NTNU

Kunnskap for en bedre verden

Bacheloroppgave

BI301305 Bacheloroppgave

Establishing procedures for 16S metagenomics analysis of urine.

10020

10001

10015

Totalt antall sider inkludert forsiden: 86

Ålesund, 06.06.2017

NTNU

Kunnskap for en bedre verden

Obligatorisk egenerklæring/gruppeerklæring

Den enkelte student er selv ansvarlig for å sette seg inn i hva som er lovlige hjelpemidler, retningslinjer for bruk av disse og regler om kildebruk. Erklæringen skal bevisstgjøre studentene på deres ansvar og hvilke konsekvenser fusk kan medføre. Manglende erklæring fritar ikke studentene fra sitt ansvar.

Du/dere fyller ut erklæringen ved å klikke i ruten til høyre for den enkelte del 1-6:		
1.	**Jeg/vi erklærer herved at min/vår besvarelse er mitt/vårt eget arbeid, og at jeg/vi ikke har brukt andre kilder eller har mottatt annen hjelp enn det som er nevnt i besvarelsen.**	☒
2.	**Jeg/vi erklærer videre at denne besvarelsen:** • ikke har vært brukt til annen eksamen ved annen avdeling/universitet/høgskole innenlands eller utenlands. • ikke refererer til andres arbeid uten at det er oppgitt. • ikke refererer til eget tidligere arbeid uten at det er oppgitt. • har alle referansene oppgitt i litteraturlisten. • ikke er en kopi, duplikat eller avskrift av andres arbeid eller besvarelse.	☒
3.	**Jeg/vi er kjent med at brudd på ovennevnte er å betrakte som fusk og kan medføre annullering av eksamen og utestengelse fra universiteter og høgskoler i Norge, jf. Universitets- og høgskoleloven §§4-7 og 4-8 og Forskrift om eksamen §§14 og 15.**	☒
4.	**Jeg/vi er kjent med at alle innleverte oppgaver kan bli plagiatkontrollert i Ephorus, se Retningslinjer for elektronisk innlevering og publisering av studiepoenggivende studentoppgaver**	☒
5.	**Jeg/vi er kjent med at høgskolen vil behandle alle saker hvor det forligger mistanke om fusk etter høgskolens studieforskrift §31**	☒
6.	**Jeg/vi har satt oss inn i regler og retningslinjer i bruk av kilder og referanser på biblioteket sine nettsider**	☒

Publiseringsavtale

Studiepoeng: 15

Veileder: Ann-Kristin Tveten og Heidi Cecilie Villmones

Fullmakt til elektronisk publisering av oppgaven

Forfatter(ne) har opphavsrett til oppgaven. Det betyr blant annet enerett til å gjøre verket tilgjengelig for allmennheten (Åndsverkloven §2).
Alle oppgaver som fyller kriteriene vil bli registrert og publisert i Brage HiM med forfatter(ne)s godkjennelse.
Oppgaver som er unntatt offentlighet eller båndlagt vil ikke bli publisert.

Jeg/vi gir herved NTNU i Ålesund en vederlagsfri rett til å gjøre oppgaven tilgjengelig for elektronisk publisering: ☒**ja** ☐**nei**

Er oppgaven båndlagt (konfidensiell)? ☐**ja** ☒**nei**
(Båndleggingsavtale må fylles ut)
- Hvis ja:
Kan oppgaven publiseres når båndleggingsperioden er over? ☐**ja** ☐**nei**

Er oppgaven unntatt offentlighet? ☐**ja** ☒**nei**
(inneholder taushetsbelagt informasjon. Jfr. Offl. §13/Fvl. §13)

Dato: 29.05.17

SUMMARY

Metagenomics is a fairly new field with many different approaches, and the need for establishing good procedures is existent. Recently, metagenomics has been used in research regarding resident flora in urine from healthy subjects. Urine, being a material with small amounts of cells, presents challenges to any method of analysis. This is why it is a good alternative in testing the quality of the procedures that are established.

In the thesis, procedures for 16S metagenomics analysis of urine are established, and changes for future application of the procedures are suggested. Urine was collected from ten healthy male subjects, and tested by 16S metagenomics analysis on the Ion Torrent PGM. Series of cultivated and uncultivated samples were set up, and the uncultivated ones provided no results, whereas the cultivated ones showed a dominance of Corynebacterium, Bacilli, Firmicutes, Lactobacilliales, and Enterococcaceae. The results proved the established procedures to be functional in cultivated samples, but metagenomics for liquid urine still needs optimization.

SAMMENDRAG

Metagenomikk er et forholdsvis nytt felt med mange ulike fremgangsmåter, og behovet for etablering av gode prosedyrer er stort. I nyere forskning har metagenomikk blitt benyttet til kartlegging av normalflora i urin hos friske mennesker. Siden urin er et materiale som inneholder små mengder med celler, opptrer den som en stor utfordring for de fleste analysemetoder. Dette gjør at det er et gunstig materiale å bruke for å utfordre kvaliteten på prosedyrene som etableres.

I oppgaven etableres prosedyrer for 16S metagenomikk analyser for urin, og eventuelle endringer til fremtidige forsøk foreslås. Urinen ble samlet fra 10 friske menn, og testet ved 16S metagenomikk analyse på Ion Torrent PGM. Serier av kultiverte og ukultiverte prøver ble satt opp, de ukultiverte prøvene ga ingen resultater, mens de kultiverte prøvene viste dominerende innhold av Corynebacterium, Bacilli, Firmicutes, Lactobacilliales, og Enterococcaceae. Resultatene viste at prosedyrene som ble etablert fungerte godt for kultiverte prøver, men metagenomikk for ukultivert urin har fremdeles behov for optimalisering.

PREFACE

This has been a challenging and educational process and we thank our supervisor at NTNU campus Aalesund, Ann-Kristin Tveten for excellent guidance. We also thank dr. Heidi Cecilie Villmones for guidance and inspiration for the assignment. A lot of gratitude goes out to the participants that provided the test material.

Table of contents

1. INTRODUCTION

Contrary to popular belief, research shows that urine in healthy humans is not sterile – but can contain a certain amount of resident flora. Since we have always been taught that urine is sterile, this project caught our eye instantly. Our interest also grew when we were presented with the opportunity to learn next generation sequencing. The specific thesis problem is; "Establishing procedures for Ion Torrent 16S metagenomics analysis of urine." The assumption that urine is sterile is no longer valid, and different studies have used metagenomics to identify what can be described as naturally occurring bacteria in urine. There are many different approaches to metagenomics, and the aim of this study is to establish procedures for pre-analytical variables of Ion Torrent 16S Metagenomics.

Material for testing will be gathered from male subjects in the age range 19-30. The selection of age group was based on the weak representation of the given age group in recent publications – and the convenience of recruiting subjects within the student environment. It has also been claimed that the resident flora in males potentially decreases with age, which supports the decision to gather samples from younger subjects.

The time frame spreads from March 20´th to June 6´th, and all written and analytical work will be done within this period. Materials will be gathered by NTNU in Aalesund and specimens will be gathered by volunteer subjects. The project is relevant not only because of our educational program, but it is also relevant for the medical laboratory scientist – and medical community. This is a project based on very new research, and it has roots in courses microbiology, biochemistry, cell biology, biomedical laboratory technology, instrumental analysis – and practical bioinformatics. It can also be used to obtain a better understanding of pathological diseases in the urinary tract, and the resident floras role in sustaining a healthy environment. It is our duty as future medical laboratory scientists to keep up with recent research, and with this thesis we wish to show the importance of that.

1.1 Thesis problem

Establishing procedures for Ion Torrent 16S metagenomics analysis of urine.

2. THEORY

2.1 16S rRNA

The ribosome is an organelle within the cells that consists of two subunits. Each unit consists of rRNA molecules and polypeptides. 16S is a part of the small ribosomal subunit which can be used for identification of different organisms. The term "S" (Svedberg) indicates the size of cell components(1).

The bacterial ribosomes consist of a small and a large subunit which is made up of proteins and one or two rRNAs. The small subunit is 30S and consists of 16S rRNA and 21 proteins, and the large subunit is 50S and consists of two rRNAs, 23S and 5S, and 34 proteins. In prokaryotes, the ribosomes are found in cytosol where they are separate when protein synthesis is not occuring. To have a functional ribosome the small and the large subunits must be bound, and the mRNA therefore binds the small subunit (30S) and makes 70S. 16S is the specific point where the anticodon gets attached in order to make the ribosome functional(2,3).

The 16S rRNA is 1500 base pairs long. On this rRNA there are three highly conserved bases, A1492, A1493 and G530. When the tRNA anticodon whit the matching bases bind to these bases, a conformational change occurs in A1492 and A1493. The matching tRNA is charged with a specific amino acid depending on the coding triplet and when it bounds to the ribosome it gives its amino acid to the growing polypeptide chain(2,3).

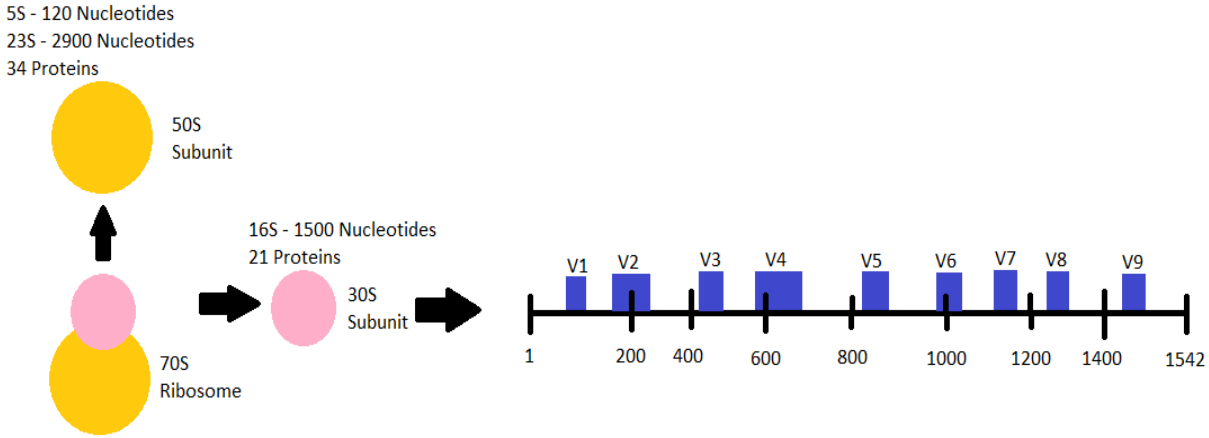


Figure 1 The ribosome illustrated in yellow and pink, and the 16S gene with the hypervariable regions illustrated in blue (Figure by authors).

As discussed above there are a few conserved bases on the 16S gene that help us find the gene sequence. We know that when the gene is found and amplified we can use it to detect which bacteria it is. To be able to do so, there must be bases on the gene that differs from each other. These bases are called hypervariable regions and there are nine different bases (V1 - V9)(4). These nine hypervariable regions are separated by nine highly conserved regions(5). To detect these bases after the 16S gene is amplified one must add different primer pairs that will detect the bases. Previous investigation has shown which hypervariable region is preferred to use for finding different bacteria families. Studies have also shown that one hypervariable region alone is not enough to identify the different bacteria. One must investigate the different regions and with the results from the different regions the bacteria findings can be determined(4).

There are different bacteria located for the different hypervariable regions, and when a region is found one can determine which bacteria it is. If the region V1 is found, it can indicate a Staphylococcus sp. and negative Streptococcus sp. The regions V2 and V3 are very good in use of finding bacterial species to genus level, except for bacteria closely related to Enterobacteriaceae. This also applies for V6. V2 and V3 are also god in use for finding Mycobacterial (V2) and Haemophilus (V3) and V6 is best for finding Bacillus Anthracis(4).

2.2 Metagenomics

There are several ways to amplify bacterial DNA. Sanger sequencing for example, is a method used frequently in amplification. So why is metagenomics the preferred method? There are microbes everywhere, many of which are beneficial to society. Microbes are essential to human life, and all other types of life on Earth. Each process of life is dependent on microbes because they convert distinguished elements into forms that can be utilized by all living things; carbon, nitrogen, oxygen and sulfur(6).

So far, all metagenomics studies are based on DNA sequencing, where the DNA is extracted from all the microbes living in the same environment. This result in a library, that contains all the genomes found in one specific environment. Further analysis is based on the content of the library, either to analyze the nucleotide sequence or to study the genes when they are expressed as proteins(6).

One of the greatest challenges with this method is that the library contains millions of clones, fragments of DNA, and analyzing this amount of information requires a wide field of knowledge. One of the factors that has contributed to this technology is the advanced computing capabilities that was needed to sort and analyze the result(6).

Metagenomics provides new access to microbes and their characteristics. Many microbes cannot be grown in a laboratory, and therefore traditional methods cannot be used in detection and identification. In metagenomics study the genomes of many organisms can be analyzed in their natural environment, and they can also be analyzed simultaneously. The focus is on the gene in the community and how they influence other genes. This will give a better understanding of the microbial world as well as the entire world. It can contribute to improving human health, food production and the production of energy. The knowledge may also contribute to a more sustainable environment, and in the long run it may contribute to solving some of today's environmental challenges(6).

PCR *(polymerase chain reaction).*

The method traditionally used for genomic amplification is PCR (polymerase chain reaction), also called Sanger sequencing. This method targets specific sequences in genome and amplifies them, to identify species of the given organism. DNA or RNA will be amplified into larger numbers that can be used in further analysis(7).

The principle in this method is that the existing DNA/RNA strand will serve as a template and be amplified. There is use of different reagents that contain enzymes that catalyse the reaction, nucleotides that will make a new strand and primers that helps targeting and starting the reaction(7). PCR method does not require vectors, like the traditional approach, and can be done in a test tube. It is very quick and many copies of a nucleotide sequence can be amplified in a few hours. But PCR is very sensitive, that may cause interference. When this method is used in diagnostics the pathological genomes are normally of interest, and it is therefore important to eliminate interference from other genomes. On the other hand it is able to detect small amounts of DNA in a sample, and it ensures that the result is accurate(8).

The use of primers is what makes this method so sensitive. The primers function as a starting point for the reaction and they also target specific sequences. This ensures that the amplified sequence is the sequence of interest. The enzyme, polymerase, will be directed by the primers to the right location on the DNA strand, where it will replicate the target sequence(8).

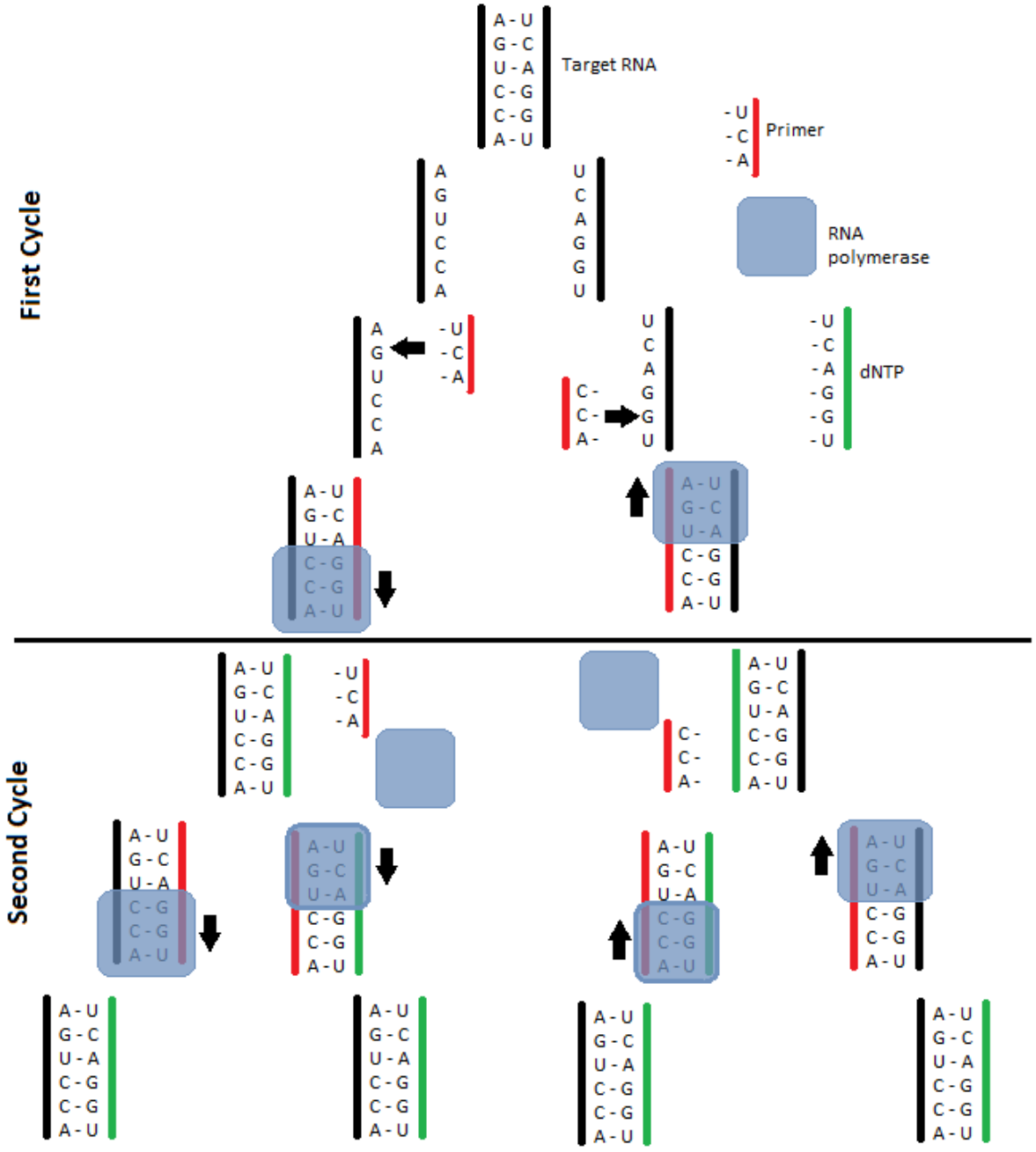


Figure 2 The polymerase chain reaction (PCR) (Figure by authors).

Figure 2 shows the course of PCR, and that each new DNA/RNA strand serves as a template for making a new strand. This process is exponential(7).

Sanger sequencing was the first method used to sequence DNA, and is termed the gold standard in DNA sequencing. It was used for decades and is a forerunner for the development of next-generation sequencing methods. After the development of the first human genome sequencing method it was convenient to search for more inexpensive and faster sequencing methods, which eventually resulted in the development of next generation sequencing(9). This method is high-throughput sequencing and is used in metagenomics research, where they are not dependent on cultivated colonies, and can analyze biological material and get both genus and species quickly(10).

By using PCR in metagenomics studies the 16S rRNA sequence becomes amplified, and the variation in this gene helps determine the species of the sample material. The method can also be used to determine characterization of bacterial populations and taxonomical analysis(11). This has led to better understanding of the advances in ecology, evolution and diversity – which is why it is important to make this method more available and standardized. With this method, it is possible to analyze a non-isolated sample and reveal its gene diversity, and this supports the claim that using metagenomics expands knowledge of the microbial world(12).

Metagenomics is a process where the entire DNA from a sample is fragmented by next-generation sequencing. The DNA material in a sample is targeted and amplified to collect information about all genes, the functions and the organism(13). The NGS (Next generation sequencing) technology has already been used to characterize the bacterial communities that is important to the human health from different places, like the mouth, airways and intestinal tract. It has also been used in discoveries of diverse bacterial population in the environment, for example as water reservoir and many others environment in the atmosphere(11). Metagenomics is already widely searched and also used in diagnostics and understanding of different human diseases(14).

2.3 Workflow in Ion Torrent PGM

This metagenomics study uses next generation platform from Thermo Fischers Ion Torrent Personal Genome Machine (PGM), which is a tool that facilitates high-throughput

sequencing. This instrument has a methodology that consists of three steps; template preparation, sequencing and data analysis (fig.3):

Figure 3 Workflow of microbial analysis (Figure by authors).

Template preparation means the production of complementary DNA/RNA, and functions as a library of bases that are amplified. The libraries consist of known fragments from the DNA/RNA, and they act as a template for the synthesis of new DNA/RNA. The DNA in the sample gets fragmented, and adapter sequences are ligated to the sample DNA fragments. When the library is made, it gets amplified before the sequencing itself takes place. Different methods are then utilized to conduct the amplification of the library. Emulsion PCR is used in PGM to amplify the fragments in the library into microbeads, and bridge amplification is used in MiSeq to create clusters of template on a flow cell(14).

When the library is amplified, it is ready to start sequencing. In order to get a nucleic acid sequence, both types of Ion Torrent sequence by synthesis of DNA. The library functions as a form of template, where new DNA fragments is synthesized based on the ”library template”. The sequencing includes several steps where the fragments are washed and flooded by known nucleotides, which are recorded in a sequential order while they are incorporated into the growing strand, using the template from the library(9). The composition of the sequence is determined by measuring pH changes that occurs while the nucleotides incorporate into the strand. The nucleotides release hydrogen ions, which will cause a change in pH value. Circuits or semiconductors are then used to register this change in pH, and this contributes to low-cost usage, instead of using expensive light-detection systems(9,14).

In this step, PGM and MiSeq also use different mechanisms in order to detect the information on the nucleotide sequence. PGM relies on semiconductor sequencing where changes in pH is detected. Hydrogen ions are released when the new nucleotides incorporate themselves into

the template DNA strand. These hydrogen ions will then change the pH, which will be registered by PGM. MiSeq detects fluorescence that occurs when nucleotides labelled with fluorescent particles, enter the DNA strand. The amount of fluorescence detected by the MiSeq therefore functions as a proportional value to the number of nucleotides incorporated in the DNA strand(9).

When the library is conducted, and the sequencing is complete – we have a sequence that reveals the identity of the test DNA. This raw sequence data has to go through some analysis steps, in order to obtain any logical information from it. In NGS, the data is pre-processed so that the adapter sequences and low-quality reads are removed. This occurs in order to get a clearer "picture" of the sequences that are to be revealed, and to remove any sort of distraction. The data is then mapped to a "reference genome" of the sequences registered, and the complied sequence is then to be analysed. Analysis of the DNA can be done in different ways, anything from genetic variant calling for detection of SNP's to identification of germline mutations or somatic mutations that may indicate disease or a genetic condition. Online tools are then used to analyse the entire data(9). The reads are sorted into the PGM software and filtered based on size and composition of the sequences(15).

During metagenomics it is important to always check your products continuously after every step. This is important due to economy and time. The process consists of many small steps that demands time and money. The quality controls that have be completed during the process of making the library is preparing a 2% Agarose Gel, Qubit and Bioanalyzer. These steps are important to ensure that there is product in the samples, and that the eventual products are of interest and completely purified. If the product is not clean enough there will be too many interfering substances that will make a poor run and therefore lead to a bad result(16,17,18).

Agarose Gel is used in electrophoresis as the supporting material. The agarose allows proteins of different sizes to migrate through the gel. The DNA is negatively charged, which is why it migrates form the negative pol to the positive pol. The main separation is based on the charge of the molecule, where the molecule with high negative charge migrates longer than the ones

with lower charge. Size and shape of the molecules also affect the separation. The small molecules migrate longer through the gel than the large ones, and the shape decides how easily and fast the molecules migrate(16).

Qubit is a quality step that measures the concentration of DNA in the sample. The Qubit uses fluorometer to measure the concentration. The kits used for Qubit contains concentrated assay reagents, dilution buffer and prediluted DNA standards. Qubit is highly sensitive for double stranded DNA and will therefore select DNA or RNA, and common interfering products as salts, free nucleotides, solvents, detergents and proteins are well tolerated in the assay. Every time Qubit is used in analysis, it gets set up with a standard. On these grounds, there is a need to calibrate the apparatus before every use. This is to ensure an accurate measurement(17).

On the bioanalyzer one can run electrophoretic assays. This procedure works with the same principles as a normal electrophoretic run only on a chip. The chip can be used for separation of DNA, proteins and RNA. When using the bioanalyzer one can reduce reagent consumption and time. The chip also provides quantitation information in a digital form and automated separation. When loading the chip, the wells are filled with a sieving polymer and fluorescence dye. As the loading is done the whole chip has become one integrated electrical circuit. The analyzing machine has 16-pin electrodes. These electrodes are placed in an arrangement that fits each well on the chip, and they are each connected to an independent power supply for maximum control and flexibility(18).

When the voltage is one, the different molecules in for example DNA will start to migrate in different speed causing a separation. The molecules will be separated by size due to mass-to-charge ratio and presence of sieving polymer matrix. The migration is detected by a laser-induced fluorescence. After the laser has detected the migration, the data is translated in to a gel-like image with bands or an electrophoretic image with peaks. The chip also contains a well specifically meant for the ladder. This ladder functions as a standard, and is necessary for the machine to analyze the samples. The ladder contains components with known sizes that helps to standardize the migration time and fragments sizes. With help from the migration time for each fragment, the samples can be calculated(18).

2.4 Limitations and advantages

Next-generation sequencing has accomplished much in a short period of time, but as any other method, it has its advantages and limitations. As mentioned earlier, NGS is a cheaper and higher-throughput sequencing system than the previous method, Sanger sequencing. With NGS the entire genome can be sequenced in a day. It can also detect targeted sequences that is associated with disease, and mutations in sequences which can cause diseases. This technique plays a major role in diagnostics of pathological conditions(9,10).

Even though NGS is a time saving low-cost method, it is still very expensive for many facilities. The method is also very sensitive for homopolymer regions, and this can lead to spans of repeating nucleotides that cause a false positive result. There is also a risk of errors occuring when sequencing short-read lengths (200-500 nucleotides). The result must be data analysed, and this is a time-consuming process. It requires knowledge about bioinformatics to secure accuracy(9,19). Another limitaiton in NGS analysis is the fact that human genome can potentially supress the bacterial genome in the sample. The 18S region in human genome can cross-react with the primers. This will construct a false-positive result. The human genome is also amplified faster than the bacterial genome, and this may cause interference in the result(28).

The selection of the Ion Torrent PGM as a machine for analyzation is naturally based upon the positive characteristics already mentioned. It is cost-effective, gives accurate results, and since it provides a lot of information from little sample-preparation – it is as timesaving as NGS analysis can get. The data analysis will still take some time(9), but the preparation and analysis in the machine itself is more effective. The machine is also very available, and though it is expensive, it is simple to get a hold of. In addition to this, the machine is fairly new on the market, and therefore presents as a good choice in regards to keeping up with recent development. Utilization for microbiology-purposes is also a fairly recent discovery, but the machine has been proven to be suitable for this. The size is also ideal for easy use, since it does not require a lot of space. It is a benchtop machine, and therefore does not compromise a lot of the laboratorys capacity(9).

In order to secure quality in the analysis, there will be use of both positive and negative controls. Familiar DNA will be used to make sure that the primers bind where they are supposed to, and that they do not cause either false negative or false positive results. Another great factor in quality control is the use of more primer pairs simultaneously. Single primer pairs may show some difficulty binding certain microbes. In a specimen of test material, some bacteria will be underrepresented, and some will be overrepresented, and this will cause a disturbed image of the actual bacterial content in the test. By using several primer pairs, this is avoided(20). Regardless of which bacteria are over – or underrepresented, the primer pairs will be able to detect them all and present accurate results, and there will be no loss of DNA as a consequence of underrepresentation or disturbed bacterial image(20).

2.5 Previous discoveries

As previously mentioned, research shows that urine is not sterile. There have been several findings of microbiota in urine, from both male and female test subjects - which suggests that there is a certain amount of resident flora in the bladder and in urine. When the human bodies resident flora was mapped, the bladder was not considered because of the complexities regarding sample collection(21). Since it is a closed cavity inside the body and the urine gets filtered by the kidneys before it reaches the bladder – this was a somewhat logical assumption. The claim that urine is sterile originated in the 1800s when Louis Pasteur, Joseph Lister and William Roberts presented the discovery that urine in a closed sterile container did not appear cloudy, whereas urine exposed to oxygen and contamination from tap water got a cloudy appearance. From that, they assumed that fresh urine from healthy people was free from microbiota(21). As more research has been done, it has been proven that microbiota exists in the bladder and in urine, but for some reason – the theory that urine is sterile still hangs on, and is still written in lecture books(21).

The lower urinary tract can in fact have a steady microbiota which like all other types of resident flora, helps the urinary tract to stay healthy and fight infectious microbes. There is a symbiotic relationship between bacterial cells and humane cells that is of extreme importance to the immune system(22). The largest difficulty when it comes to testing of microbiota from the urinary tract and in urine, is contamination obtained during sampling, from for example genitalia. Based on that, a sterile bladder-punction would provide the best material for testing,

but gathering mid-stream wash samples taken by clean-catch method also provides good material – and is less invasive and more ethical in regards to the subjects(22).

From 16S metagenomic testing, there were several individual bacterial findings, and some repeated themselves in most of the samples. There have been studies focusing on only female subjects, only male subjects, and studies working on mapping the amount and content of microbiota in urine in different age groups. Lactobacillus, Corynebacterium, Prevotella, Gardnerella and Enterococcus mainly define the differences in gender, Lactobacillus being dominant in female samples and Corynebacterium in male(23).

In research, men are underrepresented in comparison to women. There has been done far more research on the female urine, and the results are therefore much more reliable than the findings done on male. The resident flora of the male urinary tract in general is poorly described. Though Lactobacillus is one of the findings that suggestively differentiates gender – it is also a large part of the male microbiota(24). Different species have been found not only in urethral swabs, but in urine itself. Corynebacterium, Staphylococcus and Anaerococcus were also dominant in the samples, whereas Peptoniphilus, Prevotella, Finegodia, Porphyromonas, Proprionibacterium, Delftia and Pseudomonas were present, though in smaller amounts(24,24).

The female urine is far more represented in studies, and the results are therefore naturally more reliable. Undoubtedly, the most dominant discovery is Lactobacillus spp. – which is represented in most positive samples, and which also contributes to gender differentiation(24,25,26). Corynebacterium was also a frequent detection in female samples, but not as frequent as Lactobacillus. Streptococcus, Actinomyces Gardnerella, Prevotella and Staphylococcus were also well represented, and Aerococcus, Gardnerella and Bifidobacterium were relatively common(24,26). Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria, Spirochaetes and Chloroflexi bacteria were also isolated from several samples, but not as common as the species listed earlier(27). There were also findings of Enterococcus Faecialis, Rothia dentocariosa, Staphylococcus aureus and Staphylococcus epidermidis – but these only grew in thioglycolate broth(24).

3. MATERIALS AND METHODS

3.1 Sample collection and storage

The material selected for the procedure establishment was urine, and it was obtained from volunteer male candidates in the age-range 19-30. The samples were anonymous during the entire testing process. The candidates were instructed to provide a clean-catch midstream sample preferably containing morning urine, and they were also informed of the importance of following this procedure in order to eliminate preanalytical factors (see attachment 7). They were also instructed to transport the sample to the storage location within two hours after urination. The urine was collected on sterile tubes, and stored at -80 ˚C.

3.2 Urine cultivation

In samples like this, the major content is likely to consist of human genome from for example epithelial cells. Since the Ion Torrent is sensitive to all DNA/RNA, and human genome is likely to outnumber the bacterial genome – it is important to isolate the bacterial genome and thereby eliminate any interference. The non-selective sensitivity is one of the downsides to Ion Torrent, and if the samples were to contain human genome – the amplification of it would most likely suppress the bacterial genome(28).

Based on this knowledge, the urine samples were added on different culture media, and incubated for 72 hours for cultivation. 1µl of urine was used for cultivation, and all of the samples were cultivated in aerobic, anaerobic (5% CO2) and microaerophilic environments. The selected media for aerobic cultivation were blood agar (BAP), MacConkey agar, CPS (chromID CPS Elite agar), LB (luria broth) and PCA (plate count agar). For anaerobic cultivation the selected media were CPS agar, MacConkey, blood agar, PCA and LB (see attachment 7). The following medias were also cultivated in microaerophilic atmosphere; blood agar, MacConkey, CPS and LB. The original samples were also prepared to be analyzed directly on the Ion Torrent machine, in order to establish the need for cultivation. The uncultivated series of samples was named 1-10 and the cultivated series of samples was named 11-20.

3.3 Metagenomic analysis using Ion Torrent PGM.

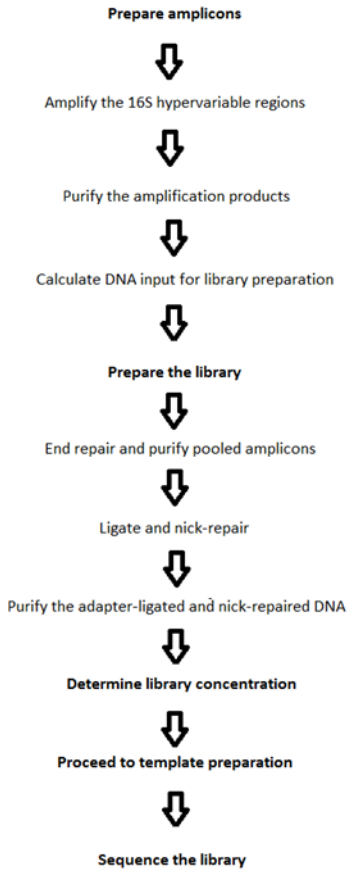


Figure 4 Metagenomics workflow

The positive samples were inserted into Thermo Fischer Ion Torrent PGM for sequencing after the preparation steps described in the procedures. The bacterial 16S rRNA genes were amplified using Ion 16S metagenomics kit (Cat.no A26216). PCR amplification product was used to create the library, in addition to the Ion Plus Fragment Library kit (Cat. No. 4471252). To organize the samples Ion Xpress Barcode Adapters 1-16 Kit (Cat. No. 4471250) was used. Using the Ion 16S Metagenomics kit ensured accurate detection and identification of bacteria down to species level. The kit contains two sets of primers, V2-4-8 and V3-6, 7-9. Template preparation is performed by Ion PGM Hi-Q View OT2 Kit (Cat. No. A27739). Sequencing is performed by Ion PGM Hi-Q View Sequencing Kit (Cat. No. A25592) on Ion PGM with Ion

318 Chip Kit v2 BC (Cat. No. 4488150), which tracks and detects the sequences – and then translates them into digital form(29).

3.4 Isolation and purification of bacterial RNA.

After cultivation, all the positive medias of each sample were used to make suspensions of 1,5 McFarland. To make sure that enough DNA material was obtained from the cultivation step, determining the use of 1,5 McFarland value was favorable.

The suspensions and the uncultivated samples were then out though a lysis - and purification process in order to break down the cell walls of the bacterial genome, free the DNA/RNA, and strip away any excess material. This was done using the DNeasy blood and tissue kit delivered by Qiagen (see attachment 3, A.1).

3.5 Quantification of DNA materials.

Before amplification, the DNA concentration was determined. Qubit dsDNA HS Assay kit (Cat.no. Q32851) was therefore used to ascertain the concentration of microbial DNA in the samples. The kit was used with the Qubit fluorometer, which detects a fluorophore attached to the DNA, and measures the quantity by photometry (see attachment 3, A.2). The determined DNA concentration then contributed to determining the number of cycles needed in the PCR amplification. It is ideal to minimize the number of cycles to avoid interferents. Excess cycles can also cause overamplification. However, the concentration must be 1-3 ng to ensure sufficient amplified materials. The PCR materials then proceeded on to usage in library preparation.

3.6 Amplifying the 16S regions.

To amplify the DNA, Ion 16S Metagenomics kit (Cat.no. A26216) was used. This was done to make amplicons of the 16S regions, and the presence of PCR product was confirmed by running a gel electrophoresis (see attachment 3, B.1).

3.7 Calculation of the DNA input.

When the 16S regions were amplified and purified, the DNA input for preparation of the library was determined. This can be done either by using Agilent 2100 Bioanalyzer wth the Agilent High sensitivity kit, or by using Qubit fluorometer with the Qubit dsDNA HS assay kit (Cat.no Q32851). The result was used to calculate the correct volume of purified PCR product needed to obtain 50 ng pooled amplicons in the library preparation (see attachment 3, B.3).

3.8 Preparation of the library.

In preparation of the library two different kits were used; the Ion plus fragment library kit (Cat.no. 4471252) and the Ion Xpress Barcode adapters 1-16 kit (Cat.no. 4471250). The preparation of the library is a procedure that purifies the fragments and attaches adapters to them. The adapters ensures a complete template preparation (See attachment 3, C.1).

3.9 Template preparation.

The library consists of known fragments that act like templates. By using Ion PGM Hi-Q OT2 kit (Cat.no. A27739), the known fragments were separated by emulsion PCR and ligated by adapters on to microbeads (see attachment 3, C.3).

Figure 5 Ion One Touch 2 Instrument by Life technologies(30).

3.10 Sequencing of the library.

New DNA fragments were synthesized by library templates using Ion PGM Hi-Q sequencing kit (Cat.no. A25592). The principle in the Ion Torrent based on pH-changes determines the rest from here on out. By using a semiconductor (Ion 318 Chip v2) that recognizes any changes in pH value, the Ion Torrent registers the change in pH that occurs whenever a new nucleotide incorporates on a new strand - and can then register the order of the nucleotides and determine the DNA detected (see attachment 3, C.4).

Figure 6 Ion Torrent Personal Genome machine by Life technologies(31).

4. RESULTS

4.1 Uncultivated samples

Concentration of DNA in the samples, determined by Qubit fluorometer:

Samples	Concentration
1	0,0143 µg/ml
2	0,0152 µg/ml
3	0,0101 µg/ml
4	0,0112 µg/ml
5	Too low
6	Too low
7	0,0059 µg/ml
8	0,0238 µg/ml
9	0,0287 µg/ml
10	0,0243 µg/ml

Gel electrophoresis to confirm the presence of PCR product:

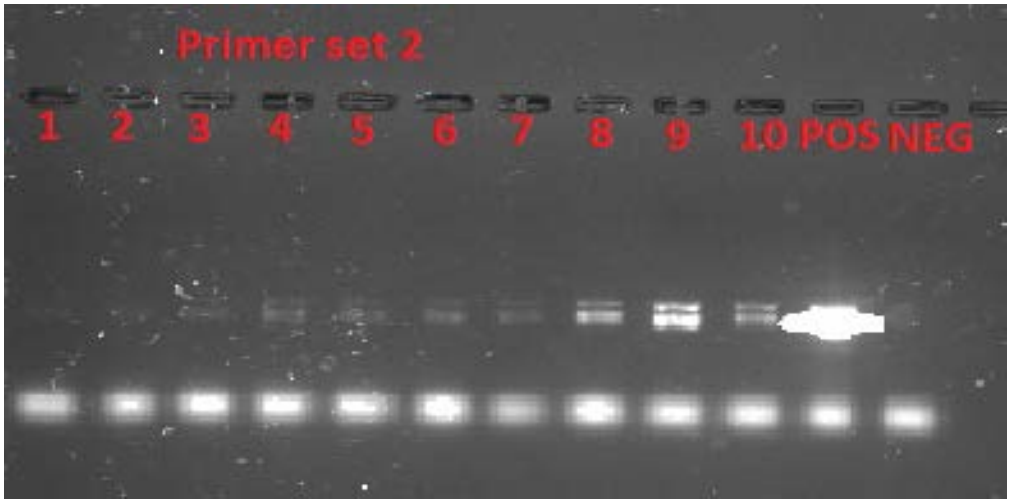


Figure 7 Agarose gel Uncultivated samples. This picture shows the PCR results from the uncultivated samples, plus positive and negative controls. E.coli was used as positive control and water was used as a negative control. This confirms the presence of PCR product in sample.

Calculating the concentration of DNA input to the library preparation using Agilent 2100 Bioanalyzer:

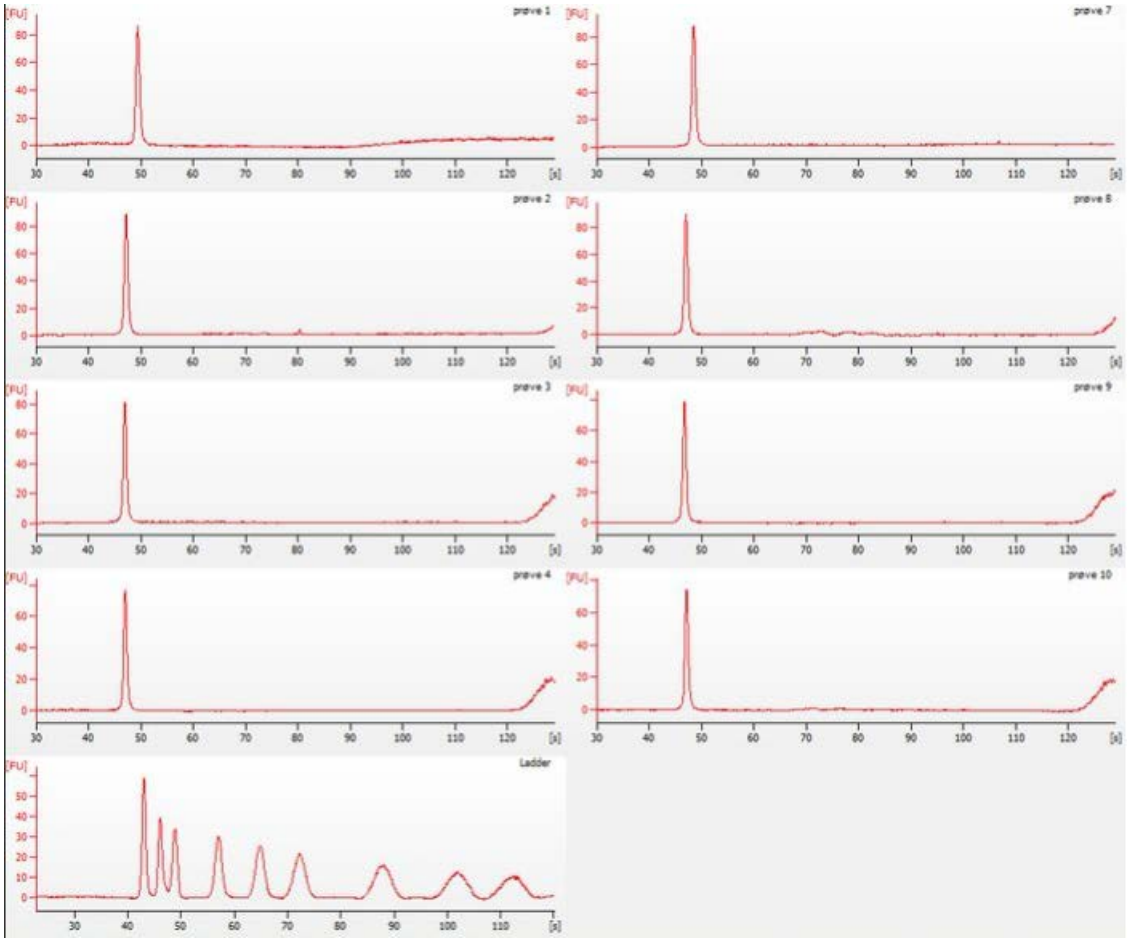


Figure 8 Bioanalyzer Uncultivated samples showing that the samples are not amplified. This picture shows the products after the initial PCR-run of the uncultivated samples. The pictures include samples 1-4 and 7-10 with the ladder as a standard.

4.2 Cultivated samples.

Concentration of DNA in the samples, determined by Qubit:

Sample	Concentration
18 A	1.16 µg/ml
18 B	1.34 µg/ml
20 A	0.93 µg/ml
20 B	0.97 µg/ml

Concentration measured on Qubit fluorometer and gel electrophoresis to confirm the presence of PCR product:

Sample	Concentration
18 A	6.53 µg/ml
18 B	7.81 µg/ml
20 A	5.93 µg/ml
20 B	5.84 µg/ml

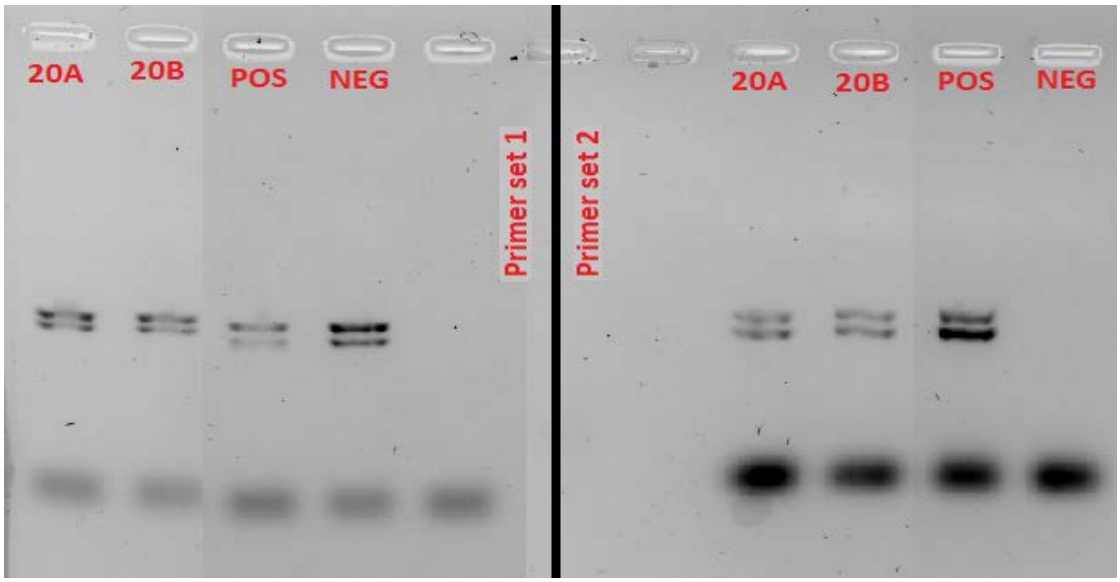


Figure 9 Agarose gel Cultivated samples. This picture shows the PCR results from the cultivated samples, 20A and 20B in addition to positive and negative controls.

Calculating concentration of DNA input to the library preparation using the measurement from Qubit fluorometer.

DNA input of purified PCR product needed to obtain 50 ng pooled amplicons was determined by the following equation:

$$Volume\ of\ PCR\ product\ = \frac{50\ \text{ng}}{\text{Concentration of PCR product}}$$

Sample 18 A:

50 ng/6.53 µg/ml = 7.65 µl DNA

79 µl - 7.65 µl DNA = 71.35 µl dd H2O —> 79 µl diluted amplicons.

Sample 18 B:

50 ng/7.81 µg/ml = 6.40 µl DNA

79 µl - 6.40 µl DNA = 72.60 µl dd H2O —> 79 µl diluted amplicons.

Sample 20 A:

50 ng/5.93 µg/ml = 8.43 µl DNA

79 µl - 8.43 µl DNA = 70.57 µl dd H2O —> 79 µl diluted amplicons.

Sample 20 B:

50 ng/5.84 µg/ml = 8.56 µl DNA

79 µl - 8.56 µl DNA = 70.44 µl dd H2O —> 79 µl diluted amplicons.

The calculated amount of PCR product previously used in library preparation was mixed with nuclease-free water to achieve a total volume of 79 µl.

Determining library concentration using the Agilent High sensitivity kit and Agilent 2100 Bioanalyzer:

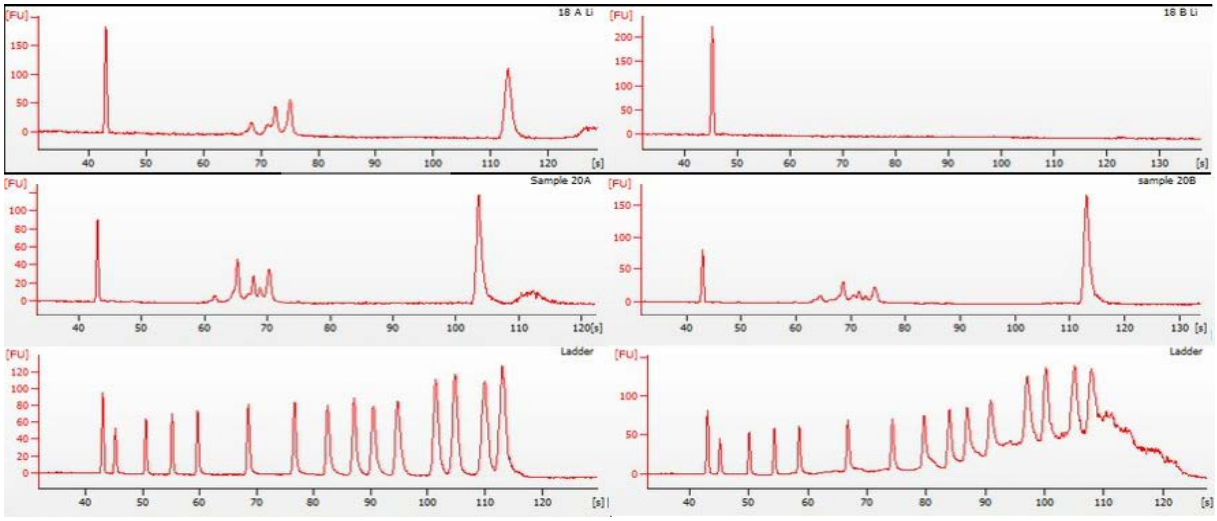


Figure 10 Bioanalyzer Cultivated samples. This picture shows the cultivated samples. The three pictures on the left side shows sample 18A, 18B and the ladder at the bottom, and the pictures to the right show sample 20A and 20B with the ladder on the bottom.

This results in the following concentrations:

Concentration of the library in pM:

Sample 18A= 400.4 pM

Sample 18B= -

Sample 20A= 1543.5 pM

Sample 20B= 812 pM

Determining the library dilution factor using the Bioanalyzer:

The library was diluted to 26 pM and the volume of library and Low-TE was determined with different total volume (it is favorable that the library volume is between 1-2 µl):

Sample 18A: 26 pM x 20 µl / 400.4 pM = 1.29 µl library

20 µl - 1.29 µl = 18.71 µl Low-TE

Sample 20A: 26 pM x 60 µl / 1543.5 pM = 1.01 µl library

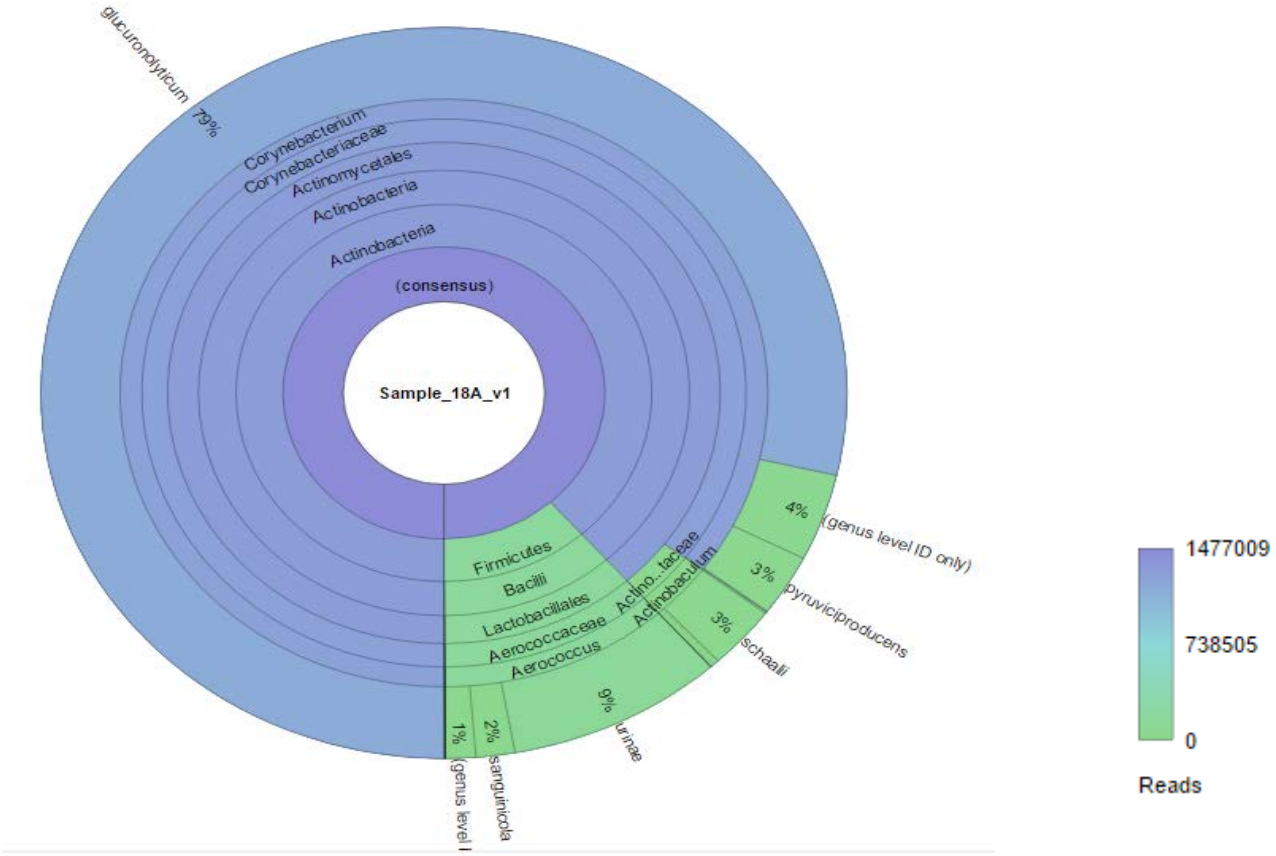
60 µl - 1.01 µl = 58.99 µl Low-TE

Sample 20B: 26 pM x 40 µl / 812 pM = 1.28 µl library

40 µl - 1.28 µl = 38.72 µl Low-TE

Sequencing on Ion Torrent PGM:

Sample18A:

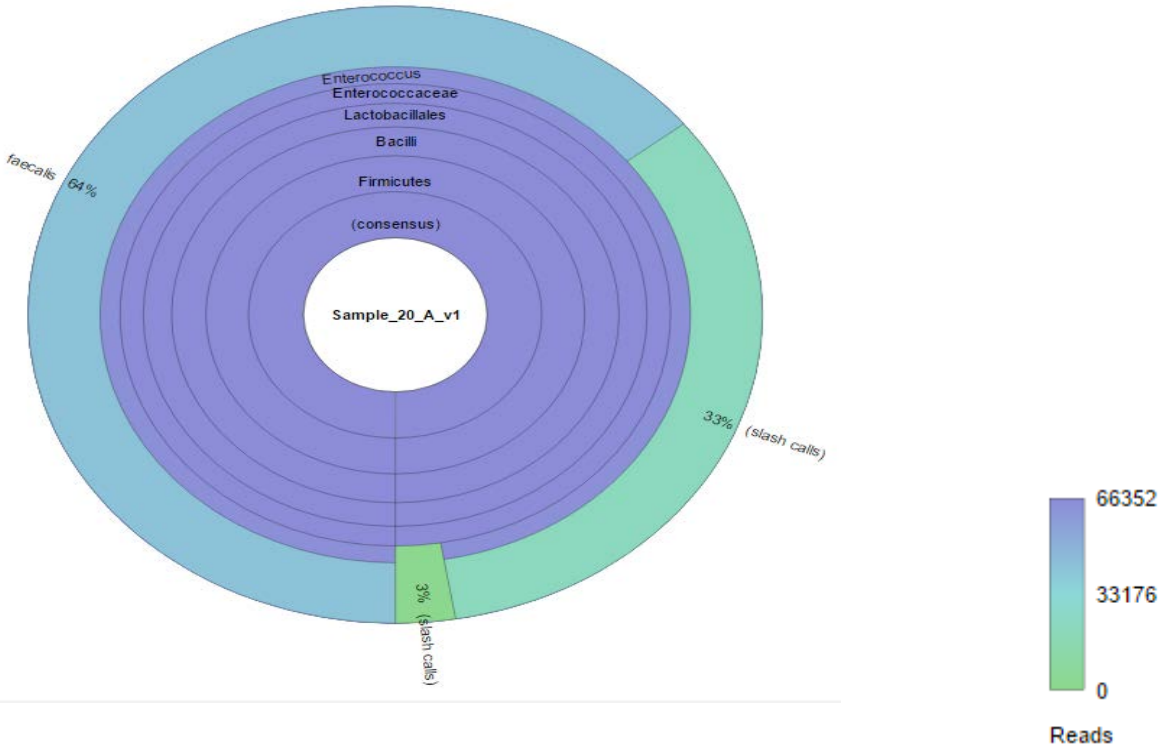


Primer	Phylum	Class	Order	Family	Genus	Species	% ID	Count	DB counters	F:R %	% of total reads	% of valid reads	% of map read
(consensus)								**1476904**	**1172567 : 304337**		**46.95**	**87.09**	**100**
	Actinobacteria							1306920	1033728 : 273192		41.54	77.06	88.4
		Actinobacteria						1306920	1033728 : 273192		41.54	77.06	88.4
			Actinomycetales					1306920	1033728 : 273192		41.54	77.06	88.4
				Actinomycetaceae				46891	0 : 46891		1.49	2.76	3.17
					(family level ID only)			65	0 : 65		0	0	0
					Actinobaculum			46826	0 : 46826		1.49	2.76	3.17
						(genus level ID only)		5615	0 : 5615		0.18	0.33	0.38
						schaalii	99.11 - 100	41211	0 : 41211	38.54 : 61.46	1.31	2.43	2.79
Corynebacteriaceae								1259905	1033604 : 226301		40.05	74.29	85.31
		(family level ID only)						1042	0 : 1042		0.03	0.06	0.07
		Corynebacterium						1258863	1033604 : 225259		40.02	74.23	85.24
			(genus level ID only)					56787	0 : 56787		1.81	3.35	3.85
			(slash calls)					237	24 : 213		0.01	0.01	0.02
			glucuronolyticum				99.08 - 100	1161269	993465 : 167804	59.78 : 40.22	36.91	68.47	78.63
			pyruviciproducens				99.35 - 100	40115	40115 : 0	70.17 : 29.83	1.28	2.37	2.72
			sp.				99.09 - 100	455	0 : 455	89.67 : 10.33	0.01	0.03	0.03
Propionibacteriaceae								124	124 : 0		0	0.01	0.01
		Propionimicrobium						124	124 : 0		0	0.01	0.01
			lymphophilum				99.55 - 100	124	124 : 0	59.68 : 40.32	0	0.01	0.01

Firmicutes							169984	138839 : 31145		5.4	10.02	11.51	11.51
	Bacilli						169348	138839 : 30509		5.38	9.99	11.47	11.47
		Bacillales					25	0 : 25		0	0	0	0
			Staphylococcaceae				25	0 : 25		0	0	0	0
				(family level ID only)			10	0 : 10		0	0	0	0
				Macrococcus			15	0 : 15		0	0	0	0
					(genus level ID only)		15	0 : 15		0	0	0	0
		Lactobacillales					169323	138839 : 30484		5.38	9.98	11.46	11.46
			Aerococcaceae				169311	138827 : 30484		5.38	9.98	11.46	11.46
				(family level ID only)			66	0 : 66		0	0	0	0
				Aerococcus			169245	138827 : 30418		5.38	9.98	11.46	11.46
					(genus level ID only)		17868	0 : 17868		0.57	1.05	1.21	1.21
					sanguinicola	99.1 - 99.55	22992	22992 : 0	41.47 : 58.53	0.73	1.36	1.56	1.56
					urinae	99.1 - 100	128385	115835 : 12550	50.32 : 49.68	4.08	7.57	8.69	8.69

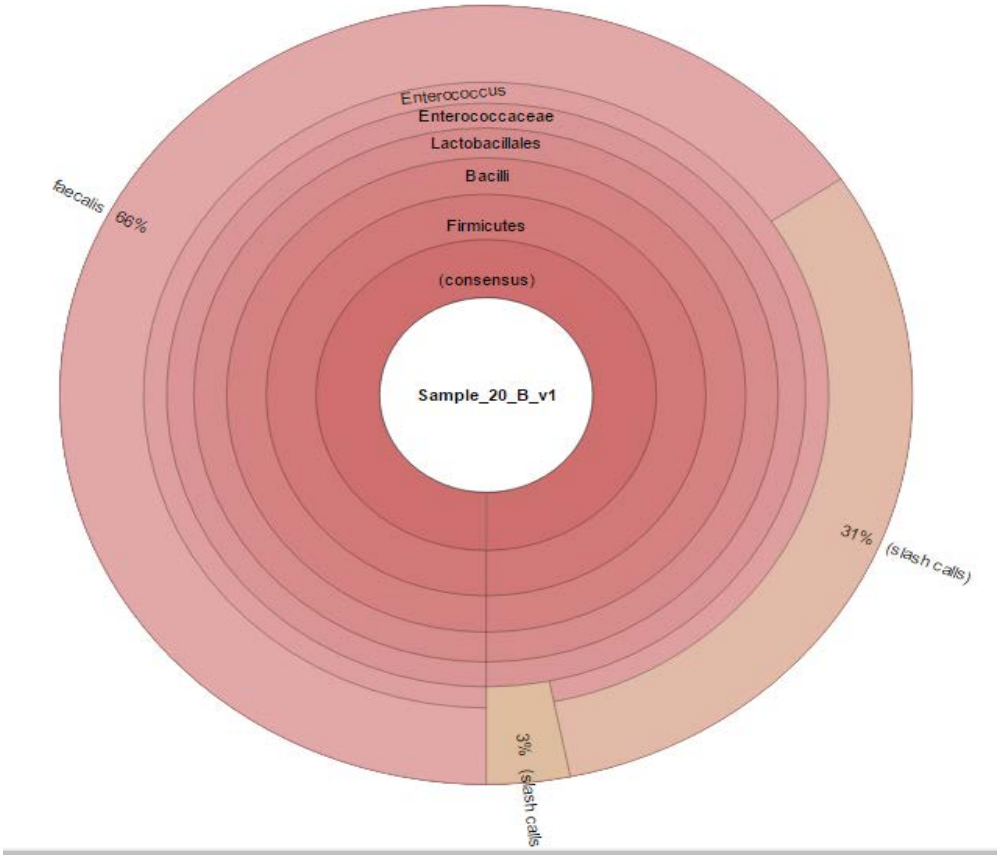
	Enterococcaceae					12	12 : 0		0	0	0	0
		Enterococcus				12	12 : 0		0	0	0	0
			(slash calls)			12	12 : 0		0	0	0	0
Clostridia						636	0 : 636		0.02	0.04	0.04	0.04
	Clostridiales					636	0 : 636		0.02	0.04	0.04	0.04
		Ruminococcaceae				636	0 : 636		0.02	0.04	0.04	0.04
			(family level ID only)			11	0 : 11		0	0	0	0
			Fastidiosipila			625	0 : 625		0.02	0.04	0.04	0.04
				(genus level ID only)		625	0 : 625		0.02	0.04	0.04	0.04

Sample 20A:



Primer	Phylum	Class	Order	Family	Genus	Species	% ID	Count	DB counters	F:R %	% of total reads	% of valid reads	% of mapped reads	% of mapped reads per primer
(consensus)								**46137**	**44703 : 1434**		**42.4**	**76.44**	**100**	**100**
	Firmicutes							46137	44703 : 1434		42.4	76.44	100	100
		Bacilli						46137	44703 : 1434		42.4	76.44	100	100
			Lactobacillales					46137	44703 : 1434		42.4	76.44	100	100
				Enterococcaceae				46137	44703 : 1434		42.4	76.44	100	100
					(slash calls)			1461	27 1434		1.34	2.42	3.17	3.17
					Enterococcus			44676	44676 : 0		41.06	74.02	96.83	96.83
						(slash calls)		14391	14391 : 0		13.23	23.84	31.19	31.19
						faecalis	99.11 - 100	30285	30285 : 0	35.45 : 64.55	27.83	50.18	65.64	65.64

Sample 20B:



Primer	Phylum	Class	Order	Family	Genus	Species	% ID	Count	DB counters	F:R %	% of total reads	% of valid reads	% of mapped reads	% of mapped reads per primer
(consensus)								**46137**	**44703 : 1434**		**42.4**	**76.44**	**100**	**100**
	Firmicutes							46137	44703 : 1434		42.4	76.44	100	100
		Bacilli						46137	44703 : 1434		42.4	76.44	100	100
			Lactobacillales					46137	44703 : 1434		42.4	76.44	100	100
				Enterococcaceae				46137	44703 : 1434		42.4	76.44	100	100
					(slash calls)			1461	27 : 1434		1.34	2.42	3.17	3.17
					Enterococcus			44676	44676 : 0		41.06	74.02	96.83	96.83
						(slash calls)		14391	14391 : 0		13.23	23.84	31.19	31.19
						faecalis	99.11 - 100	30285	30285 : 0	35.45 : 64.55	27.83	50.18	65.64	65.64

5. DISCUSSION

The method was based on knowledge obtained from the literature and from contributing parties. Early on, there was made a decision to incubate the samples in cultivation for longer than the standard 24 hours. Firstly, incubation was increased to 48 hours. This worked well in the first trial, but because too little DNA was isolated from these cultures – the samples had to be thawed and cultivated again. Incubation was then increased to 72 hours, in hopes that this would provide enough time for bacteria that are slow in cultivation. Increasing the incubation also provided more time for bacteria that existed in small amounts, and thereby also increasing the probability of more growth. The second incubation provided less cultures, this is likely because of the re-freezing of the samples which is addressed further later in this section. Increasing the incubation time in cultivation of these samples appear to be a good way to facilitate sufficient growth.

After the first trial, the volume of uncultivated urine was increased from 1ml to 2ml. This was done in an attempt to gather sufficient amounts of DNA material. A lot of the method is based on increasing volumes and expanding incubation, and these factors both contribute to more material being gathered, and may also contribute to increasing the diversity detected in the samples.

The kit that was used for lysis of the cells in the samples was DNeasy. This is an all over use kit that works on most samples, but it is best in use for animal cells in blood, or tissue. The kit does not address its effects when used on urine. In this project the approach for gram-negative bacteria was used. The kit contains no approach for both gram-negative and gram-positive bacteria. One must decide which approach to use, and one of them contains an extra buffer. These factors combined might have been a problem for the results in our project, and one can consider using a different lysis kit. Though the kit was not necessarily applicable in regards to the amount of results, it worked well for the purpose of establishing procedures.

The method appears to work well for the cultivated samples, but it still presents a couple of downsides. One is the amount of washing steps, where one may wash away too much of the

DNA material. Because the procedures that are established and the method includes a lot of manual work, it also opens up to human error. It is also a time-consuming method, because of two factors. The preparation of the samples before they enter the Ion Torrent takes a lot of time. It includes lysis, isolation, several quality controls and a lot of incubation. In addition to this, the analysis itself is time-consuming because it sequences all of the DNA-material in the sample in one run. Next generation sequencing is mentioned in the theory section as a time-saving way of analysis, and compared to other possible analysis methods such as traditional Sanger Sequencing this may be the case – but NGS definitely also consumes both time and effort. This presents as a downside if one needs samples analyzed quickly.

To obtain enough DNA material from the samples, appeared to be a challenge. From the beginning, none of the results were sufficient, and the DNA amounts were extremely small. They were ran on the first PCR for amplification to see if there was any chance of getting enough DNA material, in an attempt to open for further analysis. After the first PCR they were ran on a gel electrophoresis. The results (see figure 7) shows a bad amplification with the primer sets. Primer set one barely shows any amplification product. Primer set two is much better, but still not good enough. The samples were then ran on the Agilent Bioanalyzer, to obtain calculations of the DNA concentration in the samples. The DNA concentrations were way too small to continue to the next step. The procedure demands at least 1-3ng, and the samples were lower than this demand. Samples 1-10 were therefore discarded from further analysis. This shows that the recommended amount of DNA from the beginning is necessary for a successful metagenomic run.

From the cultivated samples there were only two samples that had enough bacterial growth to proceed with. Because of this, they were each divided into two samples to expand the material undergoing the procedure steps. The presence of DNA material in the cultivated samples was presented as early as the first step, where the starting material was determined by Qubit. After the first PCR amplification, they were analyzed with Qubit again. During these steps there was some trouble with samples 20A and 20B, and they had to be checked with electrophoresis to determine the concentration and purity of the product. This figure (see figure 9) shows that both primer sets worked just fine, and the samples were ready to proceed with.

Again the procedure demanded a concentration between 1-3ng to have enough DNA material. These samples had a much higher concentration than the demand, and the electrophoresis shows that both primer sets have worked. This resulted in a need for dilution before proceeding with the procedure. After the samples were diluted, they were again put though PCR to amplify the library. As a final quality check on the library before it was ready for Ion Torrent machines, it was ran one last time on the bioanalyzer. Figure (10) shows satisfactory results in samples 18A, 20A and 20B. Sample 18B did not show satisfactory results, and was rejected from further analysis. The final library concentration was calculated into pM by the bioanalyzer, to achieve the right amount of material for the Ion Torrent PGM. The end results after sequencing on the Ion Torrent PGM (see results) shows different bacterial detections in the urine samples.

As mentioned, sample 18B was excluded – and samples 18A, 20A and 20B were analyzed on the Ion Torrent machine. In sample 18A, there was a large dominance of Corynebacterium glucoronolyticum. This amounted to 79% of the sample, where the rest of the sample consisted of 9% Aerococcus urinae, 3% Corynebacterium pyruviciproducens, 3% Actinobaculum schaali, and 2% Aerococcus sanguinicola. Other detections were dominated by the phylum Firmicutes, including Lactobacilliales and Bacilli. Actinobaculum was also detected. The rest are presented in the result section. This was a sample with a lot of diversity, but a large dominance of Corynebacterium. This is expected, because of its occurring dominance in samples from male subjects(23). Lactobacilliales was also expected to find, though in smaller amounts than Corynebacterium. Lactobacillus presents more dominance in samples from females(25,26). This being said, Firmicutes is a phylum of gram-positive bacteria, which makes it safe to assume that the preparation kit did not exclude gram positive bacteria from the analysis, although it was meant for gram-negative.

Aerococcus has been detected as a previous finding in female urine, where Anaerococcus appeared to be more common in male samples(24). Aerococcus urinae is also pathogenic and linked to urinary tract infections(32), which can occur in both male and female subjects. The fact that some of the bacterial findings were not discussed in the literature, does not necessarily mean they are non-existent in male urine. They may appear in small amounts

which makes them less interesting to draft, and they may also be a simple case of contamination. Also, they may be more usual in analysis of cultivated samples, whereas many of the detections in the theory section are based on uncultivated samples. The fact that the method registered their presence despite the low amounts – shows that the procedures and the analysis method worked well.

Samples 20A and 20B were both derived from the same sample, and will therefore be drafted together. The findings were almost identical, where only the percentage of Enterococcus faecalis separated them. The samples presented a big dominance of Enterococcus faecalis, where 20A contained 64% and 20B contained 66%. They also contain “Slash Calls”, which means that there are multiple taxonomical assignments detected at genus level. The Slash Calls are divided into different groups, sample 20A contains one Slash Call group of 33% and one of 3%, and sample 20B contains one of 31% and one of 3%.

Other detections were again dominated by Firmicutes, including Enterococcus, Enterococcaceae, Lactobacilliales, and Bacilli. This accounted for both samples. Lactobacilliales is as previously mentioned an expected detection, though the amount is expected to be larger in urine from females(24). Enterococcus is also drafted in the theory section as an expected detection, but this again accounts mostly for urine from females.

None of the bacterial detections show signs of contamination during preparation for the analysis. When working with 16S metagenomics, contamination is a big factor that can contribute to misleading results. In contaminated samples one can for example find skin microbiota that may have entered the sample through contaminated gloves. Micrococcus is the most regular detection, and will in these situations appear dominant. Since Micrococcus did not appear as a result, and no other detections show signs of contamination during the procedure – it is safe to assume that human errors were avoided or kept to a minimum. But the fact that the samples were not contaminated in the procedures, does not mean they were not contaminated at all. Samples 20A and 20B show a large dominance of Enterococcus faecalis, which is most likely contamination from the sampling process. This may be a

consequence of for example poor washing-procedures before sampling. Cross-contamination and carry-over between the samples are also unlikely to have occurred, because of the diversity in the samples, and the differences between them.

The interesting factors in the results are whether the expected detections appear, and whether the method is able to detect the same content in two identical samples. Corynebacterium proved to be of large dominance, which was expected. Lactobacillus was also detected, but the other microbes mentioned in the theory section under samples from male subjects were not to be seen. In stead, some bacteria normal in female urine appeared, such as Aerococcus. Summed up, the samples presented some expected detections, and the most relevant microbes for male urine appeared in the results. As mentioned, sample 20A and 20B were derived from the same sample. They showed almost identical results, where only the percentage of Enterococcus Faecialis separated them. This shows that the procedures and the method works well in detection, because it is able to detect the same content in two identical samples. The percentage-variables in content can be explained by difference in the efficiency of the PCR amplification.

In the procedure of metagenomics analysis there are several steps that function as control points. This is to ensure that the method with the selected kits and instruments works, to get the best result. Each step is necessary to control the product and determine the concentration. The purification of the products was done by Agencourt AMPure XP reagent, that effectively removed unbound primers, fragment of non-16S regions and other contaminants. The purification will also remove residue of cell walls from the lysis step. This purification process was done several times to avoid interference, and to ensure that the product provided a reliable result. This is also a critical point, where there is a possibility of washing away some of the product. This is naturally unfortunate, and will decrease the amount of material that can be used for analysis. Therefore, it is important to comply the incubation time, mix thoroughly and be careful when pipetting.

Before amplifying the DNA, it is important to determine the concentration of DNA content in the samples. This can be done by measuring the concentration on Qubit fluorometer. To proceed with the samples, there must be a minimum concentration of DNA. Isolated DNA must have a concentration between 1-3 ng, and the samples directly from urine must contain 1-2 µl of extracted DNA. If the concentration is insufficient, there is no reason to proceed with the amplification (see results). The uncultivated samples 1-10 presented low concentrations as early as this step.

When the amplification is finished, it is suitable to confirm the presence of PCR product. This can be done either by running a gel electrophoresis, or by measuring the concentration on Agilent Bioanalyzer/Qubit fluorometer. In gel electrophoresis, a negative control (nuclease-free water) and a positive control (diluted DNA from E. Coli) were used, in order to have known substances to compare the samples to. When the presence of PCR product is determined, this functions as a control on the PCR itself, and shows the efficiency and whether it was successful or not. In this case, it shows that the PCR has been successful, and that the concentration measured in the beginning was correct (see results).

After amplification of the 16S region, and after purification of the product - the concentration of PCR product was determined. This is necessary before calculating the DNA input needed to make the library. First, determine the target DNA input amount (10-100 ng), and after this, the amount of PCR product can be calculated using a specific equation (see results). Measuring the concentration of the PCR product can be done by Agilent Bioanalyzer or Qubit fluorometer. The Qubit fluorometer is an effective method of measurement, unlike the bioanalyzer - which is a more time-consuming process. The concentration on the bioanalyzer was stated in proper designation (pM/µl), which is favorable for later calculation. Because of this, the bioanalyzer was often utilized instead of the Qubit.

The calculation of the PCR product determines the amount of DNA needed to prepare the library. When the library was finished, Agilent Bioanalyzer was used to determine concentration (see results), where the concentration as previously mentioned was given in

pM. The recommended library concentration for template preparation is 26 pM and the concentration of the library was diluted to the concentration of 26 pM (see results). This was considered to be sufficient, and no obstacles were presented.

In addition to all the control points in the procedures, the Ion torrent PGM also has a control system. The adapters are known fragments and the PGM machine exploits this to run an internal control. Before the sequence starts in the initializing, the instrument controls that each of the specific dNTPs will incorporate themselves on the template at a suitable time. By washing over one nucleotide at a time and registering the changes in pH value - this will confirm that the instrument function as it should. In the initialization, the instrument also performs a pH adjustment utilizing buffers. This is of great importance because the semiconductor should be able to detect all pH changes, and therefore the pH must be calibrated before sequencing(33). The ISPs (Ion Sphere Particles) were also added to the produced library before sequencing. The library enriches to the ISPs, and forms a complex with the adapters. The enriched template-positive ISPs were mixed with the library ISPs. They thereby function as a control to observe that the sequencer reads the library correctly, and they also ensure reliable results(33).

Each step in this process is equally important. They allow supervision of the product throughout the process, and ensures the best quality of the result. Because these controls were measured by the Ion Torrent, there was no possibility to impact them during analysis. The impact the executing party could have on this process is the preparation – which in this case proved to be successful. There are many preparation steps, and as mentioned – there are also many steps where errors can occur.

To make a metagenomics analysis successful, the executing party should ideally have prior experience in the field. This is a method of analysis which requires knowledge, accuracy and the ability to work with small amounts of material and keep focus. Any error along the way can contribute to disturbances in the analysis – and may even ruin the material which requires the executing party to start over. In addition to different human errors that may occur and

disturb the material, reagents and different critical points in the procedures are also great factors in regards to bias and interference. Critical points are drafted earlier in this section, and the possible errors during the procedures will now be drafted.

Possible errors can occur both before the analysis starts, in the analytical process, and after the analysis is finished. Pre-analytical errors occur in the phases before the analysis takes place, and this category involves all points from before the sample is taken, until the analysis begins. Central factors around this specific analysis is sampling method, storage of samples, medications the subjects may be on prior to taking the sample – and the conditions of the sample material.

The subjects were instructed to provide a clean-catch mid-stream sample of morning urine (see attachment 1), because this is essential to secure specimen quality. In morning urine, or urine that has been contained in the bladder for more than four hours – the concentration of matter will be higher than it is in urine that has only passed vaguely though the bladder(34). If the subjects did not wash properly before sampling, the chances of contamination increase massively and a lot of resident flora from the skin would present as results. This proved to be the case for sample 10 (analyzed as sample 10 when uncultivated and sample 20 when cultivated), which gave the highest results in both cultivated and un-cultivated samples. Naturally, the occurrence of resident flora from the urethra and skin will probably be seen, but providing a clean-catch sample reduces the amount and thereby also the interference of this matter. Even though samples 20A and 20B showed a large dominance of Enterococcus faecalis that appears to be contamination from sampling, it also provided other results of value – and contamination did not interfere too much with the results of interest.

If the subject did not provide urine that had been contained in the bladder long enough (preferably morning urine or a minimum of four hours, see attachment 1), this could also cause troubles with the analysis because of the lack of concentration(34). Based on the claim presented in the theory section on the sterility of healthy human urine, one can assume that

the concentration of the urine would reflect on the amount of results. The more concentrated, the more likely it is to contain resident flora or any bacterial flora at all.

Storage of the samples provides two critical points, whether the sample is frozen in time, and whether the sample is frozen on the right temperature. Firstly, the subjects were instructed by attachment 1 to transport the sample to the place of storage within two hours after sampling. This is an important point of handling the samples due to the loss of quality that late transport results in(34). Whether the instructions were followed or not was not possible to control, and because of that this may present as a possible explanation for unexpected - or lack of results.

Also, the temperature is an essential part of the storage. After the samples are brought to the place of storage, they must be frozen and stored at -80 °C to contain the DNA of the cells. If not, the DNA material is not contained properly, and if so, the samples would be practically useless in metagenomics analysis. Under ideal conditions, the samples should not be thawed twice. Freezing the samples within two hours will contain both the DNA material in the cells of the urine, and the quality of the urine(34). But in the timeframe where the urine is processed in room temperature, it loses quality of its contents. Re-freezing the urine will therefore not only cause more mechanical strain on the contents, but it will also make the timeframe unpredictable – and the quality of the sample becomes questionable. This occurred during the experiment, and the reasons for that will be referenced further under analytical errors.

Medication can cause interference with a lot of analysis on bodily fluids. This point can be more relevant in cases of blood tests, but may also play a role in controlling the contents of a urine sample. This naturally depends on what the medication in question is, and why the given medication is taken. If the medication is taken due to illness, microbes in conjunction with the condition may appear in the sample. Antibiotics for example, may affect the results because of its effect on all microbes and not only the pathogenic ones(7). This again may result in reduced or non-existing results, because the antibiotics may have eliminated most of the microbes. The subject who provided sample eight (analyzed as eight when uncultivated

and 18 when cultivated) reported on the questionnaire that he had been taking medication, but because of the requirements for privacy and confidentiality – no further elaboration was done. There is therefore no knowledge of any antibiotics or conditions that may cause pathogenic microbes to appear. Medication does not necessarily mean antibiotics. Sample eight did provide results in the cultivated samples, and though medication may have affected the result – the great diversity of the sample presents this as an unlikely assumption.

When the analysis starts, the category of pre-analytical errors end. From there on, a new category is entered, which includes all errors occurring during the analytical process. This is the analytical errors. They involve anything from an error with the pipette to discarding the wrong material during the washing solution. The procedures provide many steps where the same DNA/RNA material is used throughout the entire process, and analytical errors may therefore play an enormous role in the time required to perform the analysis.

The sensitivity and specificity of the primers is essential for the detection quality. The sensitivity of the primers and how often they detect material when it exists, decides how much material is bound. If too little material is bound to primers, too little material will be amplified – and there will be no material to run the analysis on. The specificity, how often the primers do not detect the material when it does not exist – helps control the amount of contamination. A high specificity is ideal in these types of analysis, especially because of the interference that can occur from the human genome.

Not all analytical errors cause large consequences, some only contribute to variables. One of these is working with living material. A lot of the reagents which are involved in the preparation work contain enzymes, which make them unstable because this is living material. The primers are also somewhat unstable, and these reagents must therefore thaw on ice in order to keep them as stable as possible. Since they are unstable, they will never react in the same way twice. The reagent will do the same job every time it is used, but it will never perform exactly the same way. This may cause problems for standardization, and marginal variations in the result.

The concentration of the material chosen from the cultivated and uncultivated urine is very important, and can also present analytical errors. If the concentration is too low, the samples cannot be processed further. The uncultivated samples were discarded as early as the first bioanalyzer step (see results). The low concentration may also have been caused by too much unamplified DNA. This could occur if many of the primers were not bound to DNA material.

Another variable to be considered is the cultivation step. Cultivating the samples is not necessarily a negative thing, it is easier to isolate and control the DNA amount through McFarland suspensions - and the possibility to see the material on the media before starting the entire process can be expedient to save both time and money. The executing party then avoids starting the process without any material to analyze. The uncultivated samples show no sign of concentration and content until the bioanalyzer/Qubit step where the concentration is measured. But the beauty of metagenomics is that it is able to detect microbes that cannot be cultivated on traditional non-selective media, and therefore it has been a big part of establishing of resident flora in the bladder(12,22). Cultivating the samples before performing metagenomics analysis on them will reduce the findings to only the microbes that grow on traditional media, but at the same time it assures that there is material present.

After the samples are prepared for analysis in the Ion Torrent machines, the control is transferred from the executing party to the machine and machine software. Any machine error that may occur also contributes as a possible error – and will naturally affect the results.

Though there are few of them, some errors can also occur post analysis. Post-analytical errors are errors that occur after the analysis is executed. When all the preparation steps and all the analysis steps are done and the result is presented, there are not a lot of possible errors left. The only error possible to make at this point is misinterpretation of the results. This is a possible error when the executing party does not have a lot of experience. Some results may be hard to interpret, especially when the software is not familiar.

The procedures that were established in this process appear to work well in some cases, and not so well in others. To increase the quality and get more accurate results, it can be necessary to make small modifications in the method. One suggestion is to increase the amount of sample material from uncultivated urine. This could provide a higher concentration of bacterial DNA. As previously mentioned, the concentration of bacterial DNA in the samples must be in a specific range, or there would be no purpose of running metagenomics analysis on them.

In the cultivated samples, 1.0 McFarland (equivalent to $300x10^6$ CFU/ml)(35) was used as a standardized concentration in the suspensions that were prepared, but this showed low concentrations of DNA later in the process. The value was therefore increased to 1.5 McFarland, which resulted in a higher concentration – and the analysis could proceed. To obtain enough DNA material from the cultivated samples, it is recommended to make suspensions of 1.5 McFarland or higher.

As mentioned, the kit that was used for preparation of the samples might not have been ideal in regards to results. Another kit that might have worked better is a lysis kit from Molzyme life science. They have series of kits under MolYsis™-Pathogen DNA Isolation. This was a recommendation during the project that was not tested out. The benefits this supplier claims to have is removal of human DNA and PCR inhibitors, sensitivity increase up to factor 40.000, broad-range lysis of gram-negative and gram-positive bacteria, DNA-free reagents and low hands-on-time(36). This could solve the issues of interference of human genome, and increase the sensitivity and thereby detect lower concentrations. It also claims to provide lysis of both gram-negative and gram-positive bacteria, and the potential limitation presented by selecting between the two is thereby excluded. The DNA-free reagents could decrease interference, and low hands-on-time could make the process less time-consuming. Shotgun-techniques may also have been better qualified for urine-studies because of the low bacterial concentration in the material, and may be ideal in future projects.

6. CONCLUSION

Urine, especially from healthy subjects - is a challenging material to perform metagenomics analysis on because of the lack of material. It contains small amounts of cells, and therefore also small amounts of DNA. The sampling method is difficult to standardize when the subjects perform the sampling themselves, and the purification steps in the procedures appear as a large threat because too much DNA material can be washed away. Because of this, there is a significant chance that some of the material will be lost during purification.

The main focus of the thesis was to establish procedures for pre-analytical variables of Ion Torrent 16S metagenomics, which involves all the steps in preparation of Ion Torrent analysis. The procedures that were established worked well for cultivated samples, where the presence of bacterial DNA was ascertained and the concentration in the samples was sufficient. For the uncultivated samples on the other hand, the procedures proved not to be optimal. It would be reasonable to apply some of the suggested changes if one was to utilize the procedures for uncultivated samples in the future. Though the established procedures did not work as well on urine from healthy subjects, they may work better on urine that contains pathogens.

7. ATTACHMENTS

1. Information for you about how to provide a urine sample.
2. Procedure for production of agar.
3. Procedure metagenomics workflow on Ion Torrent PGM.
4. Agilent high sensitivity DNA kit Quick start guide
5. Quick reference Ion PGM Hi-Q OT2 Kit
6. Quick reference Ion PGM Hi-Q Sequencing Kit
7. Prosedyre for utsåing SiV / Cultivation procedure SiV

8. REFERENCES

[1]**Sjøberg NO.** Translasjon: Molekylær genetikk. 5. ed. Norway: Vet tog Viten AS; 2013. 345 pages.
[2] **McKee T, McKee JR.** Biochemistry: the molecular basis of life. 5. ed. Oxford: Oxford University Press; 2013. 685 pages, 2(3) 56, 19(2**)** 659-660
[3] **Reece, Urry, Cain, Wasserman, Minorsky and Jacson.** Campbell Biology. 9. ed. San Francisco: Pearson Education; 2011. 1309 pages. 17(4) 385-386
[4] **Chakravorty S, Helb D, Burday M, Connell N and Alland D.** A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. 2007 [2017-05-22] 17 pages.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2562909/pdf/nihms22082.pdf
[5] **Yang B, Wang Y and Qian PY** Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. 2016 [2017-05-22] 8 pages.
https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-016-0992-y
[6] The new science of metagenomics: Revealing the secrets of our Microbial planet, National research council of the national academies, Washington DC, The national academies press, 2007, 104 s.
[7] **Tortora, Funke, Case**. Microbiology-An introduction. 11. ed. Edinburgh: Pearson Education Limited; 2014. 970 pages.
[8] **Alberts, Bray, Hopkin, Johnson, Lewis, Raff, Roberts, Walter.** Essential cell biology. 4. ed. United states of America: Garland Science; 2014. 725 pages.
[9] **Grada A and Weinbrecht K.** Next generation sequencing: methodology and application. Journal of Investigative dermatology. [2017-05-22] 2013; volume 133:4.
www.jidonline.org
[10] **Thermo Fischer scientific Inc.** Ion 16S metagenomics kit and ion reporter metagenomics workflow solution. [2017-05-22] 2016.
www.thermofisher.com/ion16smetagenomics
[11] **Life technologies.** Longer read lengths improve bacterial identification using 16S rRNA gene sequencing on the ion PGM system. [2017-05-22] 2013.
www.lifetechnologies.com/ionmicrobial
[12] **Thomas T, Gilbert J and Meyer F.** Metagenomics – a guide from sampling to data analysis. Microbial informatics and experimentation [2017-05-22] 2012 Volume 2:3.
https://microbialinformaticsj.biomedcentral.com/articles/10.1186/2042-5783-2-3
[13] Clinical-microbiomics-actionable microbiome insight. Shotgun metagenomics. [2017-05-22]
http://www.clinical-microbiomics.com/services/shotgun-metagenomics?gclid=Cj0KEQjw2fLGBRDopP-vg7PLgvsBEiQAUOnIXL5PsQeaPnxjTXfwRK_xu-7iyoRVoVZ6QKIjaPsAXe4aAtrU8P8HAQ
[14] **Su Z, Ning B, Fang H, Hong H, Perkins R, Tong W, et al.** Next-generation sequencing and its application in Molecular diagnostics. [2017-05-22] 2011. Volume 11:2011.
http://www.tandfonline.com/doi/full/10.1586/erm.11.3?scroll=top&needAccess=true
[15] **Whitley AS, Jenkins S, Waite I, Kresoje N, Payne H, Mullan B, et al.** Microbial 16S rRNA Ion Tag community metagenome sequencing using the Ion Torrent (PGM) Platform [2017-05-22] 2012. Volume 91, pages 80-88
http://dx.doi.org/10.1016/j.mimet.2012.07.008
[16] **Burtis CA, Ashwood ER, Bruns DE and Sawyer BG.** Tietz Fundamentals clinical chemistry. 6. ed. Missouri, Saunders, an imprint of Elsevier Inc 2008. 952 pages.

[17] **Life technologies.** Qubit® dsDNA HS Assay Kits. Molecular probes [2017-05-24] Catalog nos. Q32851, Q32854
https://tools.thermofisher.com/content/sfs/manuals/Qubit_dsDNA_HS_Assay_UG.pdf

[18] **Agilent technologies.** Agilent 2100 Bioanalyzer: 2100 Experts User's Guide. Germany, Agilent Technologies Inc. ed. May 2005, Manual Part Number: G2946-90004.
http://www.agilent.com/cs/library/usermanuals/Public/G2946-90004_Vespucci_UG_eBook_(NoSecPack).pdf
[19]**Yeo ZX, Chan M, Yap YS, Ang P, Rozen S and Lee AS.** Improving indel detection specificity of the Ion Torrent PGM benchtop sequencer [2017-05-22] 2012. Volume 2012:7(9). Pages 9.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3446914/
[20] The new science of metagenomics: Revealing the secrets of our Microbial planet, National research council of the national academies, Washington DC, The national academies press, 2007, 158 pages.

https://www.ncbi.nlm.nih.gov/books/NBK54006/
[21] **Thomas-White K, Brady M, Wolfe AJ, Mueller ER, Springer science + business media New York 2016**. The bladder is not Sterile: History and Current discoveries [2017-05-23] January 2016. Volume 11:18
https://link.springer.com/article/10.1007/s11884-016-0345-8

[22] **Lewis DA, Brown R, Williams J, White P, Jacobson SK, Marchesi JR, et al.** The human urinary microbiome, bacterial DNA in voided urine of asymptomatic adults [2017-05-23] August 2013. Volume 2013;3:41
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3744036/
[23]**Fouts DE, Pieper R, Szpakowski S, Pohl H, Knoblach S, Suh MJ, et al.** Integrated Next-generation Sequencing of 16S rDNA and Metaproteomics Differentiate the Healthy Urine Microbiome from Asymptomatic Bateriuria in Neurophatic Bladder Associated with Spinal cord injury. [2017-05-23] 28. August 2012. Volume 2012;10:174
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3511201/
[24] **Neloson DE, Dong Q, Pol BVD, Toh E, Fan B, Katz BP, et al**. Bacterial Communities of the Coronar Sulcus and Distal Urethra on Adolescent Males [2017-05-23] May 2012.
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0036298
[25] **Hilt EE, McKinley K, Pearce MM, Rosenfeld AB, Zilliox MJ, Mueller ER, et al.** Urine is not sterile: Use of Enhanced Urine Culture Techniques to Detect Resident Bacterial Flora in the Adult Female Bladder [2017-05-23] Mars 2014. Volume 52:3
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3957746/
[26]**Wolfe AJ, Toh E, Shibata N, Rong R, Kenton K, FitzGerald MP, et al.** Evidence of Uncultivated Bacteria in the Adult Female Bladder [2017-05-23] April 2012. Volume 50
http://jcm.asm.org/content/50/4/1376.full
[27] **Siddiqui H, Nederbragt AJ, Lagesen K, Jeansson SL and Jakobsen KS.** Assessing diversity of the female urine microbiota by high throughput sequencing of 16S rDNA amplicons [2017-05-23] November 2011. Volume 11:244
https://bmcmicrobiol.biomedcentral.com/articles/10.1186/1471-2180-11-244
[28]**Michael L. Altshuler.** PCR Trubleshooting: The essential guide. Caister Academic Press. Moscow Research Institute of Medical Ecology; 2006. 80 s.
[29]**Ion Torrent**. Ion 16S metagenomics kit and ion report metagenomics workflow solution.
[30] Ion One Touch 2 Instrument by Life technologies
https://www.thermofisher.com/order/catalog/product/4474778

[31] Ion Torrent Personal Genome machine by Life technologies
https://www.thermofisher.com/order/catalog/product/4462921?ICID=search-product

[32]**Zhang Q, Kwoh C, Attorri S**. Clarrige JE Aerococcus urinae in Urinary Tract Infections. [2017-05-27] 2000.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC86536/
[33] **Thermo Fisher scientific.** Ion PGM Hi-Q Sequencing kit user guide. Cat.no A25592.
https://tools.thermofisher.com/content/sfs/manuals/MAN0009816_Ion_PGM_HiQ_Sequencing_Kit_UG.pdf
[34]**Vinje BU.** Urinmikroskopi Praktisk Utførelse og Bildeatlas 1.ed. Norway: Cappellen Damm Høyskoleforlaget:2012. 174 pages. Pages 13-17.

[35] **Sutton S.** Measurement of cell consentration in suspension by optical density [2017-05-31] 2006.
http://www.microbiol.org/resources/monographswhite-papers/measurement-of-cell-concentration-in-suspension-by-optical-density/
[36]**Molzym life science.** Pathogen DNA Isolation, MolYsis™. [2017-05-23]

http://www.molzym.com/index.php/products/dna-isolation-products/pathogen-dna-molysis/10-products/dna-isolation/59-microbial-pathogens

Attachment 1: Information for you about how to provide a urine sample.

General information:

- The sample must be taken the same day as it is delivered. It should contain morning urine, but if this is not possible the sample should be taken four hours after last urination.
- Wash your hands before and after sampling.
- The best way to take the sample is after a shower. If so, remember to wash away all the soap residue.

Approach:

- To prevent contamination from the skin, the urine sample is taken by pulling the foreskin backwards. If the sample is taken without a shower before sampling, the area must be washed with lukewarm water and a mild soap. Make sure to remove all the soap residue.
- Start the urination and let the first drops go in the toilet, then insert the sampler in the beam whiteout stopping the urination. When collected 10-12ml remove the sampler from the beam and let the rest of the urine go in the toilet. Screw the cork firmly on.
- The sample should be delivered as soon as possible, and within two hours. DO NOT label the glass. The sample should be stored in a refrigerator until delivery.

Attachment 2: Procedure for production of agar.

Intended use:

Agar production was used to make selective – and non-selective media to utilize for cultivation of bacteria. When cultivated, the material was then used in genomic identification. The cultivation on selective – and non-selective media contributed to elimination of interference from factors such as human genome.

Principle:

MacConkey agar is a selective media used in isolation of non-fastidious gram negative rods, like species that are members of Enterobacteria and Pseudomonas. It contains essential nutrients, vitamins and nitrogenous factors that are required for growth of the relevant microorganisms.

LB agar (Lysogeny Broth agar) is a selective media for growth of Escherichia coli, and the intendent use of pure cultures achieved on the media is genomic testing. The content in LB is tryptone, sodium chloride, yeast extract and agar. Tryptone contributes with amino acids, nitrogen and carbon that is necessary for bacterial growth.

PCA (Plate Count agar) is a non-selective media for growth of aerobic plate counts. The media was used for counting the total number of aerobic bacteria in the given specimen.

Formula:

Agar	Distilled water (ml)	Powder (grams)	pH
MacConkey	1000	52 g	7.4 +/- 0.2
PCA	500	11.75 g	7.0 +/- 0.2
LB	1000	40.0 g	7.5 +/- 0.2

Directions:

1. Dissolve the powder of the wanted agar in distilled water (degree II) in an Erlenmeyer flask.
2. Mix and heat until the powder is completely dissolved in the water, providing a homogenous solution.
3. Autoclave all solutions and materials at 121 ˚C for 15 minutes.

4. Adjust pH value of the solutions by adding either HCl or NaOH when the mixture is at approximately 60-50^{o}C.
5. Pour the solutions into sterile petri dishes, and then store at 4˚C in a sterile environment.

Attachment 3: Procedure metagenomics workflow on Ion Torrent PGM.

A Preparation of pure DNA.

A.1 Purification of total DNA.

For purification of total DNA, the DNeasy blood and tissue kit was used. The kit was used for purification of DNA from both cultivated and uncultivated samples. This kit ensures rapid purification of total DNA from different sources and is suitable for PCR. The buffer system is designed to ensure optimal cell lysis followed by direct binding to the DNeasy membrane. This procedure is also time-effective, and the centrifugation process is simple.

1. Add 2 ml sample (uncultivated) or 1 ml sample (cultivated) to a microcentrifuge tube and centrifuge for 10 min at 5000 G (7500 rpm). Discard the supernatant.
2. Add 180 µl buffer ATL and 20 µl proteinase K to the pellet and vortex thoroughly. Incubate the samples at 56 ˚C overnight.

   NOTE: The lysis time is usually 1-3 hours, but the DNA is stable enough to be lysed overnight when necessary.

   IMPORTANT: After the incubation, it is important to observe the lysate. It may appear viscous, but it is important that is not gelatinous. If so, it can clog the DNeasy Mini spin column. If the material is gelatinous it can be solved by increasing g-force and/or duration of centrifugation step. In the future, it can be necessary to reduce the amount of starting material. The clogging can also come from insufficient lysis, so there can be necessary to extend incubation.

3. To ensure that the material is RNA-free genomic DNA, add 4µl RNase A, mix by vortexing, and incubate for 2 minutes at room temperature.
4. Vortex the samples for 15 seconds, and add 200 µl buffer AL. Mix thoroughly by vortexing. Add 200 µl ethanol and vortex the solution.

   IMPORTANT: It is essential that the samples are vortexed immediately to get a homogeneous solution.

5. Add the mixture from step 4 to the DNeasy Mini spin column and place the column in a collection tube. Centrifuge at 6000 x G (8000 rpm) for 1 min. Discard the eluate and the collection tube.
6. Place the DNeasy Mini spin column in a new collection tube and add 500 µl buffer AW1. Centrifuge at 6000 x G (8000 rpm) for 1 min. Discard the content in the collection tube and re-use the tube in the next step.
7. Add 500 µl buffer AW2 and centrifuge for 3 min at 20 000 x G (14 000 rpm).

   NOTE: This step will dry the membrane and ensures that no ethanol residue will be carried over and interfere with the content of the eluate in the next step. Remove the DNeasy mini spin column carefully in the next step, so that the column does not come in contact with the flow-through. This ensures that no ethanol will be carried over.

8. Place the DNeasy mini spin column in a microcentrifuge tube and add 100 µl buffer AE directly onto the DNeasy membrane. Incubate for 1 min at room temperature, and centrifuge for 1 min at 6000 x G (8000 rpm).
9. Repeat step 8 by adding 20 µl buffer AE to increase the final DNA concentration. Store the samples at 4-8˚C.

A.2 Quantification of DNA.

Before running the amplification it is important to know the concentration and the amount of DNA in the sample. This helps determine the number of amplification cycles. It can be wise to minimize the number of cycles to avoid overamplification of the 16S region, and to reduce the risk of contaminant-production. Qubit dsDNA HS Assay kit was used in this step.

10. Start by making the working solution by mixing 1990 µl Qubit dsDNA HS buffer and 10 µl Qubit dsDNA HS reagent. This is enough for 10 samples.

NOTE: All reagents from this kit were stored in room temperature. This is nessecary to avoid temperature fluctuations.

11. Load 190 µl of the working solution in to two tubes. Add 10 µl standards (S1 and S2) to their specific tubes and vortex.
12. Add the working solution to all of the tubes (20, one for each sample). The total volume must not exceed 200 µl. From the uncultivated samples, add 180 µl working solution and 20 µl sample, and from the cultivated samples, add 190 µl working solution and 10 µl sample. Vortex for 2-3 seconds. Incubate the samples for at least 2 minutes in room temperature.

IMPORTANT: To ensure that the fluorophores in the working solution will attach to all the DNA in the sample it is important to mix thoroughly.

13. Use the Qubit fluorometer to detect the DNA concentration in the solutions. Pressing "HOME" and selected "Run new calibration" and press GO.

Calibration:

Insert the tube containing standard 1 into the Qubit fluorometer, close the lid, and press GO. Remove standard 1 when "COMPLETE" showes in the display.

Insert standard 2 into the Qubit fluorometer, close the lid, and press GO. Remove standard 2 when "COMPLETE" showed in the display.

14. When the calibration is complete, insert the samples to Qubit fluorometer, close the lid, and press GO.
15. The Qubit fluorometer then calculates the concentration of the amount of DNA in the samples (see results).

NOTE: The concentration of DNA used in amplification must be 1-3 ng for pure microbial DNA (cultivated samples) or 1-2 µl extracted DNA from the uncultivated samples.

B Prepare amplicons

B.1 Amplify the 16S region.

The Ion 16S metagenomics kit (Cat.no. A26216) that contains enzymes and two primer sets is used in amplification.

16. Prepare tubes for two reactions for each sample, one for each primer set. Also include two controls.

NOTE: All reagent used in this procedure step must be kept on ice.

17. Mix the master mix and primer sets in the correct amount for 10 samples and two controls per primer set (volume given by the table below).
18. The composition for primer set 1 (V2-4-8) and primer set 2 (V3-6, 7-9) will be as followed into PCR tubes:

	Sample 1-10 Uncultivated	**Positive control**	**Negative control**
Mix consisting of master mix (195 µl) and primer 1 or primer 2 (39 µl). To each sample: 15 ul Master mix and 3 ul primer set.	18 µl	18 µl	18 µl
DNA from samples	12 µl (uncultivated)	-	-
Diluted DNA from E.Coli (positive control)	-	2 µl	-
Water (negative control)	-	10 µl	12 µl
Total volume	**30 µl**	**30 µl**	**30 µl**

	Sample 18 and 20 Cultivated	**Positive control**	**Negative control**
Mix consisting of master mix (195 µl) and primer 1 or primer 2 (39 µl). To each sample: 15 ul Master mix and 3 ul primer set.	18 µl	18 µl	18 µl
DNA from samples	3 µl (cultivated) endres til 2 ul på prøve 20 A,B	-	-
Diluted DNA from E.Coli (positive control)	-	2 µl	-
Water (negative control)	9 µl	10 µl	12 µl
Total volume	**30 µl**	**30 µl**	**30 µl**

NOTE: Before running the samples, they must be mixed and centrifuged using a pulse-spin to make sure the contents sink to the bottom of the tube.

19. All the tubes were placed into the thermal cycler, running in the following program:

Stage	Temperature	Time
Hold	95 °C	10 min
Cycle 18-25 (18 for the cultivated samples and 25 for the uncultivated samples)	95 °C 58 °C 72 °C	30 sec 30 sec 20 sec
Hold	72 °C	7 min
Hold	4 °C	Samples must be removed within 24 hours and stored at –20 °C

20. To confirm the presence of PCR product, preform a gel electrophoresis on the uncultivated samples. Measure the cultivated samples on the Qubit fluorometer or by electrophoresis (see results).

B.2 Purify the amplification product.

Agencourt AMPure XP reagent is used to purify the amplification product.

21. Load the following reagents with the amplification product:

Component	Volume
Amplification product	40 µl (20 µl of V2-4-8 reaction mixed with 20 µl of V3-6, 7-9 reaction)
Agencourt AMPure XP Reagent	72 µl

NOTE: This reagent must be stored at room temperature for at least 30 minutes before use.

22. Vortex the mixtures, pulse-spin and incubate at room temperature for 5 minutes.
23. Pulse-spin the samples one more time and place them in a magnetic rack (DynaMag-2 magnet) for 3 minutes (or until the solution is clear). Remove and discard the supernatant.

IMPORTANT: Be careful when removing the supernatant to prevent disturbing the bead pellet.

24. While the tubes are still in the magnetic rack, add 300 µl of 70% ethanol to each tube. Incubate for 30 seconds and turn the tubes around twice within the incubation time. Remove and discard the supernatant.

NOTE: This will make the magnet to move the beads around and ensure that all the beads are rinsed off by the solution.

25. Repeat step 24 for a second wash.

26. Pulse-spin the tubes to remove residual ethanol and place it back in the magnet rack. Remove the remaining supernatant with a 20 µl-pipettor.
27. Leave the tubes in the magnetic rack with the lids open, and incubate for four minutes at room temperature to let the beads air-dry.

IMPORTANT: Don't let the bead pellet dry out completely!

28. Remove the tubes from the magnetic rack and add 15 µl of nuclease-free water to the pellet. Vortex the samples for 5-10 seconds to spread the beads in the water.
29. Puls-spin the tubes and place them in the magnetic rack for at least 1 minute or until the solution is clear. Transfer the supernatant to a new tube without disturbing the pellet. The supernatant just transferred is the DNA-containing eluate. It can be stored at –30 °C to –10 °C for up to 2 weeks.

B.3 Calculate DNA input for library preparation.

Before preparing the library, the concentration of DNA input must be determined. Agilent 2100 Bioanalyzer instrument and the Agilent High sensitivity DNA kit or the Qubit dsDNA HS assay kit (Cat.no. Q32851) can be used to determine the concentration. Qubit fluorometer is ideal for measuring the concentration of the cultivated samples because of time-and cost savings (see procedure A.2, and change the volumes to 195 µl working solution and 5 µl sample).

NOTE: All reagents must be stored in room temperature for at least 30 minutes before use.

30. Dilute 2µl of each PCR product with 18µl nuclease-free water (1:10).
31. Take 1µl alicottes of each diluted PCR product, and add to a suitable chip for analysis in Agilent 2100 Bioanalyzer. The Agilent software will then determine the amount (ng) of amplicons (see user guide when applying reagent and diluted sample to the chip).
32. Use the result to calculate DNA input to the library (see result 3.2.3).
33. When the DNA input is calculated and the amount is established, add the calculated amount of pooled amplicon and the needed amount of nuclease-free water to obtain 50ng in 79 µl.

C Library template and sequencing

C.1 Prepare the library.

Ion plus fragment library kit (Cat.no. 4471252) and Ion Xpress Barcode adapters 1-16 kit (Cat.no. 4471250) was used to make the library and purify the barcoded library product.

34. Load the following components in a tube and mix thoroughly by pipettor:

Components	**Volume**
Pooled amplicons	79 µl
5X end repair buffer	20 µl
End repair enzyme	1 µl
Total volume	100 µl

35. Incubate for 20 minutes at room temperature.
36. Add 180 µl Agencourt AMPure XP reagent to each sample. Vortex, pulse-spin and then incubate for five minutes at room temperature.
37. Pulse-spin and place the sample in DynaMag-2 for at least three minutes or until the solution clears. Remove and discard the supernatant carefully.
38. While the tubes are still in the magnetic rack, add 500 µl of 70% ethanol to each tube. Incubate for 30 seconds at room temperature, and turn the tubes around twice during the incubation. Remove and discard the supernatant.

NOTE: This will make the magnet move the beads around, and ensure that all the beads are rinsed off by the solution.

39. Repeat step 38 for a second wash.
40. Pulse-spin the tubes to remove residual ethanol and place them back in the magnet rack. Remove the remaining supernatant with a 20 µl-pipettor.
41. Leave the tubes in the magnetic rack with the lids open, and incubate for four minutes in room temperature to let the beads air dry.

IMPORTANT: Do not let the bead pellet dry out completely!

42. Remove the tubes from the magnetic rack and add 25 µl Low TE to the pellet. Vortex the samples for 5-10 seconds in order to spread the beads in the Low TE solution.
43. Puls-spin the tubes and place them in the magnetic rack for at least 1 minute or until the solution is clear. Transfer the supernatant to a new tube without disturbing the pellet. This tube now contains the DNA-containing eluate. Can be stored at –30 °C to –10 °C for up to 2 weeks.
44. Load the following components to the sample in a PCR tube to make the barcoded library, using the Ion Xpress Barcode Adapters 1-16 kit (Cat.no. 4471250):

Components	**Volume**
DNA (sample)	Ca. 25 µl
10X ligase buffer	10 µl
Ion P1 adapter (barcoded libraries)	2 µl
Ion Xpress barcode X (one for each sample)	2 µl
dNTP Mix	2 µl
Nuclease-free water	49 µl
DNA ligase	2 µl
Nick repair polymerase	8 µl
Total volume	**100 µl**

IMPORTANT: Be careful not to cross-contaminate when handling barcode adapters. When making a barcoded library, use both Ion P1 adapter and the desired Ion Xpress Barcode X.

45. When all the reagents are added to the sample, place the tubes in a thermal cycler with the following program:

Stage	Temperature	Time
Hold	25 °C	15 min
Hold	72 °C	5 min
Hold	4 °	∞ *

*=This is not a stopping point, continue with the procedure.

46. Transfer the sample to a 2.0 ml Eppendorf LoBind tube.
47. Add 140 µl Agencourt AMPure XP reagent to the samples. Vortex the mixtures, pulse-spin and incubate them at room temperature for five minutes.
48. Pulse-spin the samples one more time, and place them in a magnetic rack (DynaMag-2 magnet) for three minutes (or until the solution is clear). Remove and discard the supernatant.

IMPORTANT: Be careful when removing the supernatant to prevent disturbing the bead pellet.

49. While the tubes are still in the magnetic rack, add 500 µl of 70% ethanol to each tube. Incubate for 30 seconds, and turn the tubes around twice during the incubation period. Remove and discard the supernatant.

NOTE: This will make the magnet to move the beads around and ensure that all the beads are rinsed off by the solution.

50. Repeat step 49 for a second wash.
51. Pulse-spin the tubes to remove residual ethanol and place it back in the magnet rack. Remove the remaining supernatant with a 20 µl-pipettor.
52. Leave the tubes in the magnetic rack with the lids open, and incubate them for four minutes in room temperature to let the beads air dry.

IMPORTANT: Do not let the bead pellet dry out completely!

53. Remove the tubes from the magnetic rack and add 20 µl Low TE to the pellet. Vortex for 5-10 seconds to spread the beads in the solution.
54. Puls-spin the tubes and place them in the magnetic rack for at least 1 minute or until the solution is clear. Transfer the supernatant to a new tube without disturbing the pellet. The supernatant transferred is the DNA-containing eluate. Can be stored at –30 °C to –10 °C for up to 2 weeks.

C.2 Determine library concentration using bioanalyzer.

Ion Plus fragment library kit can be used to amplified the library.

55. Add 5 µl of Low TE to the diluted adapter-ligated library (ca. 20 µl).
56. Load the following reagents to the tube containing the library:

Components	**Volume**
Uamplified library	25 µl
Platinum PCR superMix High Fidelity	100 µl
Library Amplification Primer Mix	5 µl
Total volume	130 µl

57. Split the solution into two PCR tubes, each then containing 65 µl. Place the tubes into a thermal cycler and run the following program:

Stage	Step	Temperature	Time
Holding	Denature	95 °C	5 min
5 cycles (50 ng of input)	Denature Anneal Extend	95 °C 58 °C 70 °C	15 sec 15 sec 1 min
Holding	-	4 °C	Hold for up to 1 hour

58. Combine the content in the two PCR tubes (the split solution from the same sample) in a 2,0ml Eppendorf LoBind tube.
59. Add 195 µl Agencourt AMPure XP reagent to each sample to purify the amplified library. Vortex the mixtures, pulse-spin and incubate at room temperature for five minutes.
60. Pulse-spin the samples one more time and place them in a magnetic rack (DynaMag-2 magnet) for three minutes (or until the solution is clear). Remove and discard the supernatant.

IMPORTANT: Be careful when removing the supernatant to prevent disturbing the bead pellet.

61. Keep the tubes in the magnetic rack and add 500 µl of 70% ethanol to each tube. Incubate for 30 seconds, and turn the tubes around twice during the incubation period. Remove and discard the supernatant.

NOTE: This will make the magnet move the beads around and ensure that all the beads are rinsed off by the solution.

62. Repeat step 61 for a second wash.
63. Pulse-spin the tubes to remove residual ethanol and place it back in the magnet rack. Remove the remaining supernatant with a 20 µl-pipettor.
64. Keep the tubes in the magnetic rack with the lids open, and incubate for four minutes at room temperature to let the beads air dry.

IMPORTANT: Do not let the bead pellet dry out completely!

65. Remove the tubes from the magnetic rack and add 20 µl Low TE to the pellet. Vortex for 5-10 seconds to spread the beads in the solution.
66. Puls-spin the tubes and place them in the magnetic rack for at least 1 minute or until the solution is clear. Transfer the supernatant to a new tube without disturbing the pellet. This is the library-containing eluate. Can be stored at –30 °C to –10 °C for up to 2 weeks.
67. Use the Agilent High sensitivity DNA kit (see attachment 4) to determine the library concentration.

68. Dilute 1 µl of the amplified library 1:10 (9 µl) with nuclease-free water, and analyze on the Agilent 2100 bioanalyzer instrument. Use the results to determine library concentration (see results).

C.3 Proceed to template preparation.

Use the Ion PGM Hi-Q OT2 kit (Cat.no. A27739) to separate the library fragments by emulsion PCR (see attachment 5). Load the separated fragments onto the Ion 318 Chip v2.

C.4 Sequence the library.

In the manual for the Ion PGM Hi-Q sequencing kit (Cat.no. A25592), the procedure for sequencing of the library and identification of the genome is described (see attachment 6).

Attachment 4: Quick reference guide Agilent HS DNA kit.

Agilent High Sensitivity DNA Kit Quick Start Guide

The complete High Sensitivity DNA Kit Guide can be found in the online help of the 2100 Expert software.

Agilent High Sensitivity DNA Kit (reorder-no 5067-4626)	
Agilent High Sensitivity DNA Chips	*Agilent High Sensitivity DNA Reagents (reorder-no 5067-4627)*
10 High Sensitivity DNA Chips	(yellow) High Sensitivity DNA Ladder
1 Electrode Cleaner	(green) High Sensitivity DNA Markers 35/10380 bp (4 vials)
Syringe Kit	(blue) High Sensitivity DNA Dye Concentrate [1](1 vial)
1 Syringe	(red) High Sensitivity DNA Gel Matrix (2 vials)
	2 Spin Filters (reorder-no 5185-5990)

[1] "This product is provided under a license by Life Technologies Corporation to Agilent Technologies. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product only as described in accompanying product literature. The sale of this product is expressly conditioned on the buyer not using the product or its components (1) in manufacturing; (2) to provide a service, information, or data to an unaffiliated third party for payment; (3) for therapeutic, diagnostic or prophylactic purposes; (4) to resell, sell or otherwise transfer this product or its components to any third party, or use for any use other than use in the subfields of research and development, quality control, forensics, environmental analysis, biodefense or food safety testing. For information on purchasing a license to this product for purposes other than described above contact Life Technologies Corporation, Cell
Analysis Business Unit, Business Development, 29851 Willow Creek Road, Eugene, OR 97402, Tel: (541) 465-8300. Fax: (541) 335-0354."

Research Use Only Not for use in Diagnostic Procedures. **Assay**

Principles

Agilent DNA kits contain chips and reagents designed for sizing and analysis of DNA fragments. Each DNA chip contains an interconnected set of microchannels that is used for separation of nucleic acid fragments based on their size as they are driven through it electrophoretically. Agilent DNA kits are designed for use with the Agilent 2100 Bioanalyzer instrument only.

Applications and Kits

The Agilent High Sensitivity DNA kit is designed for sizing and quantitation of fragmented DNA, DNA sequencing libraries, and DNA samples derived from ChIP.

Agilent DNA kits: DNA 1000 Kit (reorder-no 5067-1504), DNA 7500 Kit (reorder-no 5067-1506), DNA 12000 Kit (reorder-no 5067-1508) and High Sensitivity DNA Kit (reorder-no 5067-4626).

Storage Conditions

- Keep all reagents and reagent mixes refrigerated at 4 °C when not in use to avoid poor results caused by reagent decomposition.
- Protect dye and dye mixtures from light. Remove light covers only when pipetting. Dye decomposes when exposed to light.

Equipment Supplied with the Agilent 2100 Bioanalyzer System

- Chip priming station (reorder-no 5065-4401)
- IKA vortex mixer

Agilent Technologies

Agilent High Sensitivity DNA Kit Quick Start Guide

Additional Material Required (Not Supplied)

- Pipettes (10 µL, 100 µL and 1000 µL) with compatible tips (filter-free, non-autoclaved tips)
- 0.5 mL low-bind microcentrifuge tubes for sample preparation
- Microcentrifuge (> 13000 g)

Sample Preparation

NGS sheared DNA or libraries: For accurate determination of DNA concentration, the total DNA in the samples must be between 100 pg/µL to 10 ng/µL.

PCR samples: For accurate determination of DNA concentration, the total DNA in the sample must be between 5 – 500 pg/µL.

If concentration of a sample is higher, dilute or use another Agilent DNA assay (DNA 1000, DNA 7500 or DNA 12000).

Physical Specifications

Type	Specification
Analysis run time	45 min
Number of samples	11 samples/chip

Analytical Specifications

Specification	Agilent High Sensitivity DNA assay
Sizing range	50–7000 bp
Typical sizing resolution	50–600 bp: ± 10 % 600–7000 bp: ± 20 %

Sample volume	1 µL	Sizing accuracy	± 10 % (for ladder as sample)
Kit stability	4 months (Storage temperature see individual box!)	Sizing reproducibility	5 % CV (for ladder as sample)
		Quantitation accuracy	20 % (for ladder as sample)
		Quant. reproducibility	50-2000 bp: 15 % CV; 2000-7000 bp: 5 % CV (for ladder as sample)
		Quantitative range	5–500 pg/µL
		Maximum salt[1]	10 mM Tris and 1 mM EDTA

1

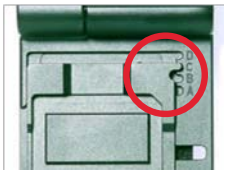
Due to the high sensitivity of the assay, different ions and higher salt concentrations might influence the performance of the assay. Water is not advised as a sample buffer. **Setting up the Chip Priming Station**

1 Replace the syringe:

a Unscrew the old syringe from the lid of the chip priming station.

b Release the old syringe from the clip. Discard the old syringe. **c** Remove the plastic cap of the new syringe and insert it into the clip.

d Slide it into the hole of the luer lock adapter and screw it tightly to the chip priming station.



2 Adjust the base plate:

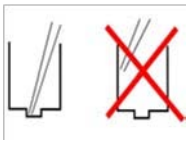
a Open the chip priming station by pulling the latch. **b** Using a screwdriver, open the screw at the underside of the base plate.

c Lift the base plate and insert it again in position C. Retighten the screw. 2

3 Adjust the syringe clip: **a** Release the lever of the clip and slide it down to the lowest position.

Essential Measurement Practices

- Handle and store all reagents according to the instructions on the label of the individual box.
- Avoid sources of dust or other contaminants. Foreign matter in reagents and samples or in the wells of the chip will interfere with assay results.
- Keep all reagents and reagent mixes refrigerated at 4 °C when not in use.
- Allow all reagents and samples to equilibrate to room temperature for 30 min before use.
- Protect dye and dye mixtures from light. Remove light covers only when pipetting. The dye decomposes when exposed to light and this reduces the signal intensity.
- Always insert the pipette tip to the bottom of the well when dispensing the liquid. Placing the pipette at the edge of the well may lead to poor results.
- Use a new syringe and electrode cleaners with each new kit.
- Use loaded chips within 5 min after preparation. Reagents might evaporate, leading to poor results.
- Do not touch the Agilent 2100 Bioanalyzer during analysis and never place it on a vibrating surface.



Agilent High Sensitivity DNA Kit Quick Start Guide

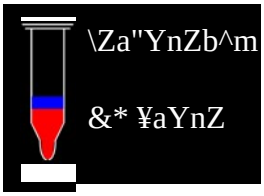
Agilent High Sensitivity DNA Assay Protocol

WARNING **Handling DMSO**

Kit components contain DMSO. Because the dye binds to nucleic acids, it should be treated as a potential mutagen and used with appropriate care.

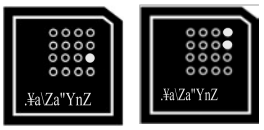
➔ Wear hand and eye protection and follow good laboratory practices when preparing and handling reagents and samples. ➔ Handle solutions with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues.

Preparing the Gel-Dye Mix



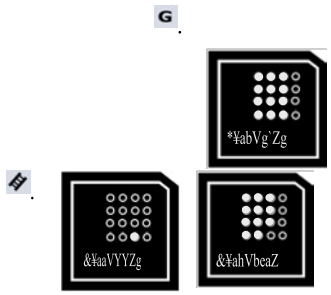
1. Allow High Sensitivity DNA dye concentrate (blue) and High Sensitivity DNA gel matrix (red) to equilibrate to room temperature for 30 min.
2. Add 15 µL of High Sensitivity DNA dye concentrate (blue) to a High Sensitivity DNA gel matrix vial (red).
3. Vortex solution well and spin down. Transfer to spin filter.
4. Centrifuge at 2240 g ± 20 % for 10 min. Protect solution from light. Store at 4 °C. Use prepared gel-dye mix within 6 weeks of preparation.

Loading the Gel-Dye Mix



1. Allow the gel-dye mix to equilibrate to room temperature for 30 min before use.
2. Put a new High Sensitivity DNA chip on the chip priming station.
3. Pipette 9 µL of gel-dye mix in the well marked G.
4. Make sure that the plunger is positioned at 1 mL and then close the chip priming station.
5. Press plunger until it is held by the clip.
6. Wait for exactly 60 s then release clip.
7. Wait for 5 s, then slowly pull back the plunger to the 1 mL position.

8 Open the chip priming station and pipette 9 µL of gel-dye mix in the wells marked



Loading the Marker

1 Pipette 5 µL of marker (green ●) in all sample and ladder wells. Do not leave any wells empty. **Loading the Ladder and Samples**

1 Pipette 1 µL of High Sensitivity DNA ladder (yellow ●) in the well marked

2 In each of the 11 sample wells pipette 1 µL of sample (used wells) or 1 µL of marker (unused wells).

3 Put the chip horizontally in the adapter and vortex for 1 min at the indicated setting (2400 rpm).

4 Run the chip in the Agilent 2100 Bioanalyzer instrument within 5 min.

Technical Support

Please visit our support web page http://www.agilent.com/genomics/contactus to find information on your local Contact Center. **Further Information**

Visit the 2100 Bioanalyzer site at http://www.agilent.com/genomics/bioanalyzer. You can find useful information, support and current developments about the products and the technology.

G2938-90322 Part Number: G2938-90322 Rev. C

G2938-90322 Edition 08/2013 Agilent Technologies

G2938-90322 **Printed in Germany** Hewlett-Packard-Straße 8

76337 Waldbronn, Germany

Attachment 5: Quick reference Ion PGM Hi-Q OT2 kit.

iontorrent QUICK REFERENCE

Ion PGM™ Hi-Q™ OT2 Kit

Catalog Number A27739

Pub. No. MAN0010903 Rev. B.0

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *Ion PGM™ Hi-Q™ OT2 Kit User Guide* (Pub. No. MAN0010902). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

IMPORTANT! If you are using the Ion OneTouch™ Dx System with Torrent Suite™ Assay Development Software[1], see the *Ion PGM™ Hi-Q™ OT2 Kit—Assay Development Mode User Guide* (Pub. No. MAN0015793).

Run the Ion OneTouch™ 2 Instrument

IMPORTANT! Ensure that the latest firmware is installed. To update the firmware to the current version, see the *Ion OneTouch™ 2 System User Guide* (Pub. No. MAN0014388).

---

1. Close the centrifuge lid, then touch **Run** on the home screen.
2. Touch the dropdown menu, then select **PGM: Ion PGM™ Hi-Q™ OT2 Kit - 200**, or **PGM: Ion PGM™ Hi-Q™ OT2 Kit 400**, depending on the read length of your library. Then touch **Next**.
3. Touch **Assisted**, then complete the setup tasks listed onscreen, or touch **Expert**, then set up the instrument according to the following instructions.

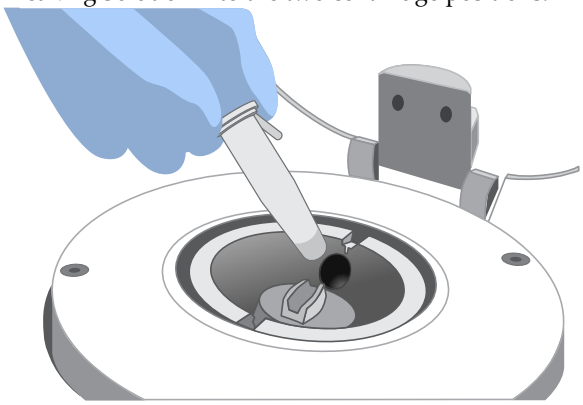
[1] The Ion PGM™ Dx System with Torrent Suite™ Assay Development Software is For Research Use Only. Not for use in diagnostic procedures.

For Research Use Only. Not for use in diagnostic procedures.

Set up the Ion OneTouch™ 2 Instrument

Install the Ion OneTouch™ Recovery Tubes and Ion OneTouch™ Recovery Router

1. Dispense 150 µL Ion OneTouch™ Breaking Solution into each of two Ion OneTouch™ Recovery Tubes.
2. Insert the Recovery Tubes containing Ion OneTouch™ Breaking Solution into the two centrifuge positions.



3. Install the Ion OneTouch™ Recovery Router into the center slot of the centrifuge.
4. Close the centrifuge lid.

Install the Ion OneTouch™ Amplification Plate

Remove the used Cleaning Adapter, insert the plate, and pull the handle to close the heat block. Thread the disposable tubing through the catch and pinch valve.

CAUTION! Hot Surface. Use care when working around this area to avoid being burned by hot components.

WARNING! Safety Hazard. Do not use the instrument with flammable or explosive materials. Use only the materials specified for use with the instrument to ensure safety.

CAUTION! PHYSICAL INJURY HAZARD. The pointed

---

end of the disposable injector can puncture your skin. Keep your hand away from the point of the disposable injector.

ThermoFisher SCIENTIFIC

Install the disposable injector

Insert the disposable injector, then confirm automatic placement of the disposable injector above the router by briefly pressing then releasing the spring-loaded top of the Injector Hub.

You should hear a click.

Install the Reagent Tubes

1. Install the Ion OneTouch™ Oil on the left front port. Invert the Ion OneTouch™ Oil bottle (450-mL size) 3 times, then fill the Reagent Tube half-full with Oil. Install the Reagent Tube. Minimize bubbles.

2. Install the Ion OneTouch™ Recovery Solution on the right front port. Invert the bottle of Recovery Solution 3 times, then fill the Reagent Tube one quarter-full with Recovery Solution. Install the Reagent Tube. Minimize bubbles.

Empty the waste container

Appropriately dispose of waste.

Prepare the amplification solution

1. Prepare the reagents as follows:

Reagents	Preparation
Ion PGM™ Hi-Q™ Reagent Mix	1. Allow the tube of reagent mix to come to room temperature before use. 2. Vortex the solution for 30 seconds, then centrifuge the solution for 2 seconds. 3. Keep the reagent mix at room temperature during use. Store unused thawed reagent mix at 2 ºC to 8 ºC.
Ion PGM™ Hi-Q™ Enzyme Mix	Centrifuge the enzyme for 2 seconds and place on ice.
Ion PGM™ Hi-Q™ ISPs	Place the suspension at room temperature.

2. Depending on your library type and concentration, dilute the library as shown in the following table. Use the library dilution within 48 hours of preparation.

	Ion AmpliSeq™ DNA Library	Ion AmpliSeq™ RNA Library	gDNA Fragment or Amplicon Library	Ion Total RNA-Seq Library
Library concentration	100 pM	100 pM	100 pM	100 pM
Volume of library	2 µL	4 µL	6.5 µL	5 µL
Volume of Nuclease-free Water	23 µL	21 µL	18.5 µL	20 µL
Total volume of diluted library to add to the amplification solution	**25 µL**	**25 µL**	**25 µL**	**25 µL**

   a. Vortex the diluted library for 5 seconds, then centrifuge for 2 seconds.

   b. Place the diluted library on ice.

3. Prepare the Ion PGM™ Hi-Q™ ISPs:

   a. Vortex the ISPs at maximum speed for *1 minute* to resuspend the particles.

   b. Centrifuge the ISPs for 2 seconds.

   c. Pipet the ISPs up and down to mix.

   d. *Immediately* proceed to the next step.

4. To a 2-mL tube (violet cap) containing 800 µL of Ion PGM™ Hi-Q™ Reagent Mix, add the following components in the designated order. Add each component, then pipet the amplification solution up and down to mix:

Order	Reagent	Cap color	Volume
1	Nuclease-free Water	—	25 µL
2	Ion PGM™ Hi-Q™ Enzyme Mix	Brown	50 µL
3	Diluted library (not stock library)	—	25 µL
4	Ion PGM™ Hi-Q™ ISPs	Black	100 µL
—	**Total**	—	**1,000 µL**

IMPORTANT! If you are performing a *de novo* sequencing experiment that does not include a reference BAM file, add 10 µL of the Ion PGM™ Calibration Standard (Cat. No. A27832) to the amplification solution, and reduce the volume of Nuclease-free Water added to 15 µL. When creating your Planned Run in the Torrent Browser, select **Enable Calibration Standard** from the **Base Calibration Mode** dropdown list in the **Kits** tab.

---

5. Vortex the complete amplification solution prepared in step 4 at maximum speed for 5 seconds.

Proceed *immediately* to "Fill and install the Ion OneTouch™ Reaction Filter.

Fill and install the Ion OneTouch™ Reaction Filter

IMPORTANT! We recommend filling the Ion OneTouch™ Reaction Filter in a room dedicated to pre-PCR activities or a controlled prePCR hood. Do not use a reaction filter assembly from any other template preparation kit.

1. Pipet 1000 µL of the amplification solution, prepared in the previous procedure, through the sample port of the Ion OneTouch™ Reaction Filter.

2. Pipet 850 µL of Ion OneTouch™ Reaction Oil (25-mL size) through the sample port.

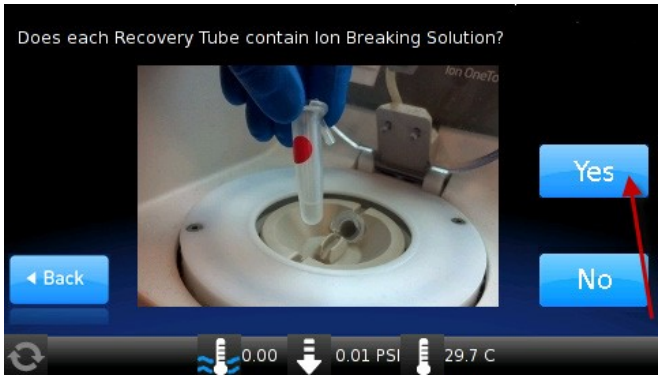
3. Change the tip and pipet an additional 850 µL of Ion OneTouch™ Reaction Oil through the sample port.

4. Invert then install the filled Ion OneTouch™ Reaction Filter into the three holes on the top stage of the Ion OneTouch™ 2 Instrument .

Start the run

1. After installing the Ion OneTouch™ Reaction Filter, touch **Next** to start the run.

IMPORTANT! Remember to add 150 µL of Ion OneTouch™ Breaking Solution to each Recovery Tube before starting the run. On the reminder screen, confirm and touch **Yes** to advance:



Note: To cancel a run, touch **Abort**, then touch **Yes**.

2. Remove the samples £16 hours after starting the run. If you touched **Next** on the centrifuge screen to centrifuge samples at the end of the run, proceed *immediately* to "Recover the template-positive ISPs".

Recover the template-positive ISPs

1. At the end of the run, follow the screen prompts to centrifuge the sample.

IMPORTANT! If you removed the Reaction Tubes at the end of the run *before* the Ion OneTouch™ 2 Instrument had centrifuged the sample or have not processed the sample after 15 minutes, centrifuge the sample on the instrument. On the home screen of the instrument, touch **Open Lid**, touch **Final Spin**, then follow the screen prompts to centrifuge the sample.

CAUTION! ROTATION HAZARD. Wait until rotation stops before opening. Rotating parts can cause injury.

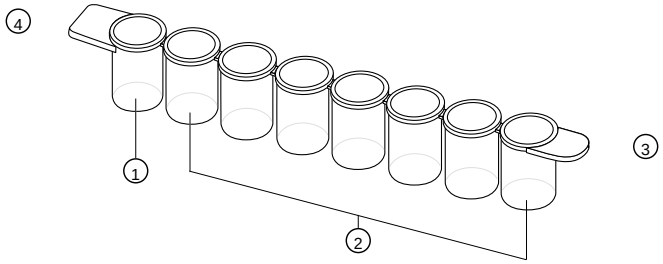
2. Immediately after the centrifuge has stopped, on the instrument display, touch **Open Lid**. Wait until the lid clicks open, then remove and discard the Ion OneTouch™ Recovery Router.

3. Remove both Ion OneTouch™ Recovery Tubes from the instrument, then put the two Recovery Tubes in a tube rack.

4. Remove all but 100 µL of the Recovery Solution from each Recovery Tube.

STOPPING POINT Add 500 µL of Ion OneTouch™ Wash Solution to each Recovery Tube and pipet up and down to disperse the ISPs. Combine the suspension from each Recovery Tube into one new labeled 1.5-mL Eppendorf LoBind™ Tube. Store the ISPs at 2°C to 8°C for up to 3 days. Before enrichment, centrifuge the ISPs at 15,500 × *g* for 2.5 minutes. Carefully remove all but 100 µL of supernatant. With a new tip, pipet up and down to resuspend the ISPs. Proceed to step 6.

5. Process the ISPs:
   a. Add 500 µL of Ion OneTouch™ Wash Solution to each Recovery Tube.
   b. Pipet the ISPs up and down to disperse the ISPs, then combine the suspension from each Recovery Tube into one new labeled 1.5-mL Eppendorf LoBind™ Tube. Note: If a precipitate is present, incubate the tube at 50°C for 2 minutes to dissolve.
   c. Centrifuge the ISPs for 2.5 minutes at 15,500 × *g*.
   d. Use a pipette to remove all but 100 µL of the Wash Solution from the tube. Withdraw the supernatant from the surface and on the opposite side from the pellet.
6. Obtain an 8-well strip from the Ion OneTouch™ ES Supplies Kit. Ensure that the square-shaped tab of an 8-well strip is on the left:



① Well 1
② Wells 2–8
③ Rounded tab
④ Square-shaped tab

7. Pipet the ISPs up and down ten times to mix, then transfer the suspension into Well 1 of the 8-well strip.

8. Retain an aliquot of the unenriched Ion PGM™ Hi-Q™ OT2 Kit from Well 1 for quality assessment. Assess the quality of the unenriched, template-positive ISPs using one of the following instruments:

   Qubit™ 2.0 or Qubit™ 3.0 Fluorometer, Guava™ easyCyte™ 5 Flow Cytometer, or the Attune™ NxT Acoustic Focusing Cytometer.

Enrich the template-positive ISPs with the Ion OneTouch™ ES". You may start the enrichment procedure while the Ion OneTouch™ 2 Instrument cleaning is in progress.

IMPORTANT! Do not store the recovered, template-positive ISPs at –30°C to –10°C. Do not store the ISPs in Ion PGM™ OT2 Recovery Solution (see step 4 of this procedure).

Clean the Ion OneTouch™ 2 Instrument

IMPORTANT! To ensure continued safe operation, visually inspect the rotor assembly and casing periodically to ensure that there are no signs of cracks or other physical damage. Follow the cleaning procedure in this section to clean the Ion OneTouch™ 2 Instrument with the Ion OneTouch™ Cleaning Adapter. *Perform the cleaning procedure after every run.*

---

1. Determine the appropriate reagents to use for maintaining the Ion OneTouch™ 2 Instrument:

If you are	
Switching to the Ion PGM™ Hi-Q™ OT2 Kit from another kit?	
Already using the Ion PGM™ Hi-Q™ OT2 Kit.	

2. Check the level of Ion OneTouch™ Oil in the Reagent Tube. If the Reagent Tube has <20 mL of Oil, pour Oil into the Reagent Tube until it is half-full.
3. Remove and appropriately discard the used Ion OneTouch™ Reaction Filter. Remove the assembly from the instrument by grasping the *filter*.

4. Firmly insert the 3 ports of a new *single-use* Cleaning Adapter into the three holes on the top stage of the Ion OneTouch™ 2 Instrument.

5. Place a 50-mL conical tube in a tube rack, then place the tube rack next to the instrument.

Note: Steps 6–9 are only necessary if you have not already removed the disposable injector before removing the Recovery Tubes from the instrument.

6. Gently pull the disposable tubing downwards on both sides of the pinch valve until the disposable tubing is out of the valve.

7. Remove the disposable injector from the Ion OneTouch™ DL Injector Hub.

CAUTION! PHYSICAL INJURY HAZARD. The pointed end of the disposable injector can puncture your skin. Keep your hand away from the point of the disposable injector.

---

8. Place the used, disposable injector into the empty 50-mL conical tube in the tube rack. The conical tube is used to collect waste.

9. On the home screen of the instrument, touch **Clean**.

10. Complete each task that is displayed on the screen, then touch **Next**. After you touch **Next** on the last task, a progress bar appears, and the cleaning starts.

11. At the end of the cleaning run, the screen displays "**Time Remaining 00:00:00, Cleaning Run Complete**". Press **Next**, then ensure that the task in bold displays: "**Remove plate, injector, conical tube, and waste**".

Note: Keep the used Cleaning Adapter on the instrument between runs.

12. Appropriately discard the waste that was collected in the 50-mL conical tube.

13. Remove and appropriately discard the used Amplification Plate, disposable injector, and tubing.

CAUTION! Hot Surface. Use care when working near this area to avoid injury from contact with hot components.

---

14. On the instrument display, touch **Open Lid**, wait until the lid clicks open, then open the centrifuge lid. Wipe the residue from the centrifuge lid with dry Kimwipes™ disposable wipers, then close the centrifuge lid.

15. Touch **Next** to return to the home screen on the instrument.

Enrich the template-positive Ion PGM™ Hi Q™ ISPs

-

Determine if a residual volume test is necessary

Condition	Action

First use of the instrument and during monthly maintenance	Perform a residual volume test (see "Ion OneTouch™ ES Instrument installation, setup, and maintenance" in the Ion OneTouch™ 2 System User Guide Pub. No. MAN0014388).
Routine use and residual volume in Well 1 and Well 8 is >5.0 µL	
Routine use and residual volume in Well 1 and Well 8 is ≤5.0 µL	Operate the instrument without performing the residual volume test. Proceed to "Prepare reagents then fill the 8-well strip".

Prepare reagents then fill the 8-well strip

Prepare Melt-Off Solution

Prepare fresh Melt-Off Solution by combining the components in the following order:

Order	
1	Tween™ Solution
2	1 M NaOH
—	

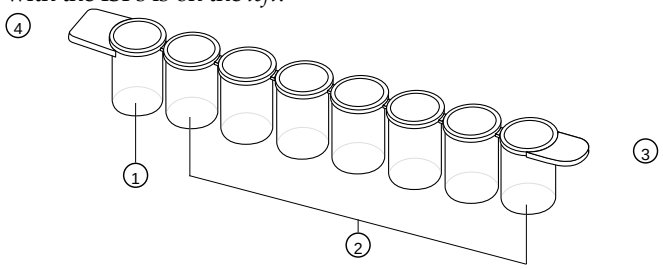
IMPORTANT! Prepare Melt-Off Solution as needed, but appropriately dispose of the solution after 1 day.

Wash and resuspend the Dynabeads™ MyOne™ Streptavidin C1 Beads

1. Vortex the tube of Dynabeads™ MyOne™ Streptavidin C1 Beads for 30 seconds to resuspend the beads thoroughly, then centrifuge the tube for 2 seconds.
2. Open the tube, then use a new tip to pipet the dark pellet of beads up and down until the pellet disperses. Immediately proceed to the next step.
3. Transfer 13 µL of Dynabeads™ MyOne™ Streptavidin C1 Beads to a new 1.5-mL Eppendorf LoBind™ Tube.
4. Place the tube on a magnet such as a DynaMag™-2 magnet for 2 minutes, then carefully remove and discard the supernatant without disturbing the pellet of Dynabeads™ MyOne™ Streptavidin C1 Beads.
5. Add 130 µL of MyOne™ Beads Wash Solution to the Dynabeads™ MyOne™ Streptavidin C1 Beads.
6. Remove the tube from the magnet, vortex the tube for 30 seconds, then centrifuge for 2 seconds.

Fill the 8-well strip

1. Ensure that the template-positive ISPs from the Ion OneTouch™ 2 Instrument are in 100 µL of Ion OneTouch™ Wash Solution and are loaded in Well 1 of the 8-well strip (see “Recover the template-positive ISPs“ on page 3). Well 1 with the ISPs is on the *left*:



① Well 1
② Wells 2–8
③ Rounded tab
④ Square-shaped tab

Note: If the template-positive ISPs were stored at 2°C to 8°C, centrifuge the ISPs at 15,500 × *g* for 2.5 minutes, then carefully remove all but 100 µL of supernatant. With a new tip, pipet up and down to resuspend the ISPs. Transfer the suspension from the tube into Well 1 of the 8-well strip.

2. If you have not done so already, assess the quality of the unenriched, template-positive ISPs using the Qubit™ 2.0 or Qubit™ 3.0 Fluorometer, the Guava™ easyCyte™ 5 Flow Cytometer, or the Attune™ NxT Acoustic Focusing Cytometer.

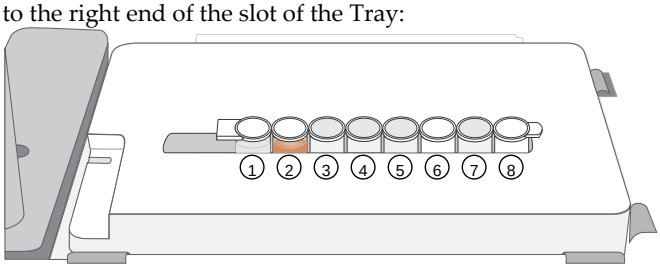
   Note: See the *Ion Sphere™ Assay on the Qubit™ 2.0 Fluorometer User Guide* (Pub. No. MAN0016387), or the *Ion Sphere™ Assay on the Qubit™ 3.0 Fluorometer User Guide* (Pub. No. MAN0016388) for detailed procedures for using the Ion Sphere™ Assay for determining the percentage of templatepositive ISPs in unenriched samples.

3. Fill the remaining wells in the 8-well strip as follows (see the figure in step 4):

Well number	Reagent to dispense in well
Well 1[1]	Entire template-positive ISP sample [100 µL; prepared in step 1 of this procedure]
Well 2	130 µL of Dynabeads™ MyOne™ Streptavidin C1 Beads resuspended in MyOne™ Beads Wash Solution] prepared in “Wash and resuspend the Dynabeads™ MyOne™ Streptavidin C1 Beads“ on page 5]
Well 3	300 µL of Ion OneTouch™ Wash Solution
Well 4	300 µL of Ion OneTouch™ Wash Solution
Well 5	300 µL of Ion OneTouch™ Wash Solution
Well 6	Empty
Well 7	300 µL of freshly-prepared Melt-Off Solution [prepared in “Prepare Melt-Off Solution“ on page 5]
Well 8	Empty

[1] Well closest to the square-shaped tab

4. Confirm that the square-shaped tab is on the left, then insert the filled 8-well strip with the 8-well strip pushed all the way to the right end of the slot of the Tray:



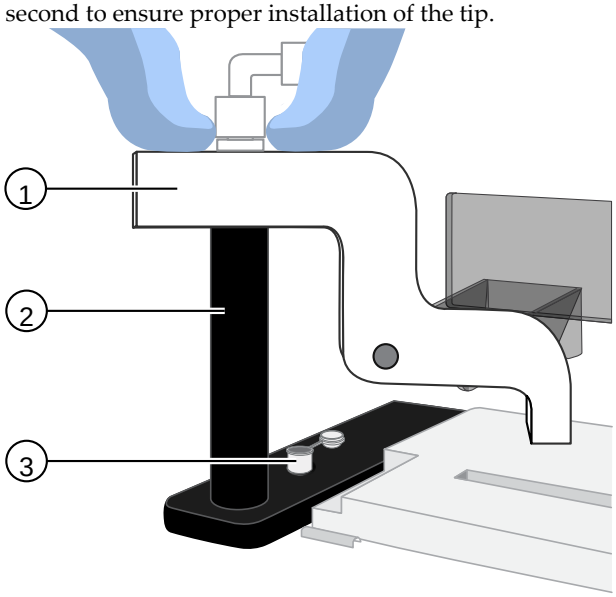
Prepare the Ion OneTouch™ ES

Before every enrichment performed on the Ion OneTouch™ ES Instrument, install a new PCR collection tube and a new Eppendorf™ LoRetention Dualfilter P300 pipette tip. 1. Add 10 µL of Neutralization Solution to a new 0.2-mL PCR tube.

2. Insert the open 0.2-mL PCR tube containing Neutralization Solution into the hole in the base of the Tip Loader, as shown in the figure in step 4.

3. Place a new tip in the Tip Loader. Remove the Tip Arm from the cradle, then align the metal fitting of the Tip Arm with the tip.

4. Keeping the fitting on the Tip Arm vertical, firmly press the Tip Arm down onto the new tip until the Tip Arm meets the Tip Loader. Hold the Tip Arm to the Tip Loader for ~1 second to ensure proper installation of the tip.



① Tip Arm
② Tip Loader
③ 0.2-mL PCR collection tube containing Neutralization Solution

5. Lift the Tip Arm *straight* up to pull the installed tip from the Tip Loader tube.
6. Return the Tip Arm to the cradle.

IMPORTANT! Ensure that the back/bottom end of the Tip Arm is not resting on top of the thumb screw, causing the Tip Arm to tilt forward.

Perform the run

Before starting the run:

- Confirm that a new tip and open 0.2-mL PCR tube have been loaded in the Ion™ OneTouch ES Instrument, and that the 8-well strip is correctly loaded.
- Ensure that Well 1 (ISP sample) is the left-most well and the8-well strip is pushed to the right-most position in the slot. 1. Pipet the contents of Well 2 up and down to resuspend the beads before starting the run. Do not introduce bubbles into the solution.

2. If needed, power on the Ion OneTouch™ ES Instrument, then wait for the instrument to initialize. The screen displays "rdy". The Tip Arm performs a series of initialization movements and returns to the home position (~5 seconds).

3. Press **Start/Stop**. The screen displays "run" during the run. The run takes ~35 minutes.

4. At the end of the run, the instrument displays "End" and beeps every 60 seconds. Press the **Start/Stop** button to silence this alarm, then reset the Ion OneTouch™ ES Instrument for the next run. The instrument can be left on between runs. 5. Immediately after the run, securely close, then remove the PCR tube containing the enriched ISPs.

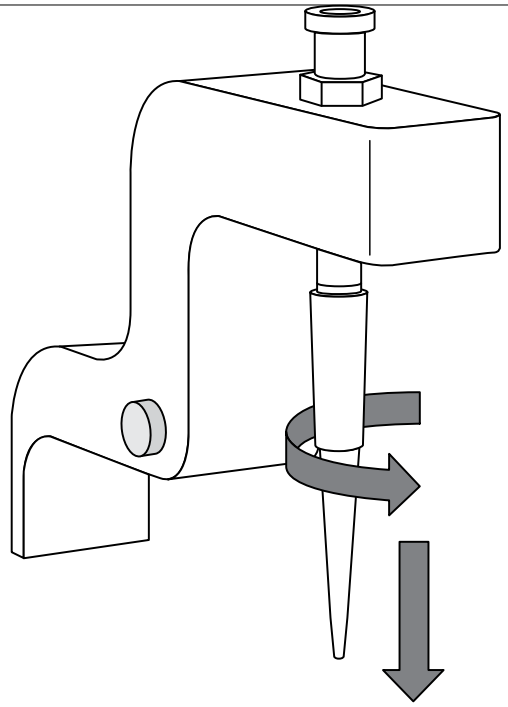
6. Mix the contents of the PCR tube by gently inverting the tube 5 times.

Note: Ensure that the 0.2-mL PCR tube has >200 µL of solution containing the enriched ISPs. After a successful run on the instrument, the sample is in ~230 µL of Melt-Off Solution, Ion OneTouch™ Wash Solution, and Neutralization Solution. If the tube has <<200 µL of solution containing the enriched ISPs, contact Technical Support.

7. Remove the used tip: with the Tip Arm in its cradle, twist the tip counterclockwise (as viewed from above), then pull it downward to remove and discard the tip.

8. Remove, then discard the used 8-well strip.

Sequence or store the template-positive ISPs



IMPORTANT! Improper removal of tips can loosen the metal tip adapter fitting on the Tip Arm and affect instrument operation.

- Proceed to sequencing using the Ion PGM™ Hi-Q™ Sequencing Kit (Cat. No. A25592). See the *Ion PGM™ Hi-Q™ Sequencing Kit User Guide* (Pub. No. MAN0009816, Rev. C.0 or later).

  or

- Store the enriched ISPs at 2°C to 8°C for up to 3 days.

The information in this guide is subject to change without notice.

Perform quality control on enriched ISPs

You can determine the enrichment efficiency using one of the following methods:

Quality assessment by	Action
Guava™ easyCyte™ 5 Flow Cytometer	Transfer a 1.0-µL aliquot of the enriched ISPs to a 1.5-mL Eppendorf LoBind™ Tube. Refer to the Ion Sphere™ Particles (ISPs) Quality Assessment Using the Guava™ easyCyte™ 5 Flow Cytometer User Bulletin (Pub. No. MAN0015799), available at: http://tools.thermofisher.com/content/sfs/manuals/MAN0015799.pdf
Attune™ NxT Acoustic Focusing Cytometer	Transfer a 1.0-µL aliquot of the enriched ISPs to a 1.5-mL Eppendorf LoBind™ Tube. Put the sample on ice, then refer to Demonstrated Protocol: Ion Sphere™ Particles (ISPs) Quality Assessment using the Applied Biosystems™ Attune™ Acoustic Focusing Cytometer User Bulletin (Pub. No. 4477181), available at: http://tools.thermofisher.com/content/sfs/manuals/4477181A.pdf

Revision history: MAN0010903

Revision	Date	Description
B.0	11 January 2017	• Illustrations enhanced • Ion OneTouch™ ES Instrument setup clarified • Web links updated • Minor clarifications and corrections made
A.0	10 March 2015	New Quick Reference

Corporate entity: Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288

Attachment 6: Quick reference Ion PGM Hi-Q Sequencing kit.

ion torrent QUICK REFERENCE

Ion PGM™ Hi-Q™ Sequencing Kit

Catalog Number A25592

Pub. No. MAN0010863 Rev. D.0

Note: For safety and biohazard guidelines, see the "Safety" and ™ ™ appendix in the *Ion PGM Hi-Q Sequencing Kit User Guide* (Pub. No. MAN0009816). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Create a Planned Run

Create a Planned Run in the following software programs for use on the following sequencing systems. In the software, be sure to specify the appropriate library, template, and sequencing kits.

Software	Instrument System
Torrent Suite™ Software, 5.0 or later	Ion PGM™ System
Torrent Suite™ Assay Development Software[1], 5.0 or later	Ion PGM™ Dx System

[1] Torrent Suite™ Assay Development Software is For Research Use Only on the Ion PGM™ Dx System. Not for use in diagnostic procedures.

For more information, see the software user documentation, or see:

- *Ion PGM™ Hi-Q™ OT2 Kit—Assay Development Mode User Guide* (Pub. No. MAN0015793), *and*
- *Ion PGM™ Hi-Q™ Sequencing Kit—Assay Development Mode User Guide* (Pub. No. MAN0015794)

Create a Planned Run

1. Open the Torrent Browser on a computer connected to your sequencing system.
2. Select the **Plan** tab, then select **Templates**.
3. Select the application in the left navigation bar (for example, **AmpliSeq DNA**). A list of existing Planned Run templates for that application will be displayed. Select one of the following options to create a new plan:
   - To create a new Planned Run without using an existingtemplate, click on **Plan New Run**.
   - To create a new Planned Run from an existing template,click the ⚙ button for the template and select **Plan Run** from the drop-down menu.
   - Other options may be available depending on the selected application, such as downloading templates from AmpliSeq.com.
4. In the wizard, make your selections on each screen, then click **Next** to proceed to the next screen.
5. When you have completed your selections, click **Plan Run**.

Clean the Ion PGM™ System

Condition the Wash 2 Bottle for first use

New Wash 2 Bottles must be conditioned with Wash 2 Bottle Conditioning Solution for at least 8 hours before first use.

To condition the Wash 2 Bottle:

1. Fill the bottle to the mold line with 18 MΩ water, add the entire container of Wash 2 Bottle Conditioning Solution, then cap the bottle and invert it 5 times to mix.
2. Allow the bottle to sit at room temperature for at least 8 hours and preferably overnight, then dispose of the contents. The bottle is now ready for use.

18 MΩ water cleaning

1. Empty any remaining solution from each cleaning bottle (two 250-mL bottles and one 2-L bottle) and rinse each bottle twice with ~100 mL of 18 MΩ water.
2. Press **Clean** on the touchscreen, and select the **18-MOhm water cleaning** checkbox. Press **Next**.

3. Using ungloved hands, secure a used chip designated for cleaning in the chip clamp.

IMPORTANT! Always make sure that both red rubber gasket port fittings are securely in place when securing chips with the chip clamp. Failure to do so can result in a spill hazard and instrument damage.

---

4. Remove all wash and reagent bottles attached to the instrument. Keep the sippers in place at all positions. Press **Next**.

5. Add 250 mL of 18 MΩ water to an empty 250-mL cleaning bottle.

6. Rinse the outside of the sipper tube in the W1 position on the instrument with a squirt bottle containing 18 MΩ water.

7. Attach the 250-mL bottle containing 18 MΩ water to the W1 position, ensuring that the W1 cap is screwed on tightly. Press **Next**.

8. Place the empty 2-L cleaning bottle in the W2 position and the empty 250-mL bottle in the W3 position, and insert the sippers into the bottles. Do not screw on the caps. 9.

   Place collection trays below the reagent sippers in the dNTP positions. Press **Next** to begin cleaning.

10. When cleaning is complete, remove the bottles and sippers from the W1, W2 and W3 positions. Leave the reagent sippers and collection trays in place. Press **Next** to return to the main menu and proceed to initialization.

Chlorite cleaning

1. Empty any remaining solution from each cleaning bottle (two 250-mL bottles and one 2-L bottle), then rinse each bottle twice with ~100 mL of 18 MΩ water.

2. Fill a glass bottle with 1 L of 18 MΩ water, then add an Ion Cleaning tablet (chlorite tablet). Allow the tablet to dissolve completely (~10 minutes).

3. When the tablet has dissolved, add 1 mL of 1 M NaOH and filter the solution using a 0.22-µm or 0.45-µm filter. Use the chlorite solution within 2–3 hours. Discard any unused solution after this time.

4. Press **Clean** on the touchscreen, then select the **Chlorite cleaning** checkbox. Press **Next**.

5. Using ungloved hands, secure a used chip designated for cleaning in the chip clamp.

IMPORTANT! Always ensure that both red rubber gasket port fittings are securely in place when securing chips with the chip clamp. Failure to do so can result in a spill hazard and instrument damage.

---

6. Remove all wash and reagent bottles that are attached to the instrument. Keep the sippers in place at all positions. Press **Next**.

7. Add 250 mL of the filtered chlorite solution to an empty 250-mL cleaning bottle.

8. Rinse the outside of the sipper tube in the W1 position on the instrument with a squirt bottle containing 18 MΩ water.

9. Attach the 250-mL bottle with the filtered chlorite solution to the W1 position. Ensure that the W1 cap is tight. Press **Next**.

10. Place the empty 2-L cleaning bottle in the W2 position and the empty 250-mL bottle in the W3 position, then insert the sippers into the bottles. Do not screw on the caps. 11.

    Place collection trays below the reagent sippers in the dNTP positions. Press **Next** to start cleaning.

12. When prompted, remove the bottle containing the chlorite solution from the W1 position.

13. Rinse the outside of the W1 sipper tube with a squirt bottle containing 18 MΩ water.

14. Fill a clean 250-mL bottle with 250 mL of 18 MΩ water, then attach the bottle in the W1 position. Ensure the cap is tight. Press **Next** to start the water rinse.

15. When cleaning is complete, remove the bottles and sippers from the W1, W2 and W3 positions. Leave the reagent sippers and collection trays in place. Press **Next** to return to the main menu, then proceed to initialization.

Initialize the Ion PGM™ System

Before initialization

1. Remove the dNTP stock solutions from the freezer and begin thawing on ice.

2. Check the tank pressure for the nitrogen gas. When the tank pressure drops below 500 psi, change the tank.

Prepare the Wash 2 Bottle

IMPORTANT! Do not let the new sippers touch any surfaces.

---

1. Rinse the Wash 2 Bottle (2 L) 3 times with 200 mL of 18 MΩ water.

2. Prepare 500 µL of 100 mM NaOH by diluting 50 µL of 1 M NaOH in 450 µL of nuclease-free water.

3. If your 18 MΩ water system has a spigot, extend it into **but not below** the neck of the Wash 2 Bottle. Otherwise, position the nozzle as close to the mouth of the bottle as possible.

4. Fill the bottle to the mold line with 18 MΩ water. The volume of water is ~2 liters. (You can mark the mold line on the bottle for clarity.)

5. Add the entire bottle of Ion PGM™ Hi-Q™ Sequencing W2 Solution to the Wash 2 Bottle.

6. Using a P200 pipette, add 70 µL of 100 mM NaOH to the Wash 2 Bottle.

7. Cap the bottle and invert 5 times to mix, and immediately proceed through the remainder of the initialization procedure.

IMPORTANT! Do not store the mixed Wash 2 Bottle.

Prepare the Wash 1 and Wash 3 Bottles

1. Rinse the Wash 1 and Wash 3 Bottles 3 times with 50 mL of 18 MΩ water.

2. **Wash 1 Bottle:** Add 350 µL of freshly prepared 100 mM NaOH to the Wash 1 Bottle, then cap the bottle.

3. **Wash 3 Bottle:** Add Ion PGM™ Hi-Q™ Sequencing W3 Solution to the 50-mL line marked on the Wash 3 Bottle, then cap the bottle.

Begin the initialization

IMPORTANT! Do not let the new sipper tubes touch any surfaces.

1. On the main menu, press **Initialize**.

2. Make the following selections in the next screen, then press **Next**:

   - Click **Enter barcode** to scan or enter the barcode on the Ion PGM™ Hi-Q™ Sequencing W2 Solution bottle, or the 2D barcode on the Ion PGM™ Hi-Q™ Sequencing Solutions box.
   - Alternatively, select the checkbox for the **Ion PGM™ Hi-Q™ Sequencing Kit** from the dropdown list.
   - In the same screen, if you routinely experience clogging during initialization, select the **Line Clear** checkbox to clear any blockage in the fluid lines before initialization. This is optional.

   After you press **Next**, the system will check the gas pressure.

3. Following the gas pressure check:

Result	Action
If the pressure is sufficient	Ensure that the cleaning chip, reagent sipper tubes, and collection trays are in place, and press Next to start the initialization.
If the pressure is low	Press Yes to re-check the pressure. If the pressure remains low, contact Technical Support.

4. Wearing clean gloves, firmly attach a new, long gray sipper to the cap in the W2 position.

5. Immediately attach the prepared Wash 2 Bottle in the W2 position, then tighten the cap. Press **Next**.

6. Change gloves and firmly install new sipper tubes (short gray) in the caps in the W1 and W3 positions.

7. Immediately attach the prepared Wash 1 and 3 Bottles, then tighten the caps. Press **Next**.

8. Following line clear, or if you did not select that option, the sequencer begins adjusting the pH of the W2 Solution, which takes ~30 minutes. After 15 minutes, check the instrument touchscreen to confirm that initialization is proceeding normally.

Prepare the 50-mL Reagent Bottles with dNTPs

1. Use the labels provided with the kit to label four new Reagent Bottles as dGTP, dCTP, dATP, and dTTP.

2. Confirm that no ice crystals are visible in each thawed dNTP stock solution. Vortex each tube to mix, and centrifuge to collect the contents. Keep the dNTP stock solutions on ice throughout this procedure.

IMPORTANT! To avoid cross-contamination in the next step, open only one dNTP stock tube at a time and use a fresh pipette tip for each aliquot.

3. Using separate filtered pipette tips and clean gloves, carefully transfer 20 µL of each dNTP stock solution into its respective Reagent Bottle.

4. Cap each Reagent Bottle and store on ice until you are ready to attach it to the instrument. Place the remaining dNTP stocks back into –20°C for storage.

Attach the sipper tubes and Reagent Bottles 1. After the wash solutions have initialized, follow the touchscreen prompts to remove the used sipper tubes and collection trays from the dNTP ports.

2. Change gloves, then firmly insert a new sipper tube (blue) into each dNTP port. Do not let the sipper touch any surfaces.

3. Attach each prepared Reagent Bottle to the correct dNTP port (e.g., the dGTP tube on the port marked "G") and tighten firmly by hand until snug. Press **Next**. 4. Follow the touchscreen prompts to complete initialization. The instrument will fill each Reagent Bottle with 40 mL of W2 Solution.

5. At the end of initialization, Ion PGM™ System will measure the pH of the reagents:
   - If every reagent is in the target pH range, a green **Passed** screen will be displayed.
   - If a red failure screen appears, see the troubleshooting section of the user guide.

6. Press **Next** to finish the initialization process and return to the main menu.

7. Proceed to the appropriate sequencing protocol for your chip type.

Load the chip and start the sequencing run

Use the following chip loading and sequencing protocol for all Ion PGM™ chip types.

2. Place the tube in a microcentrifuge with an appropriate tube adapter. Orient the tab of the tube lid so that it is pointing away from the center of the centrifuge, to indicate where the pellet will be formed.

3. Centrifuge for 2 minutes at 15,500 × *g*.

4. Keeping the pipette plunger depressed, insert a pipette tip into the tube containing the pelleted ISPs and carefully remove the supernatant from the top down, avoiding the side of the tube with the pellet (i.e., the side with the tab on the tube lid). Discard the supernatant. Leave ~15 µL in the tube (visually compare to 15 µL of liquid in a separate tube).

5. Ensure that the Sequencing Primer is completely thawed prior to use (no ice crystals should be visible).

6. Vortex the primer for 5 seconds, then pulse spin in a picofuge for 3–5 seconds to collect the contents. Leave on ice until ready to use.

7. Add 12 µL of Sequencing Primer to the ISPs, and confirm that the total volume is 27 µL (add Annealing Buffer if necessary).

Before starting • Thaw the Sequencing Primer on ice.

Optional: Prepare Ion Sphere™ Test Fragments

If you are performing an installation or troubleshooting sequencing run:

1. Vortex the Ion PGM™ Ion Sphere™ Test Fragments from the Ion PGM™ Controls Kit v2 (Cat. No. 4482010), then pulsecentrifuge in a microcentrifuge for 2 seconds before taking aliquots.

2. Add 5 µL of Ion PGM™ Ion Sphere™ Test Fragments to 100 µL of Annealing Buffer in a 0.2-mL non-polystyrene PCR tube.

Skip directly to "Anneal the Sequencing Primer". Add

controls to the enriched, template-positive ISPs

1. Vortex the Control Ion Sphere™ Particles, then pulsecentrifuge in a picofuge for 2 seconds before taking aliquots. 2. Add 5 µL of Control ISPs directly to the entire volume of enriched, template-positive ISPs (prepared using your template preparation method) in a 0.2-mL non-polystyrene PCR tube.

Proceed to "Anneal the Sequencing Primer".

Anneal the Sequencing Primer

1. Mix the tube containing the ISPs (or test fragments) by thoroughly pipetting up and down.

8. Pipet the mixture up and down thoroughly to disrupt the pellet.

9. Program a thermal cycler for 95°C for 2 minutes and then 37°C for 2 minutes, using the heated lid option. 10. Place the tube in the thermal cycler and run the program. After cycling, the reaction can remain in the cycler at room temperature (20–30°C) while you proceed with Chip Check.

Perform Chip Check

1. On the main menu of the Ion PGM™ Sequencer touchscreen, press **Run**. Remove the waste bottle and completely empty it. Press **Next**.

2. When prompted to insert a cleaning chip, use the same used chip that was used for initialization. Press **Next** to clean the fluid lines.

3. When prompted, select the instrument that you used to prepare the template-positive ISPs. Then press **Next**. 4. Remove gloves, then ground yourself by touching the grounding pad on the sequencer. Remove a new chip from its packaging, then label it to identify the experiment (save the chip package). Press **Next**.

5. When prompted, use the scanner to scan the barcode located on the new chip, or press **Change** to enter the barcode manually. Optionally, you can also enter the library kit catalog number. 6. Replace the old chip in the chip socket with the new one. Close the chip clamp, then press **Next**.

7. Press **Chip Check**. During the initial part of Chip Check, visually inspect the chip in the clamp for leaks.

8. Following a successful Chip Check, empty the waste bottle, then select the **Waste bottle is empty** checkbox on the touchscreen. Press **Next**.

Bind the Sequencing Polymerase to the ISPs

1. Remove the Ion PGM™ Hi-Q™ Sequencing Polymerase from storage and flick mix with your finger tip 4 times. Pulse-centrifuge for 3–5 seconds. Place on ice.

2. After annealing the Sequencing Primer, remove the ISPs from the thermal cycler, then add 3 µL of Ion PGM™ Hi-Q™ Sequencing Polymerase to the ISPs, for a total final volume of 30 µL.

3. Pipet the sample up and down to mix, then incubate at room temperature for 5 minutes.

Prepare and load the chip

Remove liquid from the chip

1. Following chip calibration, remove the new chip from the Ion PGM™ Sequencer. Insert a used chip in the chip clamp while loading the new chip.

2. Tilt the new chip at a 45° angle so that the loading port is the lower port.

3. Insert the pipette tip firmly into the loading port, then remove as much liquid as possible from the loading port. Discard the liquid.

4. Place the chip **upside-down** in the minifuge bucket, then transfer the bucket **with the chip tab pointing in** (toward the center of the minifuge). Balance the bucket with another chip.

5. Centrifuge for 5 seconds to empty the chip completely.

6. Remove the chip from the bucket, then wipe the bucket with a disposable wipe to remove any liquid. Place the chip rightside up in the bucket.

Load the chip

1. Place the chip in the bucket on a firm, flat surface. Following polymerase incubation, load the chip with following volume of prepared ISPs using the listed pipettes, or equivalent, depending on your chip type. We recommend using a P20 pipette for Ion 314™ Chips for optimal loading.

Chip	Volume to load	Recommended pipette[1]
Ion 316™ or Ion 318™ Chip	Entire volume (~30 µL)	Rainin™ Pipet-Lite™ LTS L-100XLS, 10–100 µL
Ion 314™ Chip	10 µL	Rainin™ Pipet-Lite™ LTS L-20XLS, 2–20 µL

[1] Alternatives from Gilson and Eppendorf can be used.

2. Insert the tip firmly into the loading port of the chip.

3. With the pipette unlocked, apply gentle pressure between the tip and chip and slowly dial down the pipette (~1 µL per second) to deposit the ISPs. To avoid introducing bubbles into the chip, leave a small amount in the pipette tip (~0.5 µL).

4. Remove, then discard any displaced liquid from the other port of the chip.

5. Transfer the chip in the bucket to the minifuge with the chip tab **pointing in** (toward the center of the minifuge), then centrifuge for 30 seconds.

6. Turn the chip so that the chip tab is **pointing out** (away from the center of the minifuge), then centrifuge for 30 seconds.

7. Remove the bucket from the minifuge, then place it on a flat surface. Set the volume of the pipettor as follows,

depending on your chip type:

- **Ion 316™ or Ion 318™ Chip**: 25 µL
- **Ion 314™ Chip**: 5 µL

8. Tilt the chip at a 45° angle so that the loading port is the lower port, then insert the pipette tip into the loading port.

9. Without removing the tip, slowly pipet the sample out and then back into the chip one time. **Pipet slowly to avoid creating bubbles.**

10. Slowly remove as much liquid as possible from the chip by dialing the pipette. Discard the liquid.

11. Turn the chip upside-down in the bucket, transfer it back to the minifuge, then centrifuge upside-down for 5 seconds. Remove and discard any liquid.

12. If some liquid remains in the chip, lightly and rapidly tap the point of the chip tab against the benchtop a few times, then remove and discard any collected liquid. Do not flush the chip.

13. When chip loading is complete, press **Next** on the touchscreen, then proceed immediately to performing the run.

Select the Planned Run and perform the run

Select the Planned Run

1. Press **Browse** next to the **Planned Run** field and select the name of the plan you created, then touch **Next.**

   Note: The Ion PGM™ Sequencer automatically populates this field for barcoded Ion chips.

2. Confirm that the settings are correct. If necessary, make any changes using the touchscreen controls.

Perform the run

1. After you enter the Planned Run, press **Next** to verify the experimental setup. Press **OK** to confirm the settings or press **Cancel** to return to the touchscreen to adjust the settings.
2. When prompted by the instrument, load and clamp the chip, then press **Next**.

3. At the beginning of the run, visually inspect the chip in the clamp for leaks before closing the cover. The instrument will flush any loose ISPs from the chip and begin calibrating the chip.

4. When the calibration is complete (~1 minute), the touchscreen will indicate whether calibration was successful.
5. After 60 seconds, the run will automatically begin, or press **Next** to begin the run immediately.

6. When the run is complete, leave the chip in place, then touch **Next** to return to the Main Menu. You can then remove the chip and proceed with another run or perform a cleaning/initializing if required.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-andconditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

 Revision history: Pub. No. MAN0010863.

Revision	Date	Description of change
D.0	8 January 2017	Updated web links and references
C.0	8 January 2016	Added information and procedures for using Torrent Suite™ Assay Development Software
B.0	13 March 2015	Unified sequencing protocol for all chips; added Planned Run sharing appendix
A.0	5 September 2014	New quick reference

Corporate entity: Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288

Attachment 7: Cultivation procedure SiV.

Sykehuset i Vestfold	*Klinikknivå - Nivå 2*	*k.nr:*
	Plassering: 2.3.4.6.2.1.5.2 *Do*	D07818
	Urin. Manuell utsed.	
Gjelder for: **Klinikk Medisinsk Diagnostikk\Mikrobiologisk avdeling**		
Hovedområde: Analyse prosesser	*Utarbeidet / revidert av*: Fagbioingeniør Kristin Jørgensen	*Revisjonsnr*: 5.00
Dokumenttype: **Prosedyre**	*Godkjent av*: Avdelingssjef Bodil Ruud / Avdelingsoverlege Dagfinn Skaare	*Godkj. dato*: 13.02.2017

Papirversjon av dokumentet finnes i prosedyreperm Utsedsplass og i tarifold.

1. HENSIKT

Sikre riktig behandling av urinprøver ved manuell utsed.

2. ANSVAR

Bioingeniør på bakteriologisk enhet.

3. FREMGANGSMÅTE Utsed

Ved manuell utsed hvor det også er bedt om TB, så prøven ut i avtrekk.

Urinprøven blandes godt ved å snu glasset et par ganger. Unngå å riste slik at det blir luftbobler.

1 µl urin sås på 1 blodskål
1 µl urin sås på 1 MAC skål
1 µl urin sås på 1 CNA skål

Når cystektomert er nevnt i kliniske opplysninger skal det alltid være utvidet dyrkning selv om det ikke er bedt om.

Tillegg ved utvidet urindyrkning

1µl urin sås på SAB
Evt. 1 µl urin sås på menadion og 1µl urin sås på blod anaerob.

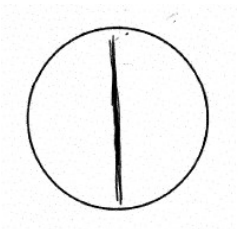
Urin fra blærepunksjon sås ut med 1µl og 100µl (tilsammen 6 skåler).

Urin fra cystoskopi sås ut med 1 µl og 10 µl (tilsammen 6 skåler).

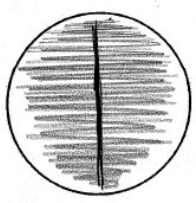
Skålene til urinprøver fra barneavd. merkes med henholdsvis B (SIV) og BT (STHF).

Utsedsteknikk:

Dypp øsa ned i urinprøven rett under væskeoverflaten. Dypp øsa der det ikke er luftbobler. Sett av væsken først på blodagarflaten, Spre godt. Se figurer.



Figur 1.



Figur 2.

Dypp øsen på nytt og sett av på MAC skål. Spre godt.

Dypp øsen på nytt og sett av på CNA skål. Spre godt.

Ved utvidet urindyrkning
Hvis det er rekvirert anaerob dyrkning spre på de anaerobe skålene før sab skålen.

Spre vist på figur.
Husk å legge på gentamicin og metronidazole lapper på menadion skålen.

Inkubering

Skålene skal inkuberes i 35□C ± 2ºC i 1 døgn.

Ved utvidet dyrkning skal skålene inkuberes i CO_2 i 2 døgn. Ses på etter 1 døgn.
Anaerob skålene inkuberes i anaerobskapet sammen med blodkulturene i 2 døgn.

Oppbevaring av ferdig sådde urinprøver:

Urinen oppbevares på kjølerom til neste dag. Hvis nødvendig kan ny utsed gjentas hvis urinen er innenfor holdbarhetsgrensen.

4. GENERELT

5. INTERNE REFERANSER

2.3.4.6.2.1.5.7 Identifikasjon av Gram negative urinveispatogener, Flytdiagram

6. EKSTERNE REFERANSER

7. VEDLEGG