström, Knutsson, Wolffs, Dahlenborg, et al. 2003; Löfström et al. 2004).

Impurities and pollutants are often collected together with the sample, as well as any buffers or sampling media that are a part of the sampling process but unnecessary once the sample has been collected. The process of separating the target DNA molecules from impurities is called DNA extraction. Since this project use pure *S. aureus* cells in solution, which are single-cell organisms with one chromosome per cell (A. Młynarczyk, G. Młynarczyk, and Jeljaszewicz 1998) DNA extraction can be applied directly without needing additional processing methods but if the sample contains a more complicated matrix, for example as with soil or mucus samples, additional processing can be necessary to first harvest the cells themselves from the sample matrix (Rådström, Knutsson, Wolffs, Dahlenborg, et al. 2003).

Simply put there are five steps to DNA extraction:

- **Harvesting cells or genetic material**

Depending on the sampling method the target molecules needs to be released from the sampling material itself. If the sample is in a liquid form, for example if it has been collecting by scraping with a buffer and collected via pipette, it can be as simple as separating the cell material and buffer liquid through centrifugation. Swabs are often shaken in liquids, or in special tubes with beads. Other methods, such as tape, can be more difficult to harvest the material from because of their properties, such as stickiness for example. Forceful mechanical harvesting methods such as bead milling and chemical or enzymatic methods can be used in combination in these cases. This step is also often combined with the next step (*DNA extraction* 2009; Chauhan 2018).

- **Releasing the DNA**
  The DNA is released from the cells by either first releasing the cells from the sampling medium and then lysing (breaking) the cells, releasing the DNA or by combining these steps into one. Lysis can be performed either mechanically, chemically or enzymatically. Bead-milling or sonication are common mechanical methods. Detergents such as SDS or Triton X can lyse the cell wall chemically and enzymes such as proteinase K and peptidase can do it enzymatically (*DNA extraction* 2009; Chauhan 2018; Dilhari et al. 2017; Eslami et al. 2017).

- **Removal of large particles**
  Large particles, such as proteins and cell debris may need to be removed. Proteins can be digested enzymatically by using proteinase K for example, and the debris removed by centrifugation, filtrating or magnetic separation (*DNA extraction* 2009; Chauhan 2018). Typical debris in skin swabs or skin samples are dead skin cells, dirt present on skin at the time of sampling and cell material from microbes on the skin (Ali et al. 2017; Dilhari et al. 2017; Eslami et al. 2017).

- **Purification of DNA**
  DNA is separated from other small particles and substances in the sample such as RNA and proteins. This step is generally broken down into multiple steps, depending on what substances needs to be removed. The DNA can be precipitated using salt or alcohol: when the salt or alcohol (preferably ice-cold ethanol or isopropanol) concentration in the sample becomes to great the DNA becomes insoluble and precipitates. It can also be extracted using a solid-phase method: the sample is put through a column with a solid phase that will bind the DNA such as silica or magnetic beads. The DNA will bind to the column while other impurities wash away (*DNA extraction* 2009; Chauhan 2018; Ali et al. 2017; Eslami et al. 2017; Dilhari et al. 2017).

- **Dissolving of DNA**
  Finally the DNA will be collected in a pure liquid sample. If precipitation has been used, the DNA is re-dissolved in an appropriate solvent such as TE buffer. If a solid-phase method has been used the DNA is released using an elution buffer, and collected as a liquid sample (*DNA extraction* 2009; Chauhan 2018; Dilhari et al. 2017; Eslami et al. 2017).

What strategies and methods will work the best varies a lot with what kind of sample they are going to be used on, therefore it is impossible to point to one method and declare it

"the best". A sample containing cells with very tough cell walls, for example gram positive bacteria such as *S. aureus*, might require a lysis strategy that would completely destroy another sample. A very pure sample, such as the cell culture used in this project, require minimal DNA purification compared to a blood sample from a crime scene. Today there are a lot of complete extraction kits available on the market, which has become popular since it is very convenient and easy for researchers and others to use. These kits are developed with a specific purpose in mind, such as purification of gram-negative bacteria in water, or mammalian tissue samples, and this is why it is important to thoroughly evaluate any kit in the situation it is planed to applied (Albertsen et al. 2015; Ali et al. 2017; Forsberg et al. 2016; Bruijns, Tiggelaar, and Gardeniers 2018).

A part of this project was to look at any detrimental effect on the analysis from skin care products. This applied to the extraction process, even though the major focus was on qPCR inhibition. A kit that performs well with a pure *S. aureus* sample might have serious problems extracting a *S. aureus* sample mixed with a skin cream for example. The cream could change the pH of the sample, or it could contain molecules that blocks or disrupts the different steps of the process. If a certain result is suspected to be caused by inhibition of the extraction process or the PCR analysis an extraction control can be added to the process to determine if there is any inhibitory forces in effect. An extraction control is a fixed amount of a particular DNA sequence, different from the target DNA of the sample, that is added to the sample before the extraction. In the PCR analysis a separate set of primers, specific to the control-DNA sequence, is added to the analysis and analysed on another detection channel. This requires that the PCR equipment used is capable of multiplexing (running multiple detection channels at the same time). The extraction control Cq results should fall within a certain value (specified by the manufacturer), in which case it confirms a successful extraction and no inhibition (See section 4.3 for more detailed descriptions of how PCR works).

4.2.3 Alternative methods to quantify bacteria.

qPCR is not the only method for quantifying bacteria. Viable count, counting cells on agar plates, have long been the gold standard in microbial quantification because it is very capable in terms of capacity for sample concentration and viability distinction. When doing a cell count on plates it is easy to adjust if the sample contains too many or too few cells for accurate counting by simply diluting or concentrating the sample. And since only viable cells grow and form colonies the risk of counting non-viable cells is very low. However cells counting takes up to several days to perform under the best of circumstances, and is therefore not a preferable alternative when performing high-throughput analysis (Hazan et al. 2012).

Cell counting with microscopy is very similar to cells counting, instead of growing colonies the microbes are stained and counted directly with the use of a microscope, getting results in minutes. It is, however, a labor-intensive method, and is therefore also not suitable for high-throughput analysis (Hazan et al. 2012).

Flow cytometry and optical density (OD) measurement both uses light to measure cell content in a sample, but both have difficulties distinguishing viable cells from non-viable. OD measurement cannot do this at all, and flow cytometry only within certain parameters. Both methods are also limited in their range of detection (Hazan et al. 2012).

4.3 Polymerase Chain Reaction.

4.3.1 Reaction mechanism.

The principle of PCR is based on a naturally occurring mechanism in the cell. In most cells there is DNA which, for the purpose of cell division and multiplication, needs to be replicated. This task is performed by a group of enzymes called DNA polymerases. A DNA strand is build out of the four nucleotides Adenosine (A), Thymine (T), Cytosine (C) and Guanine (G), which is strung together like a pearl necklace. These nucleotides form pair-bonds to each other G-C and A-T , which is what forms the DNA double helix out of two "single" DNA strands. A DNA polymerase travels the length of a DNA strand, reading it, and builds a new complimentary strand by pairing up the nucleotides of the template strand with their counterparts. To initiate replication the double helix is split apart to form two template strands, then the DNA polymerase needs a starting point in the form of a short single DNA strand that attaches to the template strand, a "primer". The primer is complementary to a specific sequence of the template strand and therefore works both as an attachment point for the DNA polymerase and as a targeting mechanism. After the primer has attached the DNA polymerase can start reading and attaching new nucleotides (Garibyan and Avashia 2013). See figure 3.

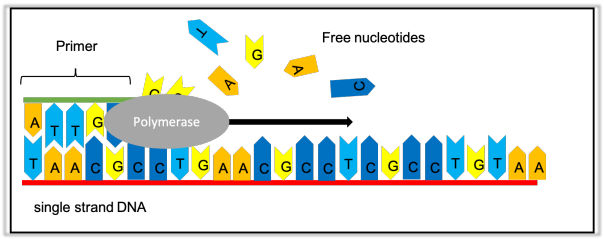


Figure 3: Visualization of a DNA polymerase reading a template DNA strand and building a new, complimentary one out of free nucleotides.

This polymerisation is the heart of the PCR reaction. A DNA polymerase is used to initiate an exponential replication of a specific DNA segment, using two sets of primers complimentary to the two strands of DNA of the segment and free nucleotides. The earliest methods of analysis of amplicon products were agarose gel electrophoresis, where DNA products are separated by size and charge on a gel, and southern blotting, where the DNA fragment in a electrophoresis gel are hybridized with specific labeled probes that can be detected (Cheriyedath 2018).

Gel electrophoresis is still used today (Garibyan and Avashia 2013), but multiple in-line or real-time analysis strategies have also been developed. They can be divided into two categories: staining, where double-stranded DNA is stained with an intercalating chemical dye such as SYTO-13, SYTO-82 or SYBR Green, and labeling, where the primers or oligonucleotides (probes) in the reaction are labeled with fluorescent markers. The chemical dye strategy utilises dyes that will nestle themselves between two DNA strands in a DNA helix (intercalation), changing the helix's light-absorbing properties which can then be measured. The primer/probe strategy utilizes primers or oligonucleotides which have been given a fluorescent tag in the process. When this primer or nucleotide attaches

to an existing DNA strand the tag fluorescence changes wavelength, and the resulting light-response can be measured (Garibyan and Avashia 2013). The most common type is a so-called hydrolysis probe, where a DNA oligonucleotide has fluorescent molecule attached to one end and a light-quenching molecule to the other end. As long as these two molecules are close to each other the light from the fluorescent molecule is caught by the quencher. But when the oligonucleotide is used in the synthesis of a new DNA strand the fluorescent molecule is detached, floats away from the quencher and starts to emit light again, which in turn becomes a signal.

In qPCR the primer/probe strategy is often used, and as the amplification proceeds the light signal from each sample is increased with each amplification cycle (theoretically). The signal is plotted and compared against a standard samples with known starting concentration, and the machine software calculates the starting concentration of the sample.

A sample is prepared for analysis by adding it to a so-called master mix, which is a mix of the reagents needed for the PCR (DNA polymerase, primers and nucleotides), and put into the PCR thermal cycler. The first step is called denaturation, the sample is heated to 94-98 degrees Celsius to make the DNA strands separate from each other, then follows annealing, where the sample is cooled down to 55-70 degrees which allows for the primers to bind to the now single DNA strands. The last step is extension, where the sample is heated again to an optimal working temperature for the DNA polymerase, 68-72 degrees, which in turn starts to synthesize a new DNA strand. When extension is complete each single DNA strand have been turned into a new double-helix pair, and the cycle can being again. the process is typically called amplification, or amplification cycle and one such cycle typically takes no more than a minute for qPCR. The cycle can be repeated 30-45, even up to 50 times in one analysis. (Valasek and Repa 2005). See figure 4.

This project have solely been using the technique called quantitative PCR (qPCR). It is also sometimes called real-time PCR (rt-PCR), but should not be confused with reverse transcriptase-PCR (RT-PCR) which enables the analysis of RNA samples through the use of reverse transcriptases (Mo, Wan, and Zhang 2012). The sample probes in these tests are labeled with FAM dye, and the extraction control probes are labeled with VIC dye, and the analyzes are run on FAM and VIC channels respectively.

4.3.2 PCR results and Cq-value.

The data from the qPCR analysis needs to be put into a context to mean anything. Most PCR software today is also equipped to do some data analysis given the right premises. By running a set of DNA standards with known concentrations, together with the samples, a standard curve can be established, and used to determine starting concentrations for the samples. The signal from a sample is measured after each amplification cycle, which enables a mapping of the signal intensity progression into an amplification curve.. In all qPCR reactions there is a measure of background noise, ambient fluorescence from the reagents in the process. This gives rise to what is called the background fluorescence threshold, or just detection threshold. The threshold is the level above which a positive result signal can be distinguished from negative result signals, however measurements can still be made under this value. The point where the signal from a sample rises above this threshold is called the Cq-value, and is often used as a point of comparison in qPCR. The

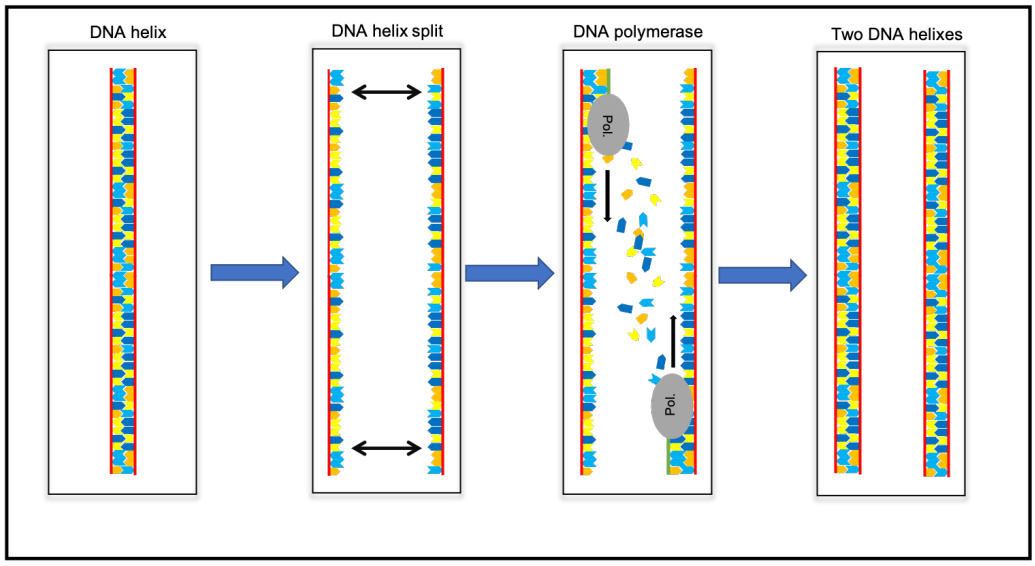


Figure 4: PCR cycle. Step 1: Denaturation of double stranded DNA into two single strands. Step 2: Primers anneal to single stranded DNA and DNA polymerase builds new complimentary strands through extension. Step 3: Primers and DNA polymerase releases and leaves two new double strands of DNA, ready for a new cycle.

Cq value is simply the cycle number when a signal becomes detectable, distinguishable, and is directly correlated with DNA amount, since a sample with a larger amount of DNA present will have a stronger signal much earlier in the process compared to a sample with a low amount of DNA. See figure 5 for a visualization of the concept (Pabinger et al. 2014).

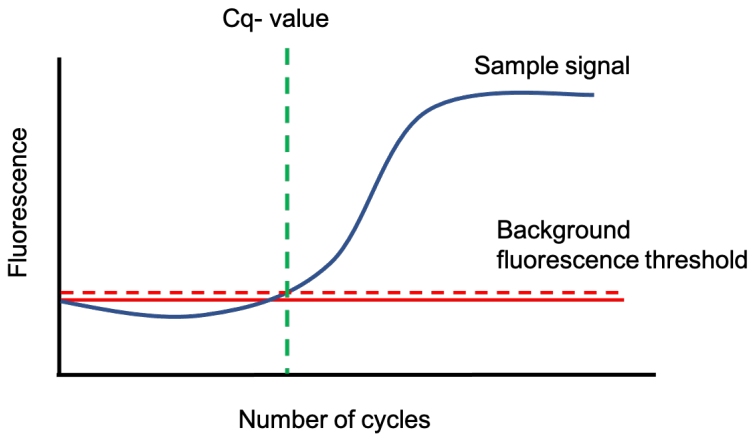


Figure 5: A visualization of what the Cq value is. The sample signal (blue) rises above the fluorescence of the detection threshold (red), and gives the Cq-value.

This project has used Cq value for easy comparison between samples, and as the main data value for analysis.

4.3.3 Inhibition.

Theoretically each amplification cycle should lead to a doubling of all single DNA strands, if all reagents (primers, DNA polymerase and nucleotides) are available in abundance, and through the cycles the amount should be increased exponentially. In reality, the efficiency of a PCR reaction never fully reaches 100 %. The reaction is dependent on primers binding to the correct DNA fragments all the time. This doesn't happen perfectly in reality, and there is almost always a small decrease in efficiency because of small variations in the activity of the primers and DNA polymerases in the reaction. Aside from the expected efficiency decrease there is inhibition, which is the problematic, unexpected decrease in efficiency caused by some compound or substance in the reaction. When talking about inhibition there's the perspective of just the PCR reaction, but it is also common to speak about inhibition in the whole process, where inhibitors can be substances that affect the extraction for example. Since extraction has already been covered in a previous section this section shall focus on inhibition of the PCR process solely. An inhibitor is a particle or substance that has a negative effect on the efficiency of the process, regardless of through which mechanism. Common inhibitory mechanisms are substances annealing to the DNA strands, hindering extension, or to primers, hindering them from annealing to DNA to start the process (Schrader et al. 2012). Other mechanisms include degradation of DNA polymerases, DNA or primers and disruption of nucleotide and probe-binding (Schrader et al. 2012). Some inhibitors work indirectly, by afflicting co-factors such as Mg^{2+}, which is an important component in the polymerization process (Hedman and Rådström 2013).

Inhibitors can be introduced in any step of the process. They can be a part of the sample matrix, any substance part of the sample other than the target substance itself. They can can be introduced in the extraction method, either by contamination or as one of the actual compounds used in the extraction process (Hedman and Rådström 2013). This proves the importance of choosing sample preparation strategy with care, and to execute the steps of the process, especially elution and washing steps of the DNA, with diligence and accuracy. Examples of common inhibitors are isopropanol left over from DNA extraction, melanin from skin samples, humic acids and EDTA (Schrader et al. 2012; Hedman and Rådström 2013).

There are multiple strategies to overcome inhibition, besides simply removing the inhibitor which might not always be possible. There are many different DNA polymerases, and they have different sensitivity to inhibition. *Taq* polymerase from the bacteria *Thermus aquaticus* is for example inhibited by melanin, while the *Tth* polymerase from the organism *Thermus thermophilus* is hardly affected by the presence of melanin (Hedman and Rådström 2013). If removing the inhibitor is not possible, it can be possible to "lower" the inhibitor amount by diluting the sample, thus lessening or removing the effect. This is encumbent on there being enough DNA in the sample, and what type of PCR is used. Another alternative is to use different facilitators, "anti-inhibitors" if you will. Bovine serum albumen (BSA) is a very common one, it binds to different organic molecules, fatty acids and phenols and acts as a alternative target for enzyme-degrading proteases (Hedman and Rådström 2013). Buffers can also be used to lessen the effects

of inhibitors, similarly to dilution. An inhibitor that is charged and active in one type of buffer solution can be neutralized by choosing another buffer (Hedman and Rådström 2013; Schrader et al. 2012).

4.3.4 PCR applications.

PCR is a very versatile tool, with a multitude of applications within microbiology research, medicine and virology amongst others. It can detect, measure and read sequences of DNA with high selectivity and sensitivity, and new ways to apply the technology keeps appearing (Dove 2018). PCR have been used extensively in virology, both as a research tool and as a diagnostic tool (Yang and Rothman 2004), in forensics, as a tool for securing and analysing DNA related to crimes (Hedman, Lavander, et al. 2018; Cheriyedath 2018; Fierer et al. 2010) and in gene research such as the Human Genome Project (HGP) by the Human Genome Organisation (HUGO) (*The Human Genome Project* n.d.; *Human Genome Organisation (HUGO) International Ltd. - About us* n.d.), where it has revolutionized DNA sequencing (Garibyan and Avashia 2013). A very current area of use is in the global struggle against the Covid-19-pandemic, where PCR-tests are being used to diagnose and trace cases worldwide, and PCR techniques are being used in the development of vaccines and treatment regimes. In short: PCR can be of use everywhere there is a need for identification, detection or quantification of DNA or RNA, and is today used extensively in the food industry, health care industry and the environmental research industry.

5 Materials and method.

5.1 General work description.

The lab work was divided into three phases or sections:

- Phase 1: Method development
- Phase 2: Inhibition study
- Phase 3: PCR optimization

In the method development different combinations of sampling methods and sample preparation were tested. Tests were done on artificial skin, inoculated with *S. aureus*. When the testing was concluded one sampling/extraction combination was chosen as the most suitable, based on which combination recovered the most DNA from the artificial skin, and it was used in all subsequent tests. In the inhibition study samples were mixed with skin care products and screened for inhibitory effects. The PCR optimization consisted of testing of different PCR master mixes, using the samples from the inhibition study as well as control samples without skin care products. All extracted samples were also measured for total DNA content after the DNA extraction. The inhibition study and PCR optimization was mostly executed together. Some methods, materials and advice was supplied by a hygiene company which does not wish to be mentioned in text, so some details have been excluded for confidentiality reasons such as the specific names of the skin care products.

5.1.1 Bacterial solution.

Bacterial solution used in all tests: 1 ml cultures of *Staphylococcus aureus* subspecies *aureus Rosenbach* ATCC 6538 (SIK 723) which RISE had in cold storage. Tube cell content had been measured to $5*10^8$ CFU/ml three days after freezing, prior to this project. No additional counting was done before testing, the number was assumed to be stable during the course of the project. Dilutions was made with physiological saline (9 g NaCl, distilled water to a total volume of 1000 ml, autoclaved). In some stand-alone tests an overnight culture was used (see section 6.1.1), it was made by inoculating a test tube of Luria broth (LB medium) with the stock *S. aureus* solution, and then left to grow overnight in 37 °C. Cell concentration was not measured, since the purpose was only to achieve as high a cell count as possible. Preparation of nutrient broth and growth of cultures was all performed in-house at RISE lab facilities.

5.1.2 Vitro-skin.

Artificial skin used: VITRO-Skin (IMS, flordia suncare testing inc.). From the company itself:

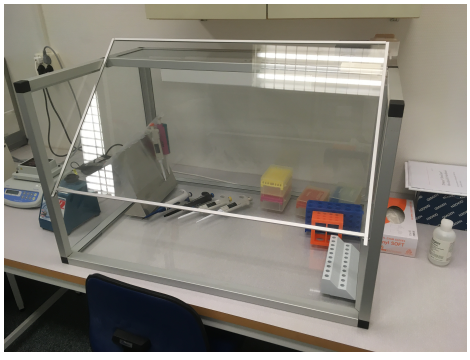
> "VITRO-SKIN® is an advanced testing substrate that effectively mimics the surface properties of human skin. It has been formulated to have topography, pH, critical surface tension, chemical reactivity and ionic strength that is similar to human skin" (IMS n.d.).

5.1.3 Primer/prob kit.

In all qPCR analyses the Primerdesign genesig *S. aureus* advanced primer/prob kit was used for primers. The kit contains primers designed to target the FEMB gene in *S. aureus*, and has 100% homology with over 95% of the NCBI database reference sequences available at the time of design (*Staphylococcus aureus FEMB gene (chromosomal gene) genesig Advanced Kit, quantification of Staphylococcus aureus genomes. Genesig Advanced kit handbook* 2018). Part of the primer kit were reagents for an DNA extraction control, pure DNA to be added to samples during the DNA extraction and separate primer/probe set to be added to the analysis process. All PCR runs were multiplexed with analysis running on a FAM channel and DNA extraction control on a VIC channel. Concentration forward primer, reversed primer and probe: 3 pmol/µL, respectively. In all qPCR reactions sample volume added to each well was 5 µL. DNA standards and DNA extraction controls came included in the S. aureus primer/probe kit. See table 5 - 8 in appendix 10.2 for detailed PCR setup instructions.

5.1.4 Lab work environment.

All lab work was performed in RISE lab facilities in Gothenburg, in Unit Process technology, safety and hygiene. Inoculation of artificial skin and sampling was performed on an ordinary lab bench. All extractions were done in a semi-open glass clean box situated in a small room separated from other, general lab work space. The box was wiped down with 70% ethanol before and after each use and sterilized with UV light after each work day. This was also were all PCR wells were prepared with genetic material. PCR master mixes and PCR wells were prepared in a special clean room, in a fume cupboard also equipped with UV light-sterilization. No genetic material, microorganisms or other possible contaminants were allowed into this room. See figure 6.



(a) Clean box where most lab work, except sampling was conducted.

(b) Clean room where all PCR master mix preparation were conducted. No microorganisms or containers that could possibly be contaminated with microorganisms were allowed inside.

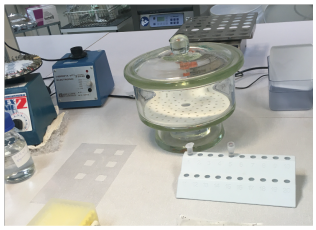
Figure 6: Lab setup

5.1.5 Analysis equipment.

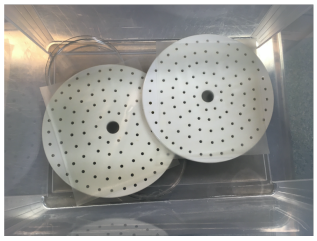
All quantification was performed using a CFX connect real-time PCR with CFX maestro software, ver. 1.1 (Bio-Rad Laboratories, Hercules, California). Total DNA content measurements were performed using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, California) and the Qubit dsDNA HS Assay Kit.

5.2 Phase 1: Method development.

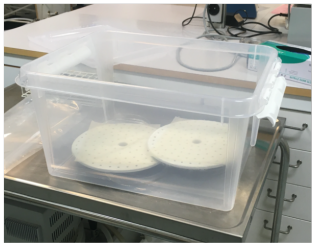
Six sampling methods and three kits were tested in the method development, they are listed in table 1, together with the artificial skin used as testing surface and qPCR master mix used for all method development tests. Before testing the artificial skin was treated as per manufacturers protocol to ensure proper hydration levels. It was placed in a glass desiccator (see figure 7a), which held an open container of 15 wt% glycerol/H_2O-solution, at room temperature, to provide a humid environment, overnight. For each sample a 2x2 cm area of artificial skin was inoculated with 20 µL *S. aureus* solution. Two different concentrations of cell solution was used: 5*10 ^{6} CFU/ml and 5*10 ^{8} CFU/ml. 5*10 ^{8} CFU/ml * 20 µL corresponds roughly to 2.5*10^{6} cells/cm^2 on a 4 cm^2 surface. The solutions were spread out with a small, plastic spatula (figure 8b) and the surface was allowed to dry for 30 minutes in the desiccator before sampling (figure 7). After collection the samples were extracted and analyzed using qPCR. After initial trials the first desiccator setup proved to be too small for all necessary vitro-skin to be sampled at one time, and a solution which could take care of larger pieces had to be found. The second setup became a plastic box, with a snap-lid. It was cleaned and sterilized with alcohol, and containers with glycerol solutions placed inside, together with vitro-skin on top of perforated desiccator trays, overnight (figure 7b and 7c).



(a) First desiccator setup for vitro-skin preparation



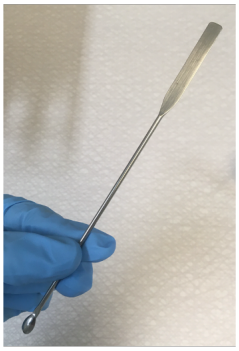
(b) Alternative "desiccator" setup for larger pieces of skin.



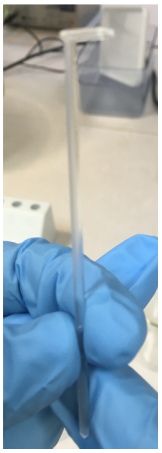
(c) Alternative "desiccator" setup for larger pieces of skin.

Figure 7: Desiccator setup

All DNA extraction kits were used according to manufacturers protocol, with the addition of a volume of extraction control DNA from the primer kit, see detailed protocol in appendix 10.3. Swabs were wetted with 50 µL physiological saline solution and swabbed at an
45 °angle to the surface, up and down, side- to side and once diagonally while rotating slightly. Tapes were dabbed 10 times on the surface. The ring had an inner diameter measurement of 2.8 centimeters. Sampling with ring was executed by placing the ring on



(a) Metal spatula used together with the ring method

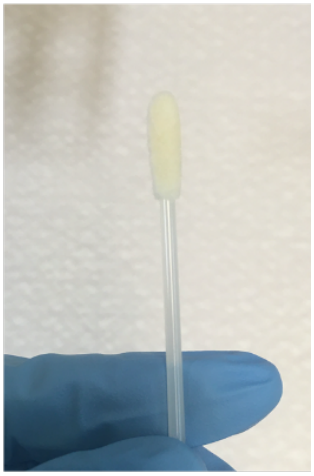


(b) plastic spatula used to spread bacterial solution.

Figure 8: additional tools used in sampling

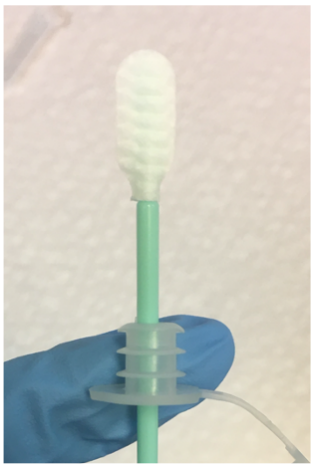
Table 1: Materials used in the method development. Sampling methods, DNA extraction kits and qPCR reagents with full name, short name used in report, source and comments

Category	Name	Short name	Company	Comments
Swabs	•Regular FLOQ Swab •ESwab 480C •SK-2S	FLOQ Nylon Isohelix	Copan diagnostic inc. Copan diagnostic inc. Cell projects Ltd	
Ring	•Metal ring	Ring		
Tapes	•D-squame Standard sampling disc •Deep cleansing porestrips	Tape Porestrips	CuDerm Corporation Kao corporation	 excluded from testing
Extraction kits	•PureLink™ Microbiome DNA Purification Kit •QIAamp® DNA Microbiome Kit •DNeasy® Blood & Tissue kit	PureLink microbiome DNA microbiome DNeasy	ThermoFischer Scientific Qiagen Qiagen	 excluded from testing

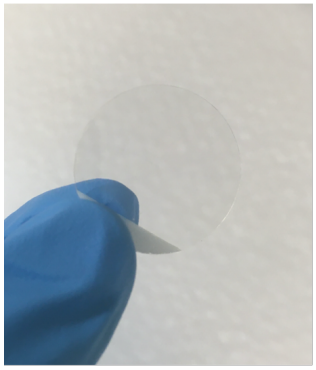


(a) The Copan Regular FLOQ swab

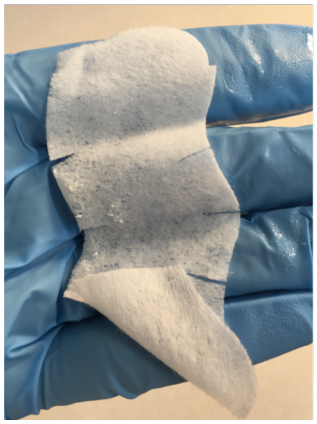
(b) The Copan ESwab 480C



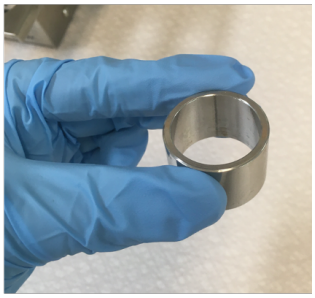
(c) The Isohelix SK-2S swab



(d) The D-squame standard sampling disc



(e) Bioré porestrip



(f) Metal ring

Figure 9: The sampling methods tested in the Method development.

target surface, adding 1 ml of physiological saline inside ring, and scraping with a sterilized metal spatula (figure 8a once horizontally, vertically and with a circular motion. The ring protocol was supplied from the same company that developed the method, and the details will not be published due to confidentiality reasons (K. Bjerre n.d.). The solution was then collected using a pipette. After sampling all samples were put in sterile eppendorf tubes and stored in freezer, -20 °C, until DNA extraction. The storage medium tubes that were included with the swabs were not used. See figure 9 for images of all methods.

When all preparations and pre-trials were done, the main set of tests were executed. Five sampling methods were collected with two concentrations of inoculate for two DNA extraction kits in triplicates, a total of 60 samples (see table 1). These were then extracted and analyzed with qPCR. For a visualization of the general workflow of the method development see figure 10.

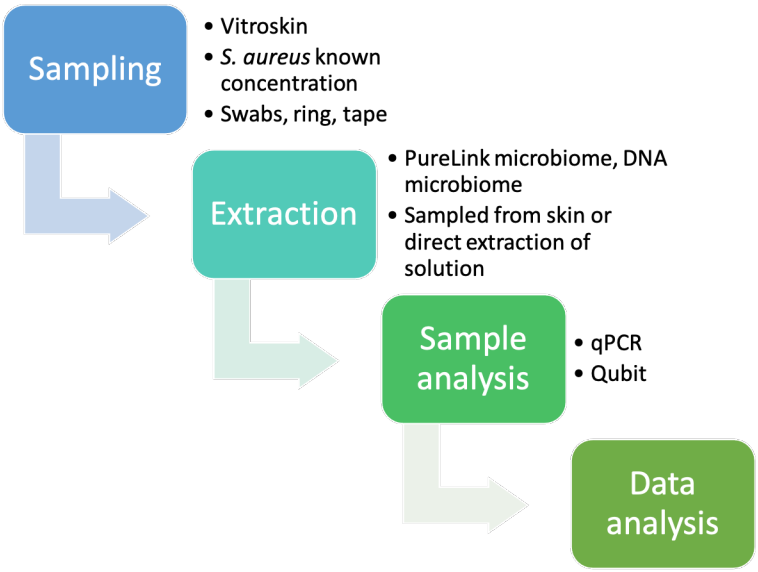


Figure 10: The general workflow description of the method development. Samples have been collected and extracted using different techniques and then analyzed and compared in this fashion

5.3 Phase 2: Inhibition study.

Four different skin care products were tested in the inhibition study, see table 2. These were supplied by the same company that developed the ring method, and the full names of these products will for confidentiality reasons not be published. Instead the products will be called by short names found in table 2. Samples were prepared with either a fixed DNA concentration and varying product concentration or fixed product concentration and varying DNA concentration, to investigate the inhibitory effects of the products. The samples with fixed product concentration were prepared with 20 µL product added to the sample before DNA extraction, and then extracted together with control samples

with water instead of products. The controls were used to ascertain whether there was any difference in results with the products present in the process. The fixed DNA concentration samples were prepared without DNA extraction, therefore a *S. aureus* DNA standard solution was used instead of cultivated cells. This was to isolate any effects of the products on the DNA extraction and the qPCR respectively. DNA standard used was standard dilution "2" of the standard from the primer kit, prepared according to instructions (copy number $2*10^4$ per µL).

Table 2: List of products used in inhibitory study. Full names are omitted due to confidentiality reasons, and products have been given generic short names instead.

Category	Name	Comments
Skin care product	Barrier cream	
	Wash cream	
	Demakup	wet wipe solution in bottle
	Moist toilet paper	wet wipe solution in bottle

For the fixed product concentration samples 20 µL product was mixed with 20 µL cell solution,($5*10^6$ and $5*10^8$ CFU/ml) and extracted, then analyzed with qPCR and qubit analysis. See figure 11

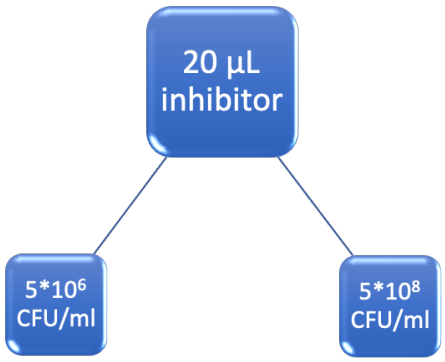


Figure 11: Product was mixed 1:1 with *S. aureus* solution, and then extracted with the PureLink microbiome kit for the "extracted" samples. These were then analyzed using both PCR master mixes.

For the fixed DNA concentration samples it was decided to test products at 10 %, 3 % an 1 % of the total sample volume for the qPCR, 20 µL. The barrier and wash cream was too viscous to measure with a pipette, and had to be portioned by weight instead, according to table 3.

Table 3: Cream measurements

Name	volume percentage inhibitor	mg per 20 µL
Barrier cream	10%	1.37
	3%	0.41
	1%	0.14
Wash cream	10%	1.98
	3%	0.59
	1%	0.19

The products were portioned into tubes first, then the PCR master mix according to protocol and standard DNA sample.

5.4 Phase 3: PCR optimization.

All master mixes were executed according to manufacturer's instructions, see 10.2 for detailed master mix recipes and protocols.

Table 4: List of PCR master mixes chosen for tests, both those that were delivered and evaluated, and those that did not arrive in time.

Category	Name	Short name	Company	Comments
Master mix	Oasig™ lyophilised 2X qPCR Master mix	Oasig master mix	Genesig	
	ToughMix® 2x master mix	Toughmix	Quantabio	
	Immolase/BSA master mix	Immolase/BSA mix	mixed in-house	Not delivered in time
	TATAA Probe GrandMaster® Mix	TATAA master mix	TATAA Biocenter	Not delivered in time

5.4.1 Data analysis.

Data analysis such as statistical calculations and graph plotting was performed using Excel, Microsoft Offices version 2016. Statistical analysis included single factor Anova, Bonferroni-corrected t-tests and Scheffe's tests, confidence interval 95%.

6 Results and discussion.

6.1 Method development.

6.1.1 DNeasy extraction kit and pore strip.

Different sampling methods (swabs, tapes and ring method) and extraction kits (PureLink microbiome, DNA microbiome and DNeasy kit) were tested by sampling artificial skin inoculated with a bacterial solution to see what combination of methods yielded the largest amount of DNA in a qPCR analysis. Data presented as Cq values from qPCR analysis.

After initial testing it was decided that the pore strip was not suitable for this project. It left a sticky resin, which made it hard to handle in a clean manner, and it was concluded that its propensity to leave material on the surface made it unfit for a method of quantification. So it was excluded from further testing.

The DNeasy kit was used for some initial "trial" tests, and was found to produce unsatisfactory results (Figure 12). Samples inoculated with less than 10^7 CFU on the artificial skin produced no detectable amplification. It was therefore tested with direct extraction of 5 dilutions of *S. aureus* solutions, ranging from 10^7 CFU to 1 CFU per sample (dilution range $5*10^8$-5*10 CFU/ml * 20 µL sample volume), as well as an overnight culture of the *S. aureus* pre-culture in storage to test the performance on very high amounts of cells compared to the samples that had been tested before (10^7 CFU). Extraction volume was 20 µL, the same as used for inoculation of the vitro skin in sampling tests. The kit only produced detectable results on 10^7 CFU or higher, any test with lower CFU amount yielded a negative result. This meant that no amplification had taken place in the PCR or no or very low levels of DNA had been extracted from the sample.

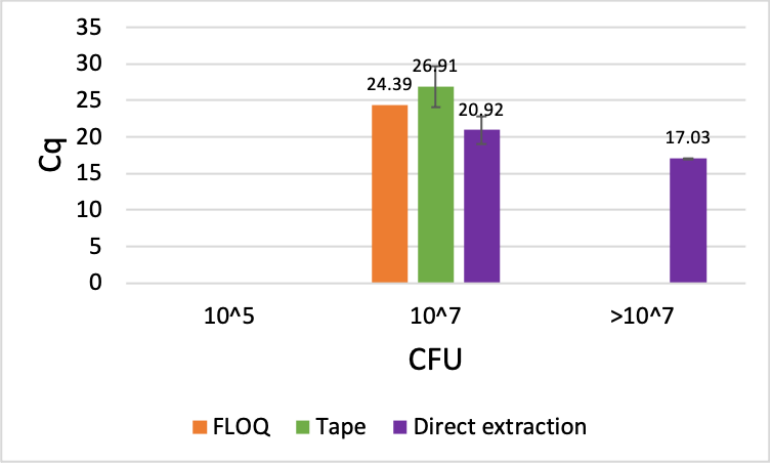


Figure 12: Average Cq values for initial tests with the DNeasy kit, using the FLOQ swab, the tape and direct extraction on cell solution without a sampling step. Tests yielded no detectable results on concentrations lower than 10^7 CFU, so these results have not been included in the graph. n=3.

In the DNeasy dilution series samples an extraction control was added from the primer/probe kit. The same amount of control DNA was added to all samples, so the Cq values for all samples should be the same, but they were not. The variation between triplicates of the same sample ranged from a difference of 10 Cq to a difference over 30 Cq, which indicates that something in the extraction process had gone wrong.

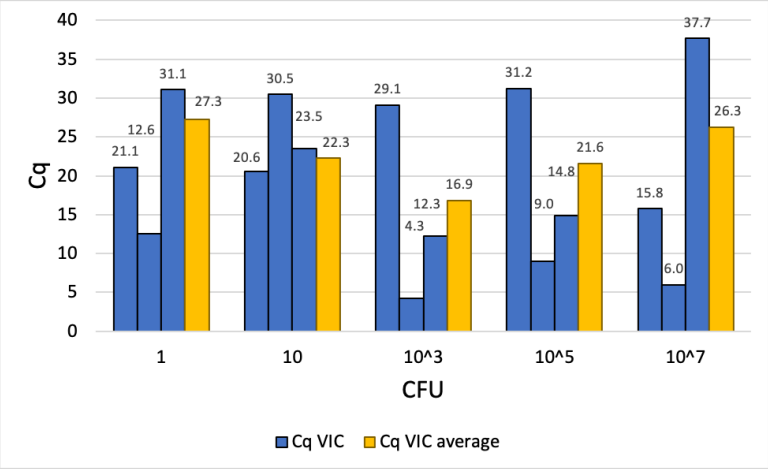


Figure 13: Extraction control results from extraction control added to a series of samples ranging from 10^7 CFU to 1 CFU per sample. The same amount of control DNA was added to all samples. n=3.

The DNeasy blood and tissue kit is very versatile, and is used to extract samples from complex matrices such as parasites found in the skin and mealybug tissue (Taslimi et al. 2017; Wang et al. 2019), and is advertised to be suitable for gram positive bacteria by the manufacturer. Since the PureLink microbiome kit and DNA microbiome at this point had yielded better results (described below) it was concluded that the DNeasy kit was unsuitable for this project, that time was better spent moving forward with the two kits that were performing instead of investigating this phenomenon further and the DNeasy kit was therefore excluded from further testing. No skin care product had been added at this stage.

6.1.2 Evaluation of different extraction kits.

After sampling, extracting and amplification, the Cq-values were averaged between triplicates of the different combinations of sampling and extraction method compared. Out of all the sampling methods the Nylon swab, extracted using the PureLink microbiome kit performed the best, resulting in low Cq values i.e. high amounts of amplifiable *S. aureus* DNA in sample compared to the other combinations.

Overall the samples extracted with the DNA microbiome kit showed a higher consistency, with very similar Cq values between the different sampling methods and little variation between the triplicates (Standard deviations below 0.73). The PureLink kit showed much more variation, both between the different methods, but also between some of the triplicates themselves as well (out of 10 groups, only two had a standard deviation below

0.5. The rest were between 0.5 and 3.90). Anova tests and subsequent Bonferroni corrected t-tests on the two populations (PureLink samples vs DNA microbiome samples, figure 15 and 14) confirmed the consistency in results in the DNA microbiome population, with only the FLOQ and Tape samples inoculated with 10^7 CFU being significantly differentiated from each other (figure 14). The statistical test also showed that the Nylon swab was the only sampling method that was significantly differentiated from the others in the PureLink population (figure 15, with the FLOQ swab being close to the Bonferroni-corrected p = 0.01. When compared with a non-corrected t-test the Nylon swab differed significantly between the two extraction kits, with the PureLink kit yielding the lowest Cq-values. The DNA microbiome kit seems to perform better with different methods than the PureLink microbiome kit, with more consistent results and smaller variation (The average Cq values for each method varied within 3 Cq from each other for the DNA microbiome kit, the same number for the PureLink microbiome kit was 10.5 Cq). However, the PureLink kit performs as good or better than the DNA microbiome together with swabs, probably because the PureLink kit was developed especially to be used together with swabs.

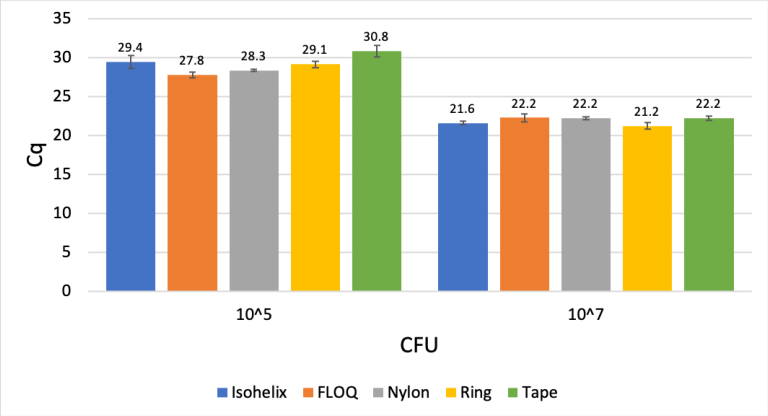


Figure 14: Average Cq-values from triplicates, for each sampling method with the DNA microbiome kit, with standard deviation error bars. n=3.

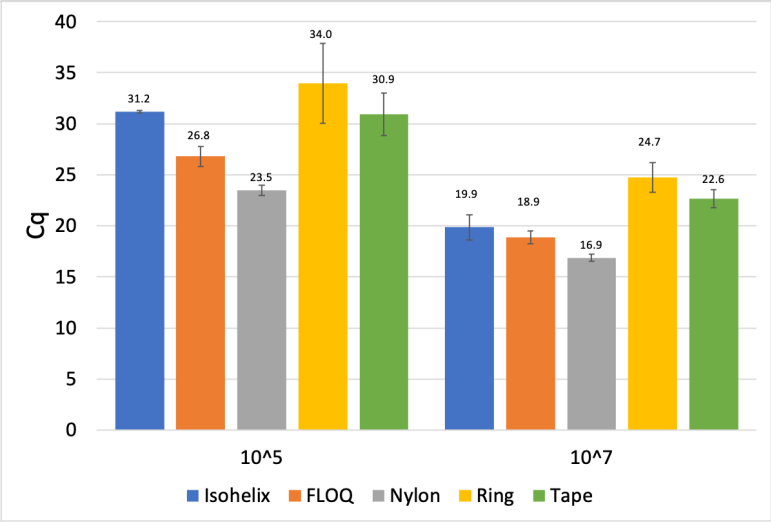


Figure 15: Average Cq-values from triplicates, for each sampling method with the PureLink microbiome kit, with standard deviation error bars. n=3.

6.1.3 Total DNA content measurements on method development samples.

All extracted samples were also screened for total DNA content using a Qubit fluorometer. These measurements further confirmed the qPCR findings of the Nylon swab and PureLink kit combination yielding the highest DNA collection, with the FLOQ swab and PureLink kit slightly behind. This strengthens the conclusion that the Nylon swab is the best at collecting sample material, and that the PureLink kit is the best option for extraction (Figure 16 and 17).

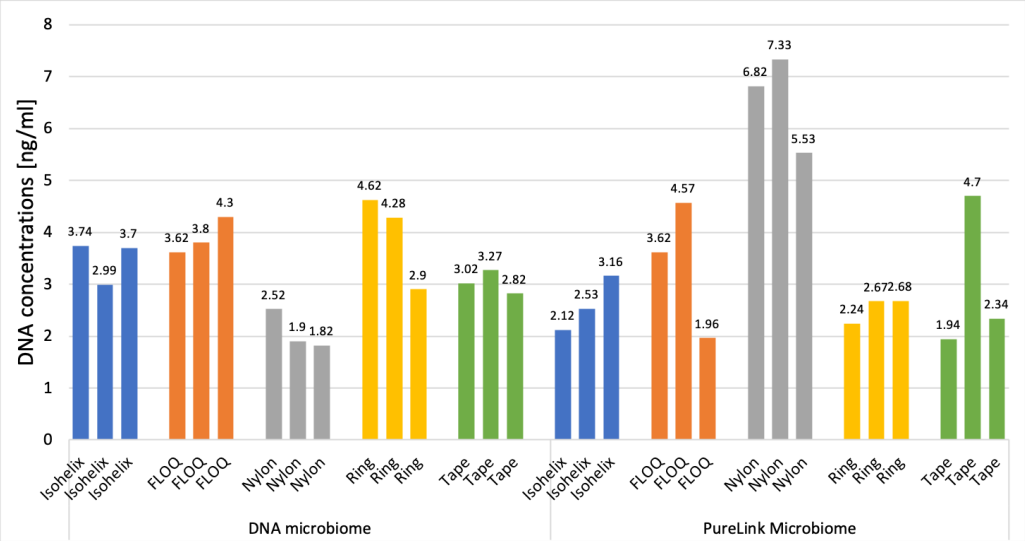


Figure 16: Total DNA measurements (Qubit) from samples extracted with the DNA microbiome and PureLink microbiome kit. Samples inoculated with 10^5 cells. Results are not averaged as earlier, but presented for each individual sample to show variations and differences between methods in detail. n=3.

All sampling methods were also tested on "clean" vitro-skin that had not been inoculated. The skin comes sterile in the package, and was opened and generally handled using gloves and on surfaces wiped with alcohol. It was stored at 6 °C, in its original packaging after opening. However, no particular measures were taken to ensure that sterility was maintained. So this test was done on skin not inoculated with cells, to estimate the effect of any background DNA that might be present and the methods propensity to collect it. The Nylon and FLOQ swap were the only ones that consistently collected DNA. One tape strip also yielded a DNA amount, while two did not.

The nylon swab yields higher background DNA than the other methods, which is consistent with it being one of the sample methods with the highest yield in earlier tests.

The Qubit results seems to confirm that the Nylon swab collected the most DNA and the extraction control results confirm that the extraction kit worked well. That the Nylon swab seemingly collects DNA on vitro skin that should be clean is concerning, but not alarming. The goal was to find a sampling method with a high collection sensitivity,

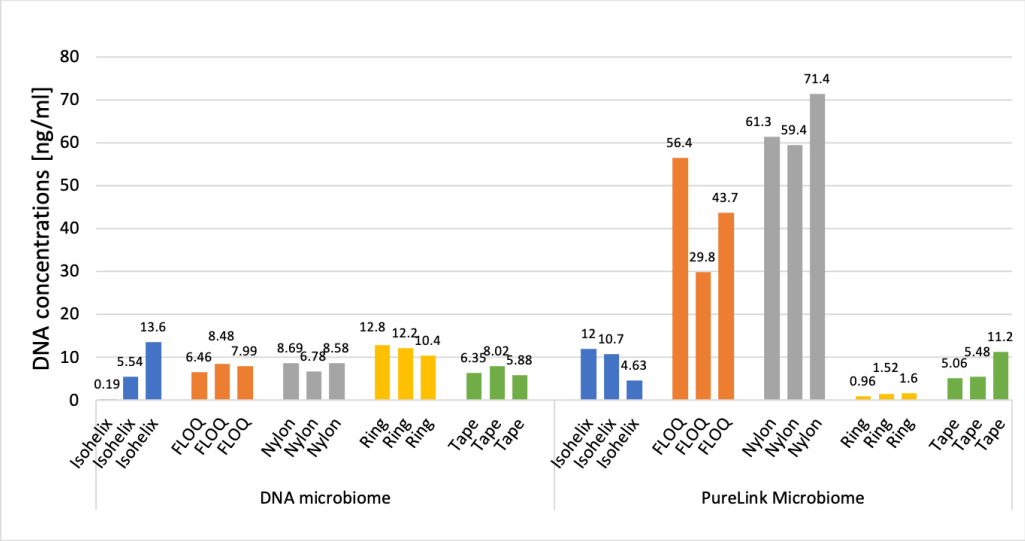


Figure 17: Total DNA measurements (Qubit) from samples extracted with the DNA microbiome and PureLink microbiome kit. Samples inoculated with 10^7 cells. Results are not averaged as earlier, but presented for each individual sample to show variations and differences between methods in detail. n=3.

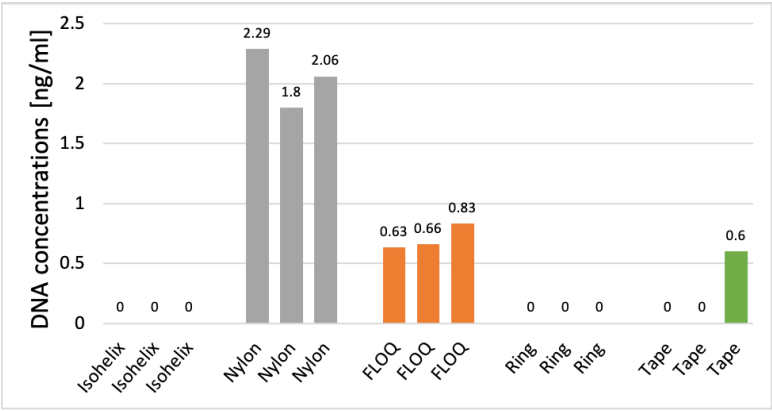


Figure 18: Total DNA measurement on samples taken from "clean" artificial skin without inoculation. Extracted using the PureLink microbiome kit.

which the Nylon swab has. However, it is an issue regarding the quality of the method if the Nylon swab is contaminated with DNA of unknown origin, and this needs to be investigated further to find out if this is the case, or if it was because of a random error such as a contamination of the Vitro-skin in this particular test.

There are benefits to both extraction kits, the DNA microbiome kit has a smaller variation between sampling methods and is independent of sampling method, while the PureLink microbiome kit is much easier to use and produced the highest DNA yield. Unfortunately logistical problems made the choice easy. Due to high demands for lab equipment, especially related to DNA isolation and PCR, only the PureLink microbiome kit was available for purchase at the time the method development was concluded. Had both kits been available the larger variation of the PureLink microbiome together with other testing methods would have needed more consideration, since it decreases the flexibility of the testing method. If there is a need to change sampling method for future tests, the quality of the results might be compromised, and the method would need to be re-optimized and validated for the new sampling method.

The aim of this project was to find a suitable method for analyzing samples of the skin microflora, that could handle large volumes of samples and at the same time produce reliable results. When choosing a method in this manner there is much to consider when making a choice for the "best" option. There is the consideration of the tools and reagents themselves, which ones gives the best results (Ogai et al. 2018; Digel et al. 2018; Digel et al. 2018)? The DNA yield can be dependent on the sensitivity of the method itself, but it can also be affected by how easy or difficult a kit is to use (Becker et al. 2016; Claassen et al. 2013; Dilhari et al. 2017). A more complicated kit poses higher demands on the skills of the staff and equipment, which increases the risk of faults or mistakes, in this case the kit with the smallest variation between methods was not the one that yielded the most DNA from sampling. The same principle applies to sampling method. As stated previously the choice of sampling method is dependent on the purpose.

A swab method is gentle on the recipient, easy to use and quick, thus very suitable for high-throughput sampling of skin (Digel et al. 2018). Tapes are also very popular within skin sampling (Fierer et al. 2010; Taslimi et al. 2017; Clausen et al. 2018), but they have a tendency to need more sample processing, and in this test setup the tapes either did not pick up as much genetic material as the swabs, or the extraction kits were not successful in extracting the DNA from the tapes. Tapes and swabs are otherwise considered comparable in terms of sample collection rates, with tapes having a slightly higher propensity to collect cultivable cells than swabs (Ogai et al. 2018). Swab material and construction is also important. The sampling method that was closest to the Nylon swab was the FLOQ, which was of the same type (flocked nylon swab), size and from the same company. The only discernible difference was in the fiber density and structure on the bud, which probably made one swab either more prone to pick up the bacterial cells or to release the cells in the extraction (Jansson et al. 2020).

6.2 Inhibition study and master mix optimization.

The chosen method combination from previous stages was tested with different master mixes to optimize the qPCR process, and the effect of skin care products on the analysis was investigated by adding skin care products to the samples.

6.2.1 Evaluation of sample DNA extraction in the presence of skin care product.

To investigate the effects of the skin care products on the DNA extraction process, and the qPCR analysis, samples were prepared where the *S. aureus* cell solution was mixed with skin care products before DNA extraction.

The PCR optimization was executed together with the inhibition study. The plan was to analyze all samples from the inhibition study with four different master mixes. However, two of them could not be delivered in time as there was a shortage of lab material due to the pandemic. So only two were used in the end; the Oasig master mix, which had been used in the method development, and the Toughmix master mix. See table 4 for all master mixes. The results were compared to see if any mix yielded lower Cq values, or if any of them were more or less affected by the presence of the products.

After analysis the Cq values for each sample category were averaged and the average Cq value for a control sample extracted without product subtracted, producing a delta Cq value to better illustrate any difference between a sample extracted with product and a sample extracted without product (figure 19 and 20). A delta Cq value of zero would indicate no difference in the PCR process compared to the control sample. A negative delta Cq value indicates a lower Cq value from the sample than the control sample, which would mean that the added skin care product has a positive effect on the amplification compared to the control sample. A positive delta Cq indicates a higher delta Cq value compared to the control, this would in turn indicate an inhibitory effect form the added skin care product (Rådström, Knutsson, Wolffs, Lövenklev, et al. 2004).

After analysis with the Oasig master mix the samples containing the Moist toilet paper and Demakup product showed consistent positive delta Cq values, with a large variation. The samples inoculated with 10^5 cells also showed a positive delta Cq for the barrier cream. This indicates an inhibitory effect from these product. The delta Cq value is very small however, and the variation between sample triplicates are considerably large (figure 19). When analysed with the Toughmix master mix there were no indications of a negative effect on the amplification from the added skin care products, although large variation was prevalent in this population as well, as is visible in the size of the error bars. 20.

Statistical analysis, Anova and Bonferroni-corrected t-test, on both the Oasig and Toughmix-samples showed that the only skin care product samples that were significantly differentiated were the Wash cream and Demakup samples in either master mix, and then only in the 10^7-populations. The wash cream and Demakup samples were differentiated from each other, and the same Wash cream sample was differentiated from the control sample (table 5). The Demakup sample was not differentiated from the control. This indicate no effect from the skin care products on the DNA extraction process, except for a decrease of 1.17 cycles when the Wash cream is present in samples of 10^7 cells. Just as different substances can be inhibitors, there is also substances that can act as PCR facilitators (Rådström, Knutsson, Wolffs, Lövenklev, et al. 2004). the decreased Cq value in the presence of the Wash cream points to the Wash cream being such a facilitator.

Data shows no significant indication of inhibition of the DNA extraction process from

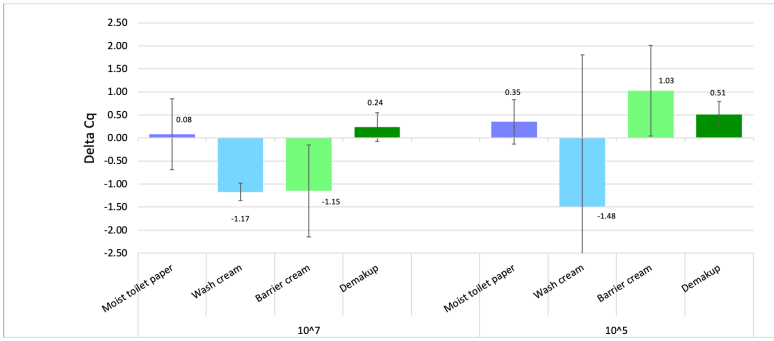


Figure 19: Average delta Cq values and standard deviation error bars of 10^5 and 10^7 cell inoculation samples extracted together with products. Analyzed with Oasig master mix. n=3.

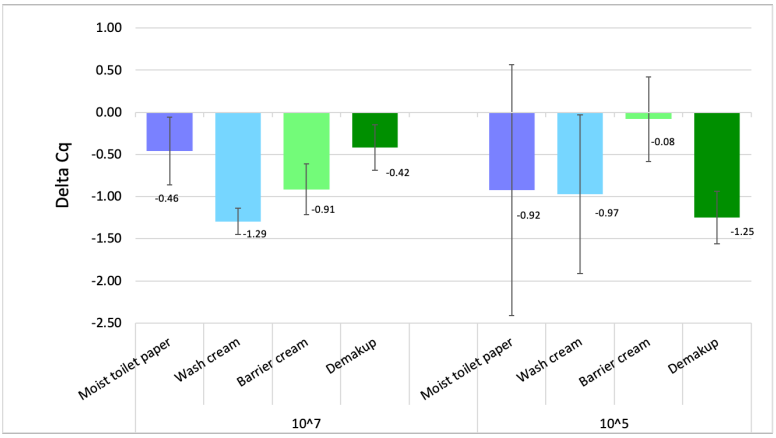


Figure 20: Average delta Cq values and standard deviation error bars of 10^5 and 10^7 cell inoculation samples extracted together with products. Analyzed with Toughmix master mix. n=3.

Table 5: The delta Cq values for the Wash cream and Demakup products, using the Oasig and Toughmix master mixes, samples inoculated with 10^7 cells. They were the only samples that were differentiated from each other.

	Oasig	Toughmix
Wash cream	-1.17	-1.29
Demakup	0.24	-0.42

these products. If anything, the Wash cream is even contributing to a lower Cq value. The Toughmix master mix shows a much greater resilience to the products than the Oasig master mix, indicated by all samples having a negative delta Cq value in the Toughmix population, while there is some variation of positive and negative samples in the Oasig population.

6.2.2 Total DNA content measurement on samples extracted with skin care products.

The extracted samples were also screened for total DNA content, results presented in figure 21. Compared to the total DNA content measurement from the method development these measurements are considerably lower, with the 10^5-population mostly showing no DNA content at all in the samples. Even the control samples with no added product are lower. It could indicate some sort of problem with the extraction caused by the addition of skin care products, although then the control samples would have been much higher. The qPCR analysis results on these samples are comparable with the ones from the method development, which would indicate a successful extraction. Then it is more likely that something has gone wrong with the Qubit analysis. According to the results from the qPCR (figure 19 and 20) there is DNA present in the samples, yet in figure 21 the fluorometer detects very small levels of DNA or not at all.

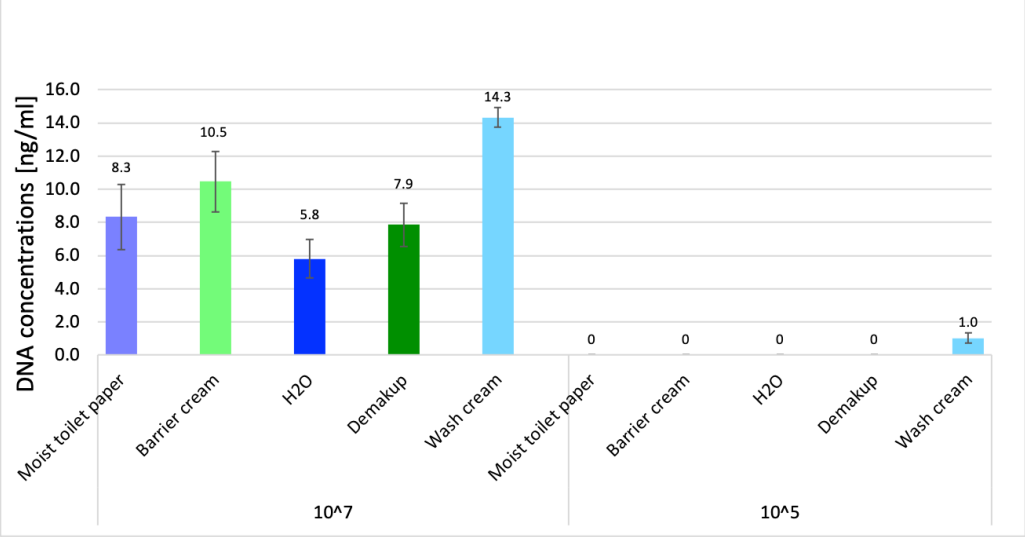


Figure 21: Total DNA content measurements averages with standard deviation error bars on samples extracted together with product, inoculated with, 10_7 and 10_5 cells. n=3.

6.2.3 Evaluation of the effects of skin care product addition to master mix.

Together with the product-extraction samples, samples were prepared where the products were added directly to the master mix. As was the case with the product-extraction samples these samples were analysed with two different master mixes, the Oasig master mix and the Toughmix master mix. A control sample without any product was also

prepared for each batch. As before the data is presented as a delta Cq value, the difference between the sample with a product and the control sample (figure 22 and 23). For the actual Cq values and delta Cq values for each individual product-master mix combination please see appendix 10.1.

The Toughmix analysis show no significant effect between samples with and without skin care products, the delta Cq values never exceeds 0.3 and is most likely due to natural variations in samples (figure 23). Samples analysed with Oasig master mix however, clearly show that the amplification is hampered in the presence of Moist toilet paper and Demakup product. Ranging from 10-1% added product both the samples with added Demakup and Moist toilet paper product indicates a decreasing delta Cq value with decreasing amount of product. This means that the inhibitory effect is decreasing with product concentration. Anova and Bonferroni-corrected t-tests confirms a significant difference between samples with added Demakup product and control samples when analysed with the Oasig master mix, meaning that the Demakup product is having an inhibitory effect on the qPCR. The Moist toilet paper product is not significantly differentiated from the control. Anova analysis yields a p value of 0.003123, which indicates a significantly differentiated group within the population (moist toilet paper samples, Oasig master mix), but subsequent bonferroni-corrected t-tests between all the samples in the population yields only p values above 0.0125 (bonferroni-correction: p=0.05/4=0.0125). However, a Scheffe's test indicate that the 10% product sample is significantly differentiated from the others, which would mean that the Moist toilet paper product does have an inhibitory effect on the qPCR at high concentrations.

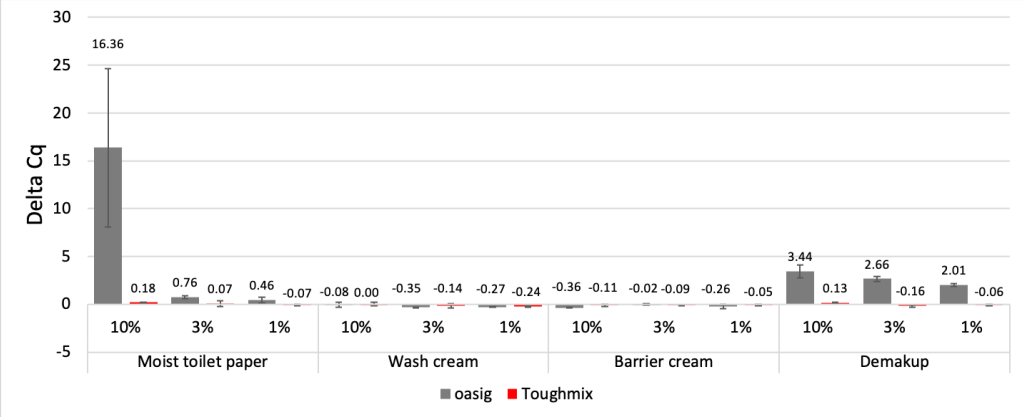


Figure 22: Average delta Cq values of samples with product mixed into master mix at different concentrations, and standard deviations.

It is apparent that the Toughmix master mix is more resilient against pollutants and potential inhibitors than the Oasig master mix. This is not strange since the Toughmix was developed for this very purpose, to be a more resilient and sturdy master mix to be used in situations where samples cannot be prepared extensively before analysis or there is a very complex matrix that is difficult to separate from the sample. When the Oasig master mix is used there is a demonstrable inhibitory effect when the Demakup

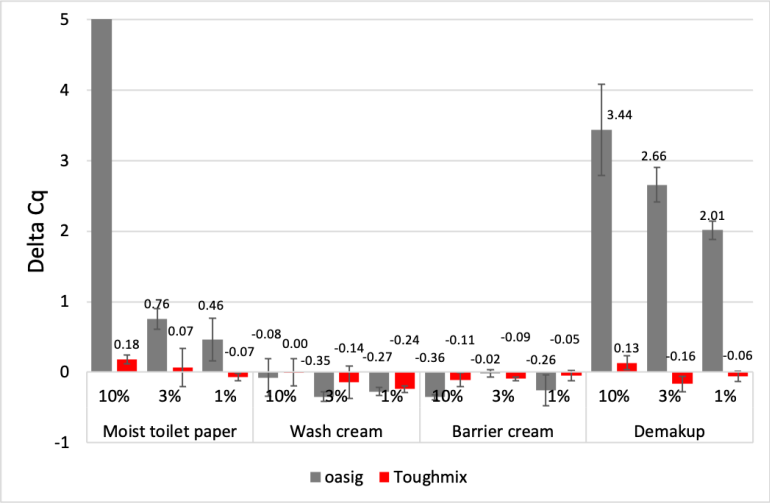


Figure 23: Figure 22, but zoomed in on the Y-axis between Y=-5 and Y=5, to show smaller delta Cq values more clearly.

product is present in the sample, and it is dependent on the concentration of product in the sample. There are also indications of a similar effect when the Moist toilet paper product is present at high concentrations.

The producer of the skin care products supplied a list of ingredients in the products, and both the Demakup and Moist toilet paper product contains sodium EDTA, which is a derivative of EDTA, which in turn is a known PCR inhibitor (Schrader et al. 2012; Hedman and Rådström 2013). The Wash and Barrier cream do not contain this ingredient. The Demakup and Moist toilet paper product also contains various polyethylene glycol (PEG)-derived molecules, PEG is also a known inhibitor of PCR (Hedman and Rådström 2013). The Wash cream also contains PEG-molecules however, and it is not clear whether molecules with PEG in their backbone would have the same inhibitory effect as a free PEG molecule.

6.3 Extraction control results.

In all samples that were extracted an internal extraction control was added, and analyzed on VIC channel in the PCR. The results are presented here. For raw data graphs please see appendix 10.1, figure 40-42.

The extraction control results from the method development confirms a successful extraction (figure 24). There is variation in the results, especially in the higher concentration series. Overall the extraction seems to have been successful, except for the Ring samples inoculated with 10^7 cells, where there was no detection of the extraction control. The Cq value for the control DNA should be around 28 $\pm$ 2 ideally. The 10^7-series consistently yields higher results than that, about Cq= 32-35. Since this is visible in the series with a higher cell concentration in the samples with a higher DNA content it is possible that this is caused by competition for DNA polymerase between the sample DNA and con-

trol DNA ((QIAGEN 2021; Science 2015). Statistical analysis shows a difference ($p< 0.05$) between the Isohelix, FLOQ, Nylon and tape samples of different concentrations (extraction kits have been disregarded). However, when the extraction kits are included (comparison method-method for each extraction kit within one concentration) those differences disappear ($p>0.05$ for all comparisons).

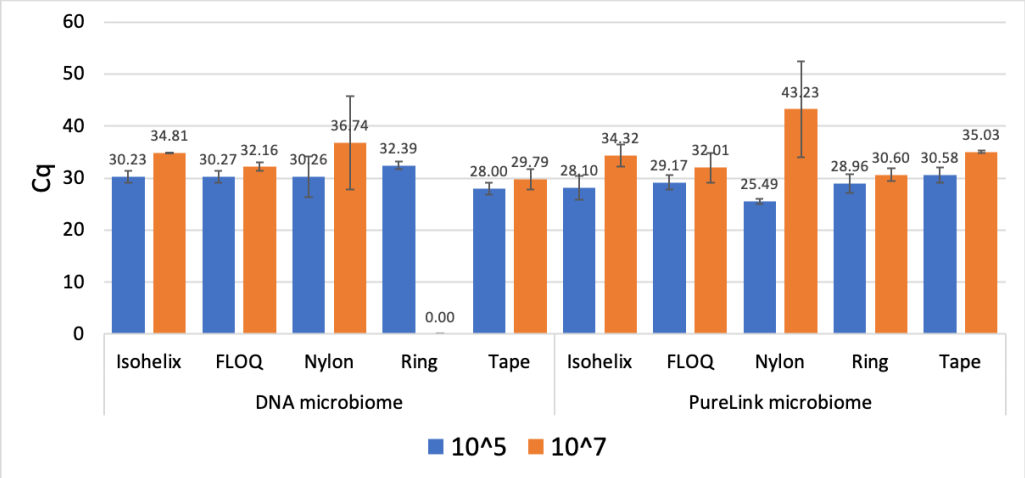


Figure 24: Average extraction results from Method development samples, figure 15 and 14. Blue series is samples inoculated with 10^7 and orange is samples inoculated with 10^5 cells. n=3.

The extraction control from the study where clean in-vitro skin was sampled yields similar results for all sampling materials except the Nylon swab, which has a significantly lower Cq average than the rest(figure 25), but still within the Cq = 28 ± 2 target. Results confirms a successful extraction.

Figure 26 Shows the collected extraction control results for the samples that were extracted together with skin care products, for both inoculate concentrations and master mixes that the samples were analysed with. All sample populations, except for the samples inoculated with 10^7 cells and analysed with the Oasig master mix, confirms a successful extraction. Since the qPCR analysis indicated similar DNA levels as earlier tests, and because it is the Oasig population with a higher amount of sample DNA it is possible that the discrepancy is caused by competition, as was suspected in the method development extraction control, figure 24.

One of the primary questions this project set out to answer was "does skin care products inhibit qPCR?". One product, Demakup, is confirmed to cause inhibition in the qPCR process, and the Moist toilet paper solution have shown indications on inhibitory effects as well. The sodium EDTA in both products is a likely culprit, although there are other compounds in both products that could possibly have this effect also. The Wash and Barrier cream does not seem to have any inhibitory effect on the qPCR process.

The Toughmix master mix, a master mix especially designed to be resilient against inhibition, does not show indication of having had been affected by the addition of these

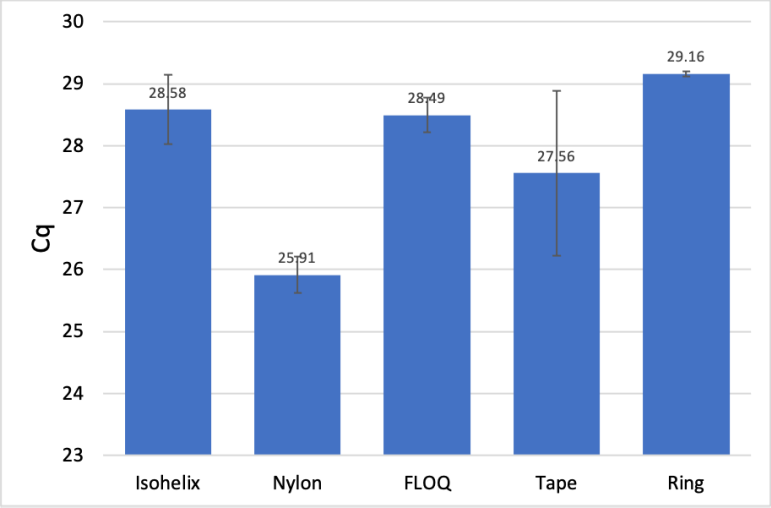


Figure 25: Average extraction control results from Method development samples from clean vitro skin, figure 18. n=3.

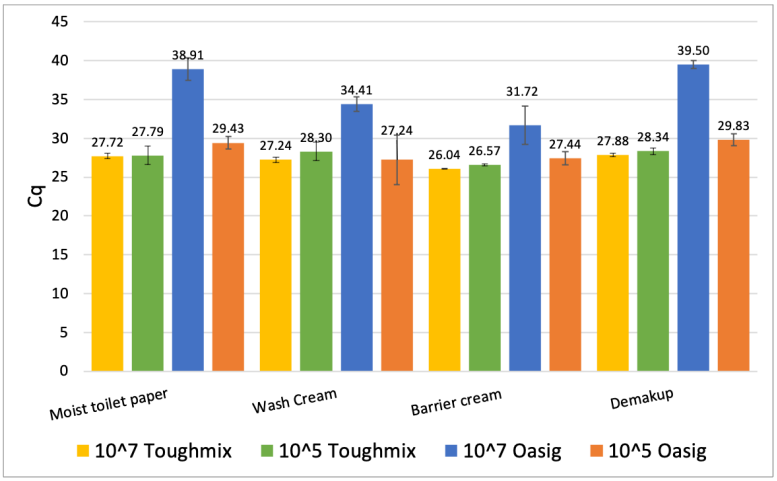


Figure 26: Average extraction control results from samples extracted together with inhibitors from the inhibition study, figure 19 and 20, in two concentrations. n=3.

products to the samples. The total DNA quantification results assessed from the samples extracted together with product (figure 21) were significantly lower than the samples extracted without product. When comparing the results the samples from the method development (figure 16 and 17) the product-extraction samples shows a generally lower level of DNA present, even though the samples should be of the same concentration. However, the extraction control results from the product-extraction samples seems to indicate a satisfactory extraction, with the exception of the Oasig 10^7-series. The quantification results from those samples also seem to indicate similar levels of *S. aureus*-DNA as in previous samples. It could be a problem completely unrelated to the skin care products, some sort of disturbance of the Qubit working solution created by the addition of the skin care products, or on the equipment itself.

6.4 General discussion.

These tests show the complexity of skin micro flora sampling and the importance of optimizing and adapting a method for its intended purpose. The tests in this project have been executed with a prepared, isolated, homogeneous bacterial solution, to test a method meant to sample actual test subjects, sampling their complex and diverse micro floras. There is more work to be done in terms of quality testing and validation of this method, but this has been a start. The limit of detection (LOD) and limit of quantification (LOQ) needs to be established for the test setup, and the setup validated against a more complex target, a heterogeneous culture perhaps (Hedman, Lavander, et al. 2018).

This method have been developed to be used for quantification, but as the research project progresses beyond the scopes of this master thesis it is supposed to be adapted for sequencing as well. Just as a method needs to be tried and validated for quantification it needs to be for its suitability to collect target genomes at a quality adequate for sequence analysis. A method that have a high collection rate, but breaks the genetic material into unreadable fractions at the same time is useless for sequencing purposes (R. D. Bjerre et al. 2019; Meisel et al. 2016). Swabs are generally perceived as very good for this purpose, and in terms of being able to generate high-quality libraries from samples. eSwabs (like the Nylon swab used in this project) have been shown to perform very well in this regard (R. D. Bjerre et al. 2019; Meisel et al. 2016). The PureLink microbiome kit works well with swabs, but it does have a significant flaw compared to the DNA microbiome kit in this regard. The DNA microbiome kit contains measures to remove any human host-DNA which might be collected with the sample. By removing the human DNA present in the sample the sequencing sensitivity and library quality is greatly increased, which is something to consider for the future uses of this method (R. D. Bjerre et al. 2019).

7 Conclusions.

In the introduction three questions were stated as the framework for this project:

- What is the best Sampling method/extraction method combination?
- What master mix is most suitable?
- Does skin care products have an inhibitory effect on the qPCR analysis?

This report has come to the conclusion that the best sampling/extraction combination, of the alternatives tested, are the Copan ESwab 480C together with the ThermoFischer PureLink Microbiome DNA Purification Kit and the best master mix is the Quantabio Toughmix. As to the question on inhibition of the qPCR analysis caused by skin care products, I find that there are indications that some skin care products will have an inhibitory effect on a PCR or qPCR reaction. This effect can be mitigated by the choice of master mix, and there is a need for further research. The effect can probably be connected to one or multiple ingredients in these products, and when they are known they can be anticipated in the testing process.

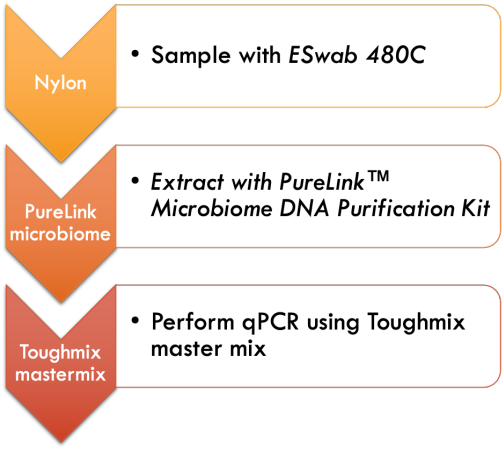


Figure 27: Finalized method. Sampling method, extraction kit and master mix.

7.1 Future research needs.

The TATAA Grandmaster mix and Immolase/BSA master mix should be evaluated as well, so first order of business should be to run the same analysis set up with those master mixes as was done with the Oasig and Toughmix master mix. The reason behind the inhibitory effects of the Demakup and Moist toilet paper products needs to be investigated further, particularly if they can be linked to specific compounds that might be present in many skin care products. The testing protocol (Sampling, extraction and PCR) needs to be validated and evaluated for sensitivity, LOD and LOQ, tested with a bacterial culture that is more representative of the microbial community present on the skin rather than just with a homogeneous culture of one type of bacteria. Finally the protocol should be adjusted for other types of PCR which it might be used for, such as sequencing.

8 Acknowledgements.

I would like to thank RISE Research Institutes of Sweden for giving me this opportunity, and for the trust in letting me conduct this work at your facilities. I would especially like to direct a warm thank you to Ingela Persson, lab engineer at RISE, for answering all my questions and showing me around the premises, as well as Unn Tjörnstrand, lab engineer, and Linnea Qvirist, lab engineer, for welcoming me in your lab.

To my fantastic supervisors: Karin Bjerre, researcher, Charlotta Löfström, researcher, and Erik Nygren, researcher, from RISE and Linda Jansson, researcher, and Johannes Hedman, researcher, from LTH. Thank you for all the input and guidance along the way.

Lastly, I would like to thank Lund University and LTH for giving me the tools to perform this kind of work, and for helping me in this, the final step of my journey of becoming an engineer.

9 Popular Scientific summary: How to investigate the effects of skin care products on skin bacteria.

It has been said that the human body consists of more bacterial cells than human. Whether this is true or not it is a fact that we are landlords to millions of tiny tenants living on and inside our bodies. Most people have heard of the gut microbiome, the microbial community of the intestines, but it is not the only bacterial community paying rent to us. The skin is also home to a vast selection of bacteria, and it is becoming more and more clear that it might equal the role the gut microbiome plays in our health and well-being. From arming our budding immune systems as babies to acting as security guards against pathogenic bacteria colonizing our skin, the skin microflora fulfills a lot of different functions that help keep us healthy throughout our entire lives.

However, there is still much to learn about the skin micro flora, how is it affected by the hosts' diet and hygiene routines? Which bacteria are responsible for certain skin conditions and which helps keep others away? To answer these questions more research and investigation is needed. This project has focused on developing a method which can be used for that purpose, by testing different sampling and analysis techniques. To assess the different techniques samples were collected and the amount of bacterial cells collected with the method counted, quantified, using PCR-technique. The resulting method uses the ESwab 480C from Copan, PureLink™ Microbiome DNA Purification Kit from Thermofischer and the ToughMix® 2x master mix from Quantabio for sampling, sample processing and qPCR analysis. The method was then tested with samples containing various skin care products to investigate what effects the presence of skin care products would have on the method. It was shown that skin care products can have an adverse effect on the qPCR analysis, but that the Toughmix master mix is very adapt at mitigating these effects, producing accurate results anyway.

The analysis did only involve quantification at this stage, although PCR is a very powerful and versatile tool for microbiological analysis, and could be adapted to produce more results than simple quantification of bacteria present in a sample.The PCR protocols can be adapted to enable detection and sequencing of bacteria with very high accuracy, meaning that bacteria present even in very small quantities could be detected and identified in a sample. By doing this bacterial profiles can be generated for different subjects, and then screened for changes with time, change in hygiene routine, etc. By understanding the skin flora we might find treatments for various skin conditions, find ways to prevent infections caused by antimicrobial-resistant bacteria and mechanisms to boost weak immune systems. To do this we need to have a better understanding of the interactions between ourselves and the microorganisms living on our skin. Hopefully this research method can contribute to the improvement of that understanding and help uncover more knowledge in the future. Illustration created by author from sourced image, see ref. list. (Origimm 2021)

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10 Appendices.

10.1 Complementary data and result graphs.

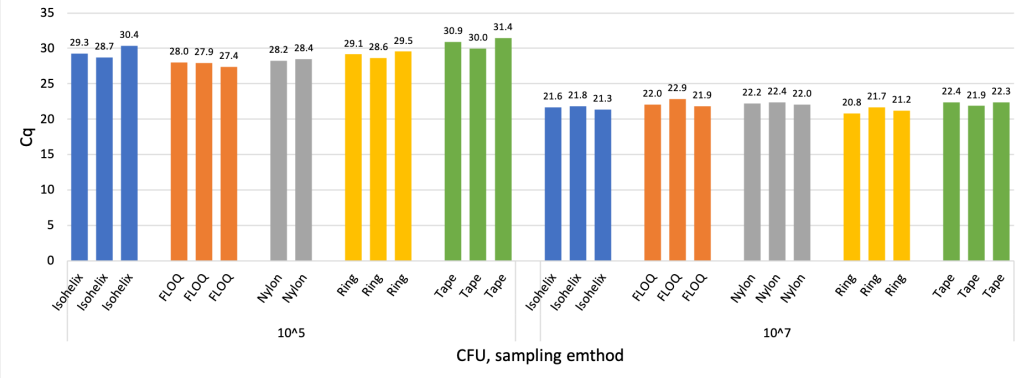

Figure 28: Raw data to figure 14, method development samples extracted with DNA microbiome kit. X-axis is labelled with sampling method and inoculate concentration on artificial skin (CFU/ml).

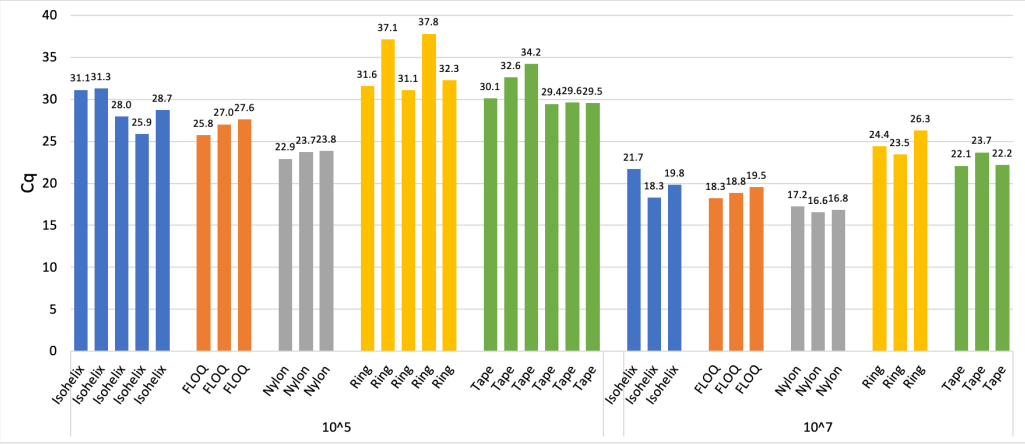

Figure 29: Raw data to figure 15, method development samples extracted with DNA microbiome kit. X-axis is labelled with sampling method and inoculate concentration on artificial skin (CFU/ml).

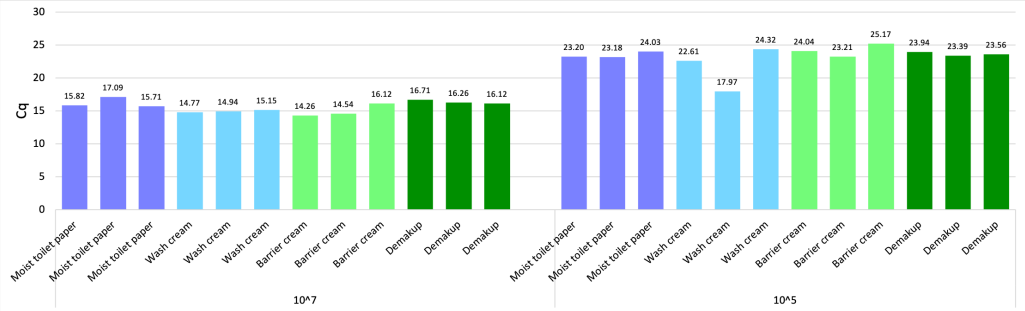

Figure 30: Raw data to figure 19, master mix optimization and inhibition study samples, extracted together with skin care products. X-axis is labelled with skin care product and inoculate concentration on artificial skin (CFU/ml).

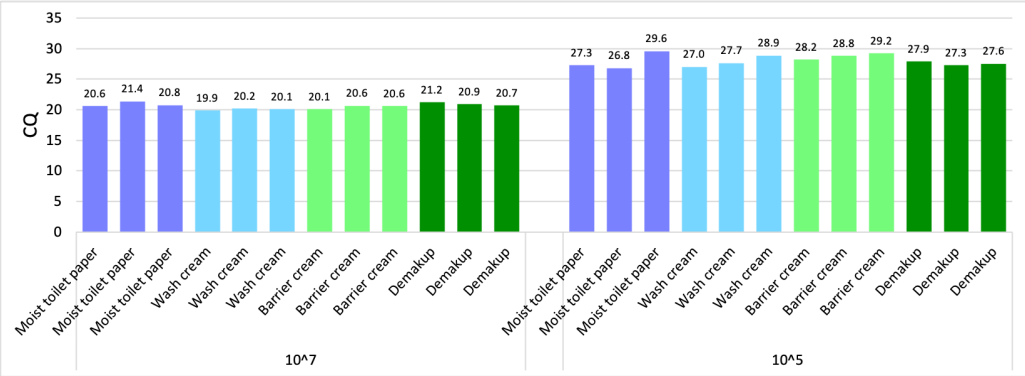

Figure 31: Raw data to figure 20, master mix optimization and inhibition study samples, extracted together with skin care products. X-axis is labelled with skin care product and inoculate concentration on artificial skin (CFU/ml).

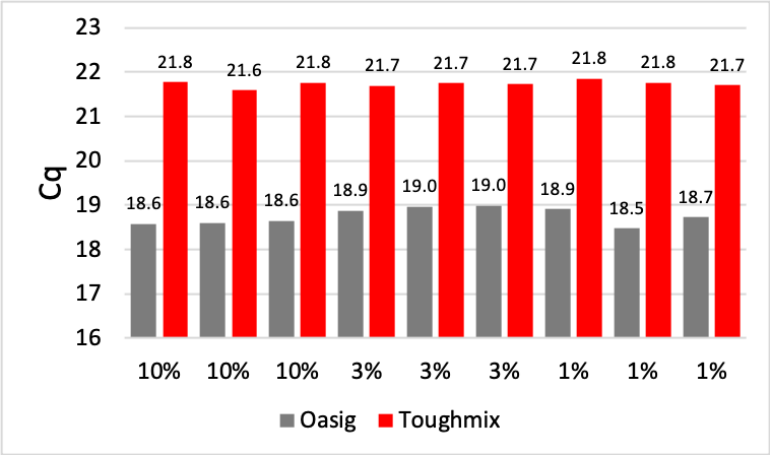

Figure 32: Barrier cream. Raw data to figure 37, master mix optimization and inhibition study samples, where skin care products have been mixed together with master mix before PCR well preparation. X-axis is labelled with volume percentage skin care product added to PCR well.

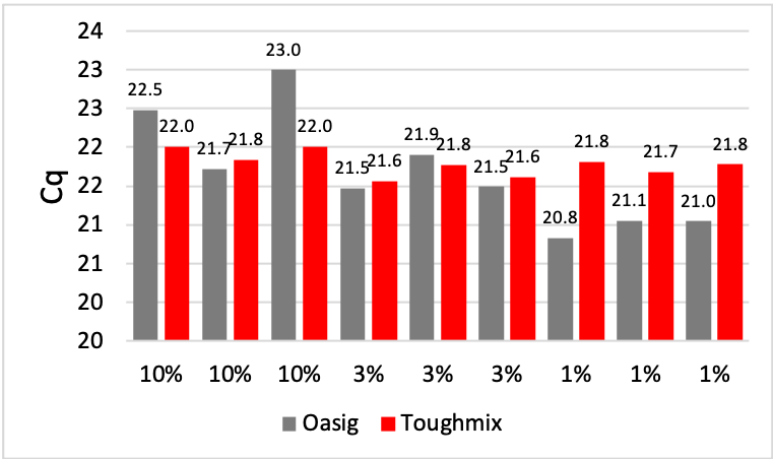

Figure 33: Demakup. Raw data to figure 38, master mix optimization and inhibition study samples, where skin care products have been mixed together with master mix before PCR well preparation. X-axis is labelled with volume percentage skin care product added to PCR well.

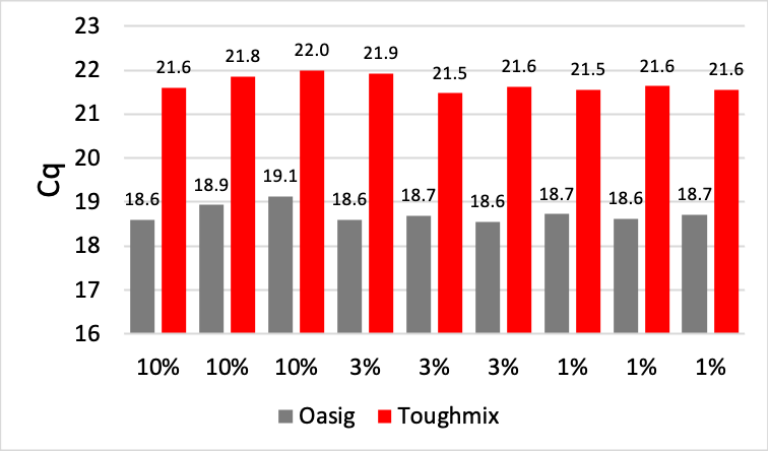

Figure 34: Wash Cream. Raw data to figure 39, master mix optimization and inhibition study samples, where skin care products have been mixed together with master mix before PCR well preparation. X-axis is labelled with volume percentage skin care product added to PCR well.

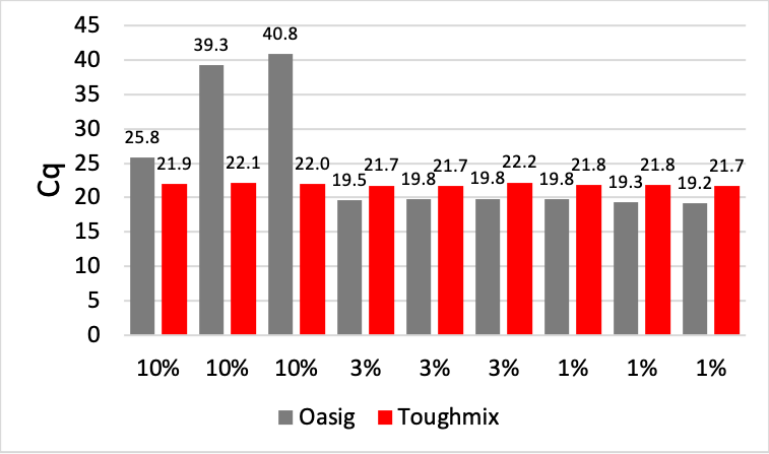

Figure 35: Moist toilet paper. Raw data to figure 36, master mix optimization and inhibition study samples, where skin care products have been mixed together with master mix before PCR well preparation. X-axis is labelled with volume percentage skin care product added to PCR well.

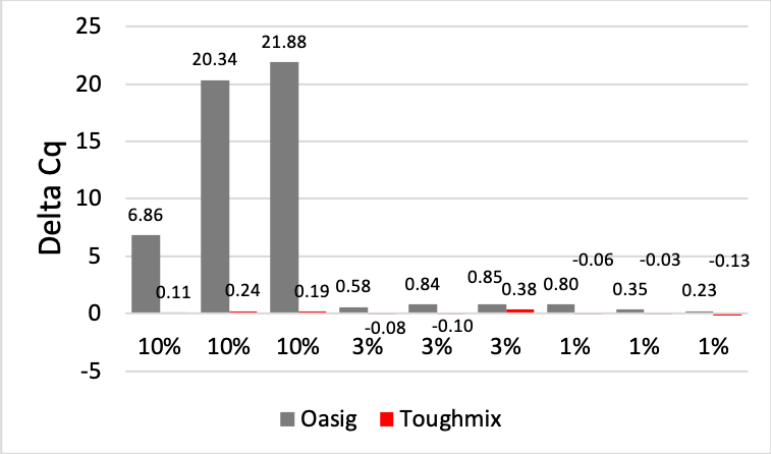

Figure 36: Delta Cq values for Moist toilet paper product vs. samples with no product. product was added at 10-, 3- and 1% of total reaction volume. 0% samples are the control samples without any product. n = 3.

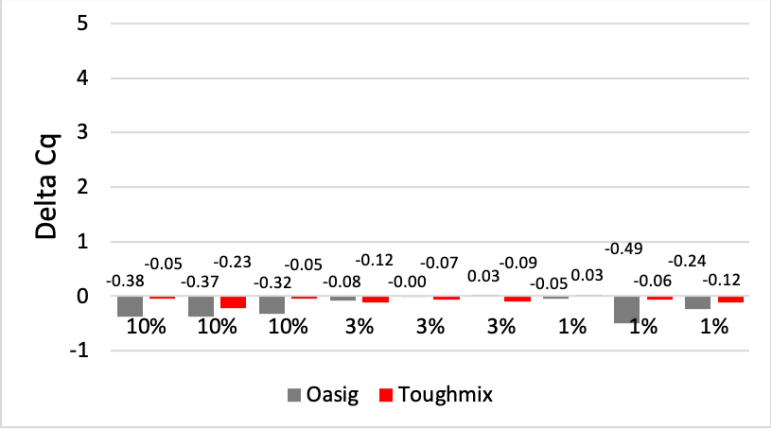

Figure 37: Delta Cq values for Barrier cream product vs. samples with no product. product was added at 10-, 3- and 1% of total reaction volume. 0% samples are the control samples without any product. n = 3.

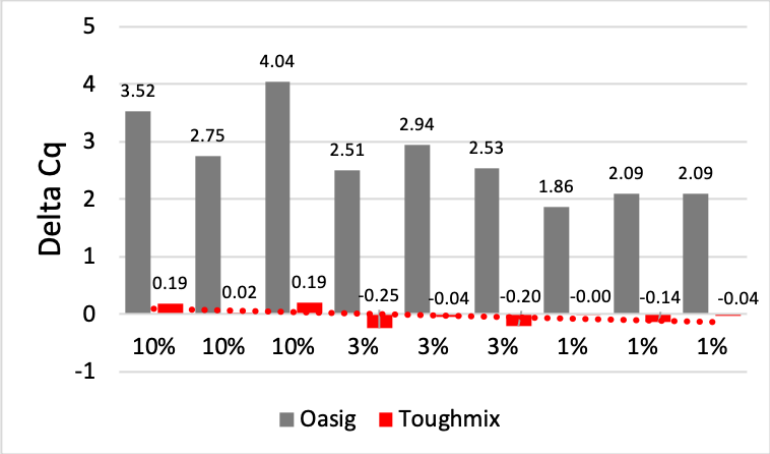

Figure 38: Delta Cq values for Demakup product vs. samples with no product. product was added at 10-, 3- and 1% of total reaction volume. 0% samples are the control samples without any product. n = 3.

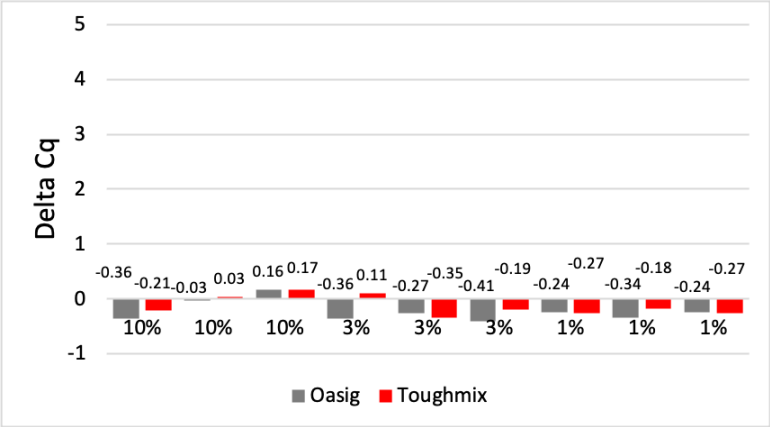

Figure 39: Delta Cq values for Wash cream product vs. samples with no product. product was added at 10-, 3- and 1% of total reaction volume. 0% samples are the control samples without any product. n = 3.

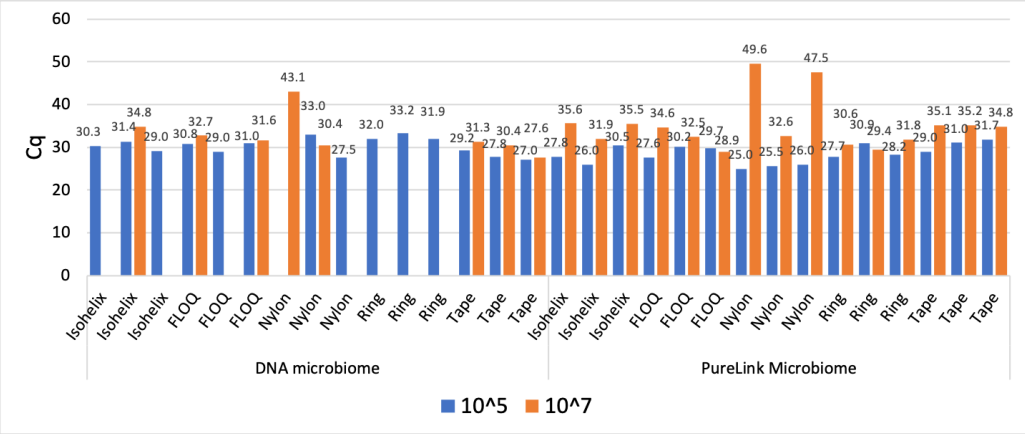

Figure 40: Raw data to figure 24.

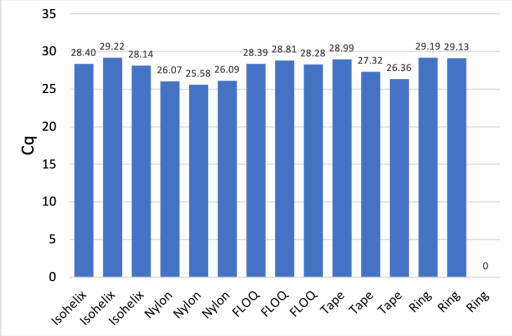

Figure 41: Raw data to figure 25

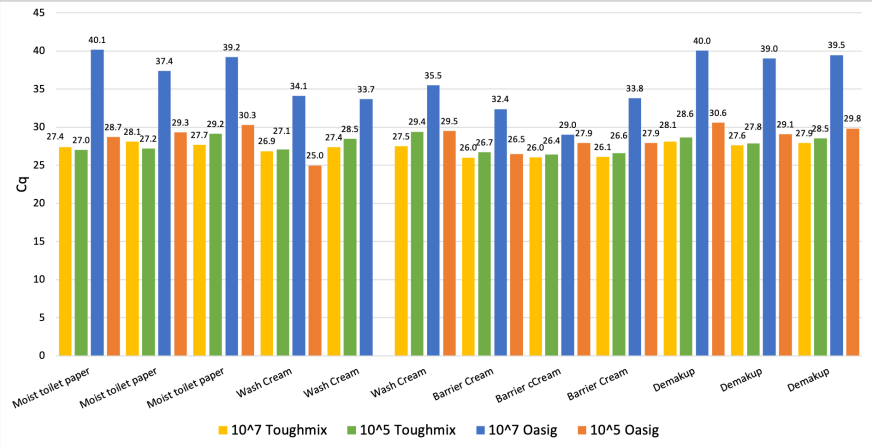

Figure 42: Raw data to figure 26

10.2 PCR setups and protocols.

10.2.1 OasigTM lyophilised 2X qPCR Mastermix

Table 6: Reaction setup, per well. Reaction volume 20 µL.

Sample type	Reagents	Volume [µL]
Standards and controls	Master mix	10
	Primer/prob-mix	1
	RNas/DNAs free water	4
	final volume	**15**
samples with internal extraction control	Master mix	10
	Primer/prob-mix	1
	Internal extraction control	1
	RNas/DNAs free water	3
	final volume	**15**
samples with 10% product	Master mix	10
	Primer/prob-mix	1
	Product	2
	RNas/DNAs free water	2
	final volume	**15**
samples with 3% product	Master mix	10
	Primer/prob-mix	1
	Product	0.6
	RNas/DNAs free water	3.4
	final volume	**15**
samples with 1% product	Master mix	10
	Primer/prob-mix	1
	Product	0.2
	RNas/DNAs free water	3.8
	final volume	**15**

Table 7: Oasig mastermix cycle protocol. Cycle step 2 and 3: denaturation and data analysis a total of 50 times

Step	Time [s]	Temp [°C]
Enzyme activation	120	95
Denaturation	10	95
Data analysis	60	60
total time (hh:mm)	1:00	-

10.2.2 ToughMix® 2x master mix

Table 8: Reaction setup, per well. Reaction volume 20 µL.

Sample type	Reagents	Volume [µL]
Standards and controls	Master mix	10
	Primer/prob-mix	2
	RNas/DNAs free water	3
	final volume	**15**
samples with internal extraction control	Master mix	10
	Primer/prob-mix	2
	Internal extraction control	2
	RNas/DNAs free water	1
	final volume	**15**
samples with 10% product	Master mix	10
	Primer/prob-mix	2
	Product	2
	RNas/DNAs free water	1
	final volume	**15**
samples with 3% product	Master mix	10
	Primer/prob-mix	2
	Product	0.6
	RNas/DNAs free water	2.4
	final volume	**15**
samples with 1% product	Master mix	10
	Primer/prob-mix	2
	Product	0.2
	RNas/DNAs free water	2.8
	final volume	**15**

Table 9: Toughmix mastermix cycle protocol. Cycle step 2 and 3: denaturation and annealing/extension a total of 45 times

Step	Time [s]	Temp [°C]
Initial denaturation	180	95
Denaturation	10	95
Annealing/extension	50	60
total time (hh:mm)	0:48	-

10.3 Extraction kits protocols with modifications Qubit protocol.

These are the standard protocols of the extraction kits with any modifications made during the experiments included.

10.3.1 PureLink™ Microbiome DNA Purification Kit

1. Mix lysis buffer and internal extraction control-DNA from primer/probe-kit, 800 µL and 4 µL respectively into a lysis buffer-mix.
2. Add 804 µL lysis buffer-mix to the beadtube and add sample.
3. Add 100 µL lysis enhancer, close tube and vortex briskly.
4. Incubate in 65 °C, for 10 minutes.
5. Put in beadshaker for 10 minutes on max speed.
6. Centrifuge 14000 x g for 1 minute.
7. Transfer 500 µL of the supernatant in the beadtube to a clean eppendorf tube, be careful not to transfer any beads or debris).
8. Add 900 µL binding buffer, vortex briskly.
9. Load 700 µL of this binding buffer- sample-mix into the spin column with collection tube and cetrifuge 14000 x g for 1 minute.
10. Discard the flow-through and repeat the centrifugation with the remaining binding buffer-sample-mix.
11. Place the column in a clean eppendorf tube and add 500 µL wash buffer and centrifuge 14000 x g for 1 minute.
12. Discard the flow-through and repeat the centrifugation for 30 seconds.
13. Place the column in a clean eppendorf tube, add 50 µL elution buffer and incubate in room temperature for 1 minute.
14. Centrifuge 14000 x g for 1 minute, sample DNA is now collected in the eppendorf tube.

10.3.2 QIAamp® DNA Microbiome Kit

1. Put sample in a 2 ml eppendorf tube.
2. Shake sample in 1 ml PBS or transport medium for at least 20 seconds and press all liquid from the sample collector before discarding it.
3. Add 500 µL AHL buffer to the 1 ml sample. Incubate for 30 minutes at room temperature during end-to-end rotation. A thermomixer 600 rpm can be substituted.
4. Centrifuge the tube 10 000 x g for 10 minutes and remove the supernatant carefully without disturbing the pellet.
5. Add 190 µL RDD buffer and 2.5 µL benzonase. Mix well and incubate in thermomixer, 37 °C and 600 rpm, for 30 minutes.

6. Add 20 µL proteinase K and incubate in thermomixer, 56 °C and 600 rpm, for 30 minutes.
7. Quickly centrifuge tube on low speed to collect any condensation.
8. Mix ATL buffer and internal extraction control-DNA, 200 and 4 µL respectively, into a lysis buffer-mix.
9. Add 204 µL lysis buffer-mix to tube. Mix well and transfer to a pathogen lysis tube L.
10. Place the tube in a horizontal shaker for 10 minutes, max speed.
11. Centrifuge the tube 10 000 x g for 1 minute
12. Carefully transfer the sample supernatant into a new tube. Take care not to include any beads.
13. Add 40 µL proteinase K, mix and incubate in 56 °C, 600 rpm, for 30 minutes.
14. Add 200 µL APL2, mix by pulsing for 30 seconds.
15. Incubate the tube in 70 °C for 10 minutes, then spin down the contents.
16. Add 200 µL ethanol (90-99%), mix thoroughly for 15-30 seconds.
17. Transfer 700 µL supernatant to a UCP mini column, close the lid and centrifuge 600 x g for 1 minute.
18. Discard the flow-through and put the column back into the same tube. Transfer the rest of the sample supernatant into the column and repeat the centrifuging.
19. Put the column in a new collection tube, add 500 µL AW1, centrifuge 6000 x g for 1 minute.
20. Put the column in a new collection tube, add 500 µL AW2, centrifuge 20 000 x g for 3 minute.
21. Place the column in another new collection tube and centrifuge 20 000 x g for 1 minute to dry the filter.
22. Place the column in a clean eppendorf tube and add 50 µL AVE buffer, incubate for 5 minutes at room temperature.
23. Centrifuge 6000 x g for 1 minute, sample DNA is now collected in the eppendorf tube.

10.3.3 DNeasy® Blood and Tissue kit

1. Before extraction prepare an enzymatic lysis buffer-mix:
   - TE lysis buffer 160 µL per sample
   - Lysozyme (20 mg/ml) 40 µL per sample
   - RNasA (100 µg/ml) 1.4 µL
   - Internal extraction control DNA 4 µL per sample

2. Release the bacterial cells from the swab by shaking it in 1 ml sterile distilled water or mili-Q water or PBS for 20 minutes. Remove swab.
3. Centrifuge 10 000 xg for 10 minutes.
4. Discard supernatant, and keep pellet. Add 184 µL enzymatic lysis buffer-mix and re-suspend pellet.
5. Incubate for 30 minutes in 37 °C.
6. Heat heating block to 56 °C.
7. Add first 25 µL proteinase K, then 200 µL AL-buffer. Vortex and transfer to an eppendorf tube, before placing in the heating block.
8. Incubate for 30 minutes.
9. Add 200 µL ethanol (96-100%) to each sample, vortex thoroughly
10. Transfer the suspension to a DNeasy mini spin-column and centrifuge 600 x g for 1 minute.
11. Transfer the column to a clean collection tube, discard the old tube.
12. Add 500 µL AW1 buffer and centrifuge 600 x g for 1 minute.
13. Transfer the column to a new collection tube and discard the old tube.
14. Add 500 µL AW2 buffer and centrifuge 1700 x g for 3 minutes.
15. Transfer the column to an eppendorf tube. Add 50 µL elution buffer (Buffer AE)and incubate for 1-2 minutes at room temperature.
16. Centrifuge 600 x g for 1 minute.
17. Discard the column, sample DNA is now collected in the eppendorf tube.

TE lysis buffer: For 250 ml buffer mix together

- 20 mM Tris-CL 0.605g
- 2 mM Na EDTA 0.186g
- 1.2 % TritonX-100 3 µL

10.3.4 Qubit fluorometer protocol

Instructions are valid for both the broad range (BR) and high sensitivity (HS) assays available in the fluorometer. To apply protocol, simply choose BR- or HS reagent as desired.

- Mix working solution (WS) 1:200 Qubit reagent and Qubit buffer to a total volume of 200 µL per sample and two calibration standards.
- Mix 190 µL WS with 10 µL sample or calibration standard in special Qubit-particular micro tubes.
- incubate for 1 minute at room temperature.

- Run analysis according to equipment operating instructions.