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Extraction of DNA from difficult samples with an automatable portable system for early detection of intestinal cancer

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LIST OF ABBREVIATIONS

APC	Adenomatous Polyposis Coli
β-actin	Beta-actin
CsCl	Cesium chloride
CTAB	Cetyltrimethylammonium bromide
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EtBr	Ethidium bromide
FOBT	Fecal occult blood test
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
LB	Lysogeny broth
POC	Point-Of-Care
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
TB	Terrific broth

1 INTRODUCTION

1.1 Extraction of DNA

In molecular biology, one of the most important methods is the extraction of biomolecules, proteins, RNA and DNA [1]. There are various sources from which proteins, DNA and RNA can be isolated, for example cells (prokaryotic / eukaryotic), viruses and living or conserved tissues [1] [2].
In general, DNA is typically extracted by performing the following three steps [3]:

1.) Disruption of cells
2.) Separation of (soluble) DNA from cell debris and other (insoluble) unwanted substances and source material (medium, blood, soil, etc.)
3.) Purification of DNA from other nucleic acids and soluble proteins

As the quality of extracted DNA has direct influence on the results of all following scientific research [4], the extracts should be as free of contaminants as possible.

1.1.1 History

Friedrich Miescher (1844 – 1895), a Swiss physician, performed the very first DNA isolation in 1869 [5]. While trying to show that proteins were the major components of cell cytoplasm, in his experiments a substance precipitated when acid was added and dissolved again after alkali addition. The precipitate, as was later discovered, was DNA [2]. After several attempts, he succeeded in development of a protocol for isolation of the newly discovered substance, which was later called “nucleic acid” by Richard Altman, Miescher’s student [5].

Figure 1: Friedrich Miescher [8]

With time passing by, more and more advancements to DNA extraction procedures were made. The first routine extraction methods for DNA were based on density gradient centrifugation. This method was used by Meselson and Stahl in 1958 [9], who demonstrated the semiconservative replication of DNA. Newer techniques utilized the different solubility of large chromosomal DNA, plasmids and proteins in alkaline buffer [9].

1.1.2 Current methods

Most of the protocols invented so far have been developed into commercial kits and can be divided into two groups: solution-based or column-based protocols [2]. The most common contamination of DNA samples is salt, which should be removed using desalting steps prior to downstream processing or analysis [10]. A widely used method for extraction of DNA, solution-based, is the phenol-chloroform extraction [2]. With this method, proteins, cell debris, carbohydrates and lipids are removed from the sample [6] [7]. After addition of the organic solvent solution, two phases start to show up: The hydrophobic layer on the bottom and the hydrophilic layer on top [9]. The upper phase is isolated and ethanol or isopropanol is added together with a high concentration of salt, causing the DNA to precipitate. After centrifugation, the precipitated DNA pellet is isolated, washed with 70% ethanol and dissolved again in TE buffer or water [9]. A variation of this protocol was developed by Chomczynski and Sacchi in 1987 [6], who established the guanidinium thiocyanate-phenol-chloroform extraction. This is a single-step method which extracts DNA with phenol / chloroform at reduced pH, while guanidinium thiocyanate, a chaotropic agent, is used for protein degradation. With this method, RNA can be separated from DNA [7]. Another method, mostly used for isolation of plasmid DNA from bacteria, especially *Escherichia coli (E. coli)*, is the alkaline extraction method [2] [6]. Here, sodium dodecyl sulfate (SDS) is used for isolation via selective alkaline denaturation of chromosomal DNA. Circular DNA, covalently closed, remains untouched, while cell debris and denatured chromosomal DNA are bound in large complexes with dodecyl sulfate [11]. After centrifugation, DNA can be recovered from the supernatant. Other common solution-based extraction methods include the cetyltrimethylammonium bromide (CTAB) extraction method [6] and the ethidium bromide (EtBr) – cesium chloride (CsCl) gradient centrifugation [2] [4].

Most of the commercially available extraction kits on the market are based on solid-phase purification methods, which are quicker and more efficient, compared to conventional methods [12]. DNA is bound to a solid phase depending on the pH and salt concentration, based on one of the three principles:

1.) Hydrophilic matrix, hydrogen-binding under chaotropic conditions
2.) Anion exchanger, ionic exchange under aqueous conditions
3.) Affinity and size exclusion

In most cases, a spin column is used for purification, driven by centrifugal force [13]. The solid phase is made of silica matrices, diatomaceous earth (also known as kieselguhr), glass particles, anion-exchange carriers or similar materials. Purification with this method is based on the negatively charged DNA backbone that has a high affinity to the positively charged silica particles [14]. There are, in general, three steps during purification [15]:

1.) Cell lysis
2.) Binding of nucleic acids to solid phase
3.) Washing and elution of DNA

A variation of this method is the mixed-bed solid phase nucleic acid extraction. This method uses at least two different phases, solid, semisolid, porous or non-porous, which bind and release DNA under different solution conditions [16]. There are also other materials besides silica matrices, such as nylon matrices, nitrocellulose and polyamide membranes, but these are rather used as solid-phase nucleic acid transfer and hybridization matrices due to their lower specificity [17].

A newer principle often used today is the magnetic bead-based purification, where charged particles are used [2]. In some variations the charge of the particles can be removed by application of a magnetic field using a permanent magnet [18]. In other cases, the beads are covered with a solid matrix the DNA binds to, as mentioned in the previous paragraph. Such a bead system was used for extraction of DNA within this work.

Out of this principle various automated extraction systems have been developed in recent years, thereby simplifying isolation of nucleic acids [19]. Such a system is usually large, expensive and complex, but very beneficial at the same time, as it reduces working time, decreases labor costs and increases reproducibility and quality of results [20]. Such systems have been developed for medium to large laboratories and have become more and more common during recent years [21]. Even in forensic laboratories such a system is in use nowadays, as it meets all quality standards for forensic laboratories [22].

1.2 Extraction of DNA from difficult samples

Although extraction of DNA has become a standard lab method over the last few decades [1], there are still many sample types where DNA is difficult to extract from, causing problems in clinical diagnostics, food analytics, environmental analysis and molecular forensics [23]. Some examples for these sources are body fluids (urine), stool, fatty foods or soil. DNA that has been extracted from such difficult samples using conventional methods is usually unstable, difficult to analyze and not suitable for further downstream processing like PCR. These effects are caused by mutagenic and DNA-destructive compounds and inhibitors that interfere with analytical enzymes (DNA polymerases, restriction enzymes), negatively influencing further processing of DNA. Some of these substances are DNases, metal ions, bacterial carbohydrates and bile salts [24] [25]. Most of these contaminants are not removed using conventional DNA extraction methods, but are purified together with the DNA instead.

Depending on the sample type and DNA concentration, in some cases dilution of the extracts can reduce the negative effects mentioned before [26]. However, DNA is diluted as well, making the final DNA concentration in the eluate too low for further analysis and / or processing in many cases. Additionally, substances that are binding directly to the DNA are not diluted in relation to the DNA. Thus, it is better to separate these substances from the DNA during the extraction process or to inactivate these. There are various protocols available for this purpose, all based on one of the three following principles [1] [27] [28].

Principle 1

Insoluble substances can be separated from the homogenized sample by centrifugation or filtration through a cellulose filter membrane. After this, DNA is usually separated from the homogenate by binding to a silica matrix, usually in the form of a spin-column. Own preliminary works and data from other research groups [29] have shown that DNA extracted with one of these protocols is not any more stable than DNA extracted with common extraction methods. This is due to the solubility, DNA-like properties and direct binding to DNA of these interfering substances. Thus, these substances are not separated upon centrifugation, bind to the silica matrix and are eluted together with DNA.

Principle 2

Many of the interfering substances can be precipitated in organic solvents [1]. Even though many substances, e.g. some salts, can be separated, this method is depending on the insolubility of the unwanted substances and, thus, only applicable for some sample materials.

Principle 3

This principle is based on the removal of unwanted substances by precipitation and adsorption of DNA to an insoluble matrix. This method, developed by our work group and others [30], was the first one suitable for the isolation of DNA from difficult samples where DNA can still be analyzed afterwards and is still one the most dependable to date. This method has two additional steps, compared to common ones. Homogenization is performed in a specific high-salt buffer causing precipitation of many unwanted substances which can be removed by centrifugation. Secondly, a carbohydrate-matrix is used to which many DNA-damaging and PCR-inhibiting substances bind to [31]. This matrix, patented under the name “Inhibit-Ex”, is separated via centrifugation together with the unwanted substances bound to it. The remaining DNA is then isolated using a silica matrix. This method as well as a commercially available kit based on this method [98] is now well-established for extraction of DNA from difficult samples like stool, soil or food [32].

1.3 Difficult samples

As mentioned in the previous chapter 1.2, there are various types of samples that are difficult to extract and / or analyze, mostly because of their composition and the presence of inhibitors. Within this work, two sample types are further analyzed: Soil and stool.

1.3.1 Soil

Soil samples were used for development of the extraction system due to better availability, easier handling and ethical reasons. However, soil is similarly difficult to extract DNA from and analyze as stool samples are. Reason being is mainly the presence of humic acids that affect downstream processing of DNA by binding to analytical enzymes and chelating of Mg^{2+} ions, an important cofactor for many enzymes [33]. Other possible impurities and inhibitors are discussed later in detail in chapter 4.1.2. Although in some cases dilution of the raw DNA extract acquired by

standard methods may lead to a successful PCR [26] [34], it is not the solution to the initial problem. Thus, specialized methods and kits have been developed for this purpose. However, none of these techniques are suitable for all soil types [37] and they are neither portable nor easy to use.

Soil has not only been used as a substitute for stool in this work, it is also of significant interest for research. Extraction of soil DNA and subsequent analysis of the complete soil genome allows conclusion on the composition of the soil's microflora in the area the sample was taken from. This is of great importance, as today the analysis of microbial communities is still based on isolation of bacteria and subsequent cultivation in laboratory conditions prior to extraction of DNA and subsequent analysis [35]. However, as the vast number of bacteria present in soil all have individual growth conditions and requirements, only the ones for which the cultivation criteria have been chosen correctly are cultivatable. Due to this problem a significant number of bacteria have not been analyzed yet or are even unknown. It has been shown that less than 1% of the microorganisms present in various environments are cultivatable [36].

That being said, downstream processing like PCR or – especially – sequencing requires clean DNA samples without impurities or inhibitors. Thus, pure and high molecular weight DNA is of great importance for studies of microbial diversity, metagenomic analysis etc. [37].

1.3.2 Stool

Stool is a sample material similarly difficult to extract DNA from like soil, as mentioned in the previous chapter. There are even more inhibitory components present in stool samples than in soil, some of which are macromolecules, low molecular substances and, especially, salts that are destructive against DNA or inhibit analytical enzymes. Most of these substances are not separated during extraction of DNA, but are purified together with the desired DNA instead. Examples for such compounds are DNase, bile acids, metal ions and bacterial carbohydrates [24] [25]. Table 1 gives an overview of components present in stool that may have an influence on DNA and PCR.

Table 1: Components in stool with possible influence on DNA and PCR (own previous works and [38])

Component	Percentage of dry mass	DNA-destructive	PCR-inhibiting	Removal / inactivation with
Fibers, bulk materials	< 30%	Yes	No	Cellulase
Bacteria	< 30%	Yes	Yes	Lysozyme
Fats and lipids	< 20%	Yes	Yes	Lipase
Salts	< 10%	Yes	Yes	EDTA
Proteins	5%	Yes	Yes	Proteinase
Mucopolysaccharides	2%	No	Yes	Amylase
Other carbohydrates	5%	No	Yes	Amylase
Fungi	< 1%	Yes	Yes	Chitinase
Free DNA	< 0.1%	No	Yes	DNase
Eukaryotic cells	< 0.1%	No	No	Chaotropic salts

To eliminate these problems, various methods and techniques have been developed, as previously mentioned in chapter 1.2.

Extraction of DNA from stool is very important, as fecal DNA has high diagnostic relevance. It is composed of DNA from various sources, like the (gut) microbiome, blood or intestinal mucosa. Thus, conclusions on the presence of (intestinal) diseases can be made upon analysis of fecal DNA. The most popular method used today for this kind of purpose is the fecal occult blood test (FOBT), which was introduced by Sidransky et al. [39]. Today, various versions of the FOBT exist for different applications: Guaiac based tests, heme-porphyrine tests and immunochemical tests [40]. However, the FOBT is not very accurate, as it tends to deliver false positive results [41]. Moreover, most tests cannot distinguish between benign and malignant lesions [42]. As this test only detects the presence of occult blood in a stool sample, it is not more than a first signal for a disease rather than a concrete indicator. In contrast, the presence of occult blood in stool is not necessarily a sign of disease [40].

A more specific approach to the detection of diseases from stool samples was made by Deuter and Müller [43], who analyzed mutations in the tumor suppressor gene Adenomatous Polyposis Coli (APC) in stool DNA obtained from patients with colorectal carcinomas. This type of cancer is the second most cause of deaths among cancers worldwide and is the third most common form of cancer [44]. The mutation mentioned earlier usually occurs in early stages of cancer and does not increase over time [45] [46], making early detection feasible. As several studies have shown that screening programs may reduce cancer mortality [47] [48] [49], this is very beneficial. The heteroduplex-PCR method developed by Deuter and Müller utilizes the electrophoretic mobilities of heteroduplex DNA double strands as well as amplification by PCR,

resulting in a non-radioactive method to detect mutations in DNA with a high background of non-mutated DNA fragments [43].
Another approach was made by Kutzner et al. [50], who combined the FOBT mentioned earlier with molecular diagnosis to detect alterations in three tumor-relevant markers: APC, BAT26 and L-DNA. With the combination of both methods they achieved a sensitivity of 93% and an overall specificity of 89%, which is similar to invasive methods.
Rengucci and colleagues [51] introduced a very promising new approach where they combined a semiautomatic DNA extraction method with real-time PCR analysis, which made extraction and analysis of fecal DNA significantly easier and faster.

1.4 Microchips and microfluidic devices

Microchips using microfluidic techniques have been developed in the past into complete so-called Lab-On-Chip systems for various applications, e.g. for medical diagnosis. They have been available since the 1960s with more and more systems being developed every year [52] [67]. The idea for microfluidic systems came up earlier, in the 1950s, when researchers needed systems for dispensing of small amounts of liquids in nano- and sub-nanoliter scale [53]. A great breakthrough for microfluidic platforms, especially when it comes to fluid propulsion, was made in 1979, when a miniaturized gas chromatograph was developed on a silicon wafer [54]. After this, more and more innovations were presented, such as the first micro-valves [55] and micro-pumps [56] [57]. During the last 30 years, more and more microfluidic components have been developed, including for fluid transport, valving, fluid mixing and concentration or separation of molecules in small quantities [59].
More and more techniques and protocols have been combined, striving into the Point-Of-Care (POC) setting. There are many different principles these systems are based on, one of which is the lateral flow bioassay, one of the most common systems [58] [59]. This type of system utilizes capillary forces for fluid transport and uses dried biomarkers, pre-stored on the chip, to trigger a color reaction when a desired molecule is present in the sample. Although systems of this type are very limited to specific sample types and reactions, they are inexpensive, generate results quickly and are easy to apply, because even non-trained operators can use them [52].
Diagnostic microfluidic devices can generally be divided into two groups: Disposable diagnostic cards that require instrumentation [60] and those with little to no

instrumentation required [61]. The latter group is a great improvement, as it is not place bound due to its independency from other instruments and / or power supply. An example for a device of this group is a sequencing system on a disposable card that uses pressurized gas stored in a micro-reservoir on the card as an energy source [62].

1.5 Aim of this work

As already mentioned before in chapter 1.2, analysis of difficult samples is a huge problem in clinical diagnostics, food analytics, environmental analytics and molecular forensics [23]. With the arising of big data, autonomous systems etc. with a constantly rising amount of data generated and analyzed, DNA extraction has slowly become a painful bottleneck in the process [63]. Although various methods and techniques have been developed during recent years, not all methods are reliable and applicable for all samples due to sample heterogeneity. A method for extraction of DNA can deliver good results on one day, but fail on the other day. Additionally, no method available today can be used in daily routine for analysis of huge sample numbers in clinical diagnostics, food analytics or environmental analytics.

The majority of methods are based on single steps which have to be executed manually, taking a significant amount of time for each sample. Plus, in most cases laboratory equipment as well as skilled personnel is needed for extraction and analysis, making extraction and analysis of DNA on-site, e.g. bedside in hospital or in field, nearly impossible. This is one of the reasons why non-invasive tumor diagnosis using tumor-DNA from stool samples is not cost-effective yet [64]. Costs for molecular analysis are still too high to be used in cancer screening programs [65]. This was shown by Song et al. [66], who compared the costs of fecal molecular tests with colonoscopies. They estimated the costs of the tests between $350 and $795, while a colposcopy is between $1,200 and $1,800. Thus, development of an easy-to-use automatable and portable system for extraction of DNA from difficult samples would be very beneficial.

The aim of this work is the development of an easy-to-use, automatable and portable system for the extraction of DNA from difficult samples like soil or stool. The DNA gained with this method should be stable and clean enough for subsequent analysis, for example for PCR. The system should be small enough to allow the use in remote areas outside the laboratory, e.g. bedside or in field. Additionally, the system should require a minimal number of manual steps and should be automatable, so that only

addition of the raw sample would be required. With such an easy protocol, the system could be used by untrained personnel without the need for additional equipment. Plus, the system should be quick enough for cost-effective use and should be cheap to build and operate in routine analysis, once mass-produced and fully automated. After this project is finished, the microsystem could be developed further into a simple-to-use analysis system with build-in PCR and / or biomarkers (see chapter 4.7 for an outlook with more ideas). At this stage a POC-setting, for example for early non-invasive disease detection, could possibly be realized, with multiple application fields.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments

- Syringe pump HLL LA-100 (Landgraf Laborsysteme HLL GmbH, Germany)
- Thermocycler Biometra® Tgradient (Biometra GmbH, Germany)
- Gel documentation system Vilber Quantum ST4 (Vilber Lourmat Deutschland GmbH, Germany)
- Mini and Midi electrophoresis chambers (Carl Roth GmbH + Co. KG, Germany)
- Power supply (Bio-Rad Laboratories GmbH)
- Photometer Nanodrop 2000 (Thermo Fisher Scientific GmbH, Germany)
- Spectrophotometer Perkin Elmer® LambdaBio+ (Perkin Elmer, Germany)
- Hot-glue gun Pattex® Hotmelt Supermatic (Henkel AG & Co. KGaA, Germany)
- Ultra-low temperature freezer HeraFreeze (Thermo Fisher Scientific GmbH, Germany)
- Autoclave VE-75 (Systec GmbH, Germany)
- Single channel pipettes (Eppendorf AG, Germany, and Gilson International B.V., Germany)
- Fine scale (Kern & Sohn GmbH, Germany)
- Cooling centrifuge Hermle Z323K (Hermle Labortechnik GmbH, Germany)
- Mini centrifuge MiniSpin (Eppendorf AG, Germany)
- Microwave exquisit MW1780G (GGV Handelsgesellschaft mbH & Co. KG, Germany)
- PH meter PB-11 (Sartorius AG, Germany)
- Pipette filler Pipetboy acu2 (Integra Biosciences Deutschland GmbH, Germany)
- Shaking incubator Ecotron (Infors GmbH, Germany)
- Sterile bench MSC Advantage (Thermo Fisher Scientific GmbH, Germany)
- Thermo mixing block MB-102 (Biozym Scientific GmbH, Germany)
- Vortexer Vortex-Genie 2 (VWR International GmbH, Germany)

2.1.2 Expendable items

- Cellstar® tubes, 25 mL and 50 mL (Greiner bio-one International GmbH, Austria)
- Reaction tubes, 0.5 mL, 1.5 mL and 2.0 mL (Greiner bio-one International GmbH, Austria)
- Pipette tips 0.5 – 20 µL, 10 – 200 µL, 100 – 1,000 µL (Greiner bio-one International GmbH, Austria)
- PCR SingleCap SoftStrips, 0.2 mL (Biozym Scientific GmbH, Germany)
- Versilic® silicone tubes, Ø = 1.0 mm (Carl Roth GmbH + Co. KG, Germany)
- Cryo.s™ freezing tubes, 1.0 mL (Greiner bio-one International GmbH, Austria)
- Vis Cuvettes (Eppendorf AG, Germany)
- Single-use pipettes, 5 mL, 10 mL, 25 mL (Greiner bio-one International GmbH, Austria)
- Petri dishes (Greiner bio-one International GmbH, Austria)

2.1.3 Microchips

Microchips were produced and customized by thinXXS Microtechnology AG, Germany.

2.1.4 Microchip accessories

- Tube connectors Rotilabo® mini (Carl Roth GmbH + Co. KG, Germany)
- Tube connectors Rotilabo® Luer (Carl Roth GmbH + Co. KG, Germany)
- Pinch clamps “Quetsch-Fix” (Esska.de GmbH, Germany)
- Neodymium magnets, 0.5 x 0.5 x 0.5 cm (Chemicell GmbH, Germany)
- Hot glue sticks Pattex “Hotmelt sticks” (Henkel AG & Co. KGaA, Germany)
- Sticky tape Tesa Tesafilm® (Tesa SE, Germany)

2.1.5 Sealing foils

- PeqLab qPCR seal (VWR International GmbH, Germany)
- PeqLab Adhesive PCR film (VWR International GmbH, Germany)
- Viewseal sealer (Greiner bio-one International GmbH, Austria)
- Ampliseal sealer (Greiner bio-one International GmbH, Austria)
- Easyseal sealer (Greiner bio-one International GmbH, Austria)

2.2 Solutions

2.2.1 Bacteria culture

- Lysogeny broth (LB) medium (Carl Roth GmbH + Co. KG, Germany)
- Lysogeny broth (LB) agar (Carl Roth GmbH + Co. KG, Germany)
- Terrific broth (TB) medium (Carl Roth GmbH + Co. KG, Germany)
- Ampicillin sodium salt (100 mg/mL) (Carl Roth GmbH + Co. KG, Germany)

2.2.2 DNA extraction

- GeneMAG-RNA/DNA extraction kit (Chemicell GmbH, Germany)
- 70% Isopropanol or ethanol
- Double-distilled H_2O

2.2.3 PCR

- MyTaq™ Red Mix (Bioline GmbH, Germany)
- Double-distilled H_2O

The primers shown in Table 2 have been used within this work.

Table 2: Primer sequences

Primer	Sequence 5' → 3'
8F	AGA GTT TGA TCC TGG CTC AG
926R	CCG TCA ATT CCT TTR AGT TT
EGFP fwd.	GAT CTA TGG TGA GCA AGG GC
EGFP rev.	CTT GTA CAG CTC GTC CAT GC
hGAPDH_P2 fwd.	CCG CAT CTT CTT TTG CGT CG
hGAPDH_P2 rev.	AAA TGA GCC CCA GCC TTC TC
hGAPDH_P5 fwd.	CCG CAT CTT CTT TTG CGT CG
hGAPDH_P5 rev.	GAT GGC ATG GAC TGT GGT CA
h-bActin_P1 fwd.	GTG CTA TCC CTG TAC GCC TC
h-bActin_P1 rev.	CAG CTC AGG CAG GAA AGA CA
h-bActin_P2 fwd.	CCA CCA TGT ACC CTG GCA TT
h-bActin_P2 rev.	AGC TCA GGC AGG AAA GAC AC

The 8F [1] + 926R [73] primer pair codes for the prokaryotic 16S rRNA gene [74], the EGFP primer pair codes for the EGFP gene [75] used in the transfected *E. coli* bacteria. The hGAPDH_P2 and hGAPDH_P5 primer pairs code for the human glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), a housekeeping gene commonly used

as marker for human DNA [76]. The h-bActin_P1 and h-bActin_P2 primer pairs code for the human beta-actin gene, one of two non-muscle cytoskeletal actins, also a housekeeping gene commonly used as marker for human DNA [77].

2.2.4 Agarose gel

- 50x TAE buffer
  - 121 g Tris base in 250 mL H_2O
  - 28.6 mL acetic acid
  - 50 mL 0.5 M EDTA (pH 8.0)
  - H_2O added to final volume of 500 mL
- 1% agarose gel
  - 0.5 g agarose
  - 50 mL 1x TAE buffer
- Midori Green Advance DNA stain (Biozym Scientific GmbH, Germany) (2 µL per 50 mL gel)
- BlueJuice™ Gel Loading Buffer (10X) (Thermo Fisher Scientific GmbH, Germany) (2 µL + 20 µL sample)
- HyperLadder™ 1kb DNA ladder (Bioline GmbH, Germany)
- HyperLadder™ 100bp DNA ladder (Bioline GmbH, Germany)

2.3 Methods

2.3.1 Collection of soil samples

All soil samples were collected from a lawn in Zweibrücken, Germany, using a small garden shovel. All collection conditions (date, time, weather conditions (rain, snow etc.) and sample qualities (moisture, color)) were noticed, as factors like these may influence the microflora, bacteria count [1] [79] and the soil sample itself (moisture, salinity, nutrients) [80]. Samples were acquired from a hole with a depth of approximately 10 – 15 cm and collected in 50 mL falcon tubes while avoiding stones and other solid matter (e.g. roots, worms) upon collection where possible. Soil samples were stored at 4°C subsequently and were used the same day, if not stated otherwise.

2.3.2 Bacteria culture

E. coli bacteria were grown either in terrific broth (TB) or lysogeny broth (LB) medium, usually overnight, if not stated otherwise. Cultivation took place with varying volumes of medium, depending on the number of bacteria needed for the subsequent experiment, in 10 mL or 25 mL falcon tubes or 25 – 100 mL Erlenmeyer flasks using a shaking incubator at 37°C. In later experiments transfected *E. coli* were used with a vector containing an EGFP gene as marker and Ampicillin resistance for selection (see chapter 3.3.2). Selection was achieved by addition of 1 µL Ampicillin (100 mg/mL) per mL medium.

2.3.3 Collection of stool samples

Human stool samples were collected with as little fluid as possible in 50 mL falcon tubes and frozen at -20°C subsequently. The next day, samples were divided into smaller aliquots to avoid multiple unnecessary freezing / thawing processes leading to possible DNA loss [81]. These aliquots were used for the following experiments.

2.3.4 PCR

The PCR programs used are shown in the following Table 3. Depending on the amount of DNA expected, 20 – 30 PCR cycles were performed.

Table 3: PCR programs

Step		Temp. [°C]	Time [sec]	Cycles
1	Initial Step	94	120	
2	Denaturation	94	60	20-30
3	Annealing	See Table 4	45	
4	Elongation	72	60	
5	Final Elongation	72	240	
6	Cooling	4	Pause	

The annealing temperatures of the primer pairs are shown in Table 4 below.

Table 4: Annealing temperatures

Primer pair	Annealing temperature [°C]
8F + 926R	49
EGFP	58
hGAPDH_P2	54
hGAPDH_P5	
h-bActin_P1	
h-bActin_P2	

2.3.5 Agarose gel

For a small gel with 2 x 8 lanes (mini electrophoresis chamber) 50 mL of agarose gel are prepared using the following protocol: 0.5 g of agarose are transferred into 50 mL 1X TAE buffer in a glass bottle. To get it into solution, the bottle is microwaved at 600 W for 1 min and mixed afterwards. Then the solution is microwaved again for 1 min at 600 W, hereafter the solution should be clear and agarose dissolved completely. 2 µL Midori Green Advance DNA stain are added (if PCR products are to be run on the gel), mixed and gel is poured into the gel pouring chamber. After 10 – 20 min the gel should be solid and can be transferred into the mini electrophoresis chamber, which is then filled with 1X TAE buffer. For a medium gel the volumes given are scaled up to 100 mL.

The lanes are loaded with 2 µL DNA ladder and 5 µL PCR products. For direct DNA staining without previous PCR, samples are mixed with BlueJuice™ Gel Loading Buffer (10X) in a 1:10 volume ratio and loaded onto a gel directly without Midori Green Advance DNA stain.

2.3.6 DNA extraction

DNA was extracted using the geneMAG-RNA/DNA kit (chemicell, Berlin, Germany). The principle is shown in Figure 2 below.

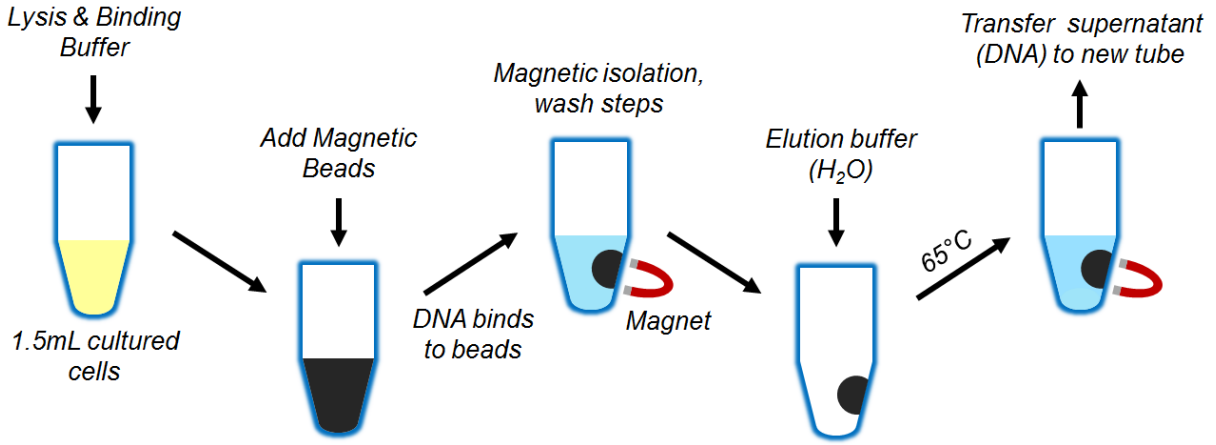

Figure 2: DNA extraction
Schematic drawing of DNA extraction protocol: Lysis & binding buffer is added to centrifuged bacteria pellet; magnetic beads are added; magnet is applied, supernatant is discarded; wash buffers are added, magnet applied, supernatants discarded; elution buffer (H_2O) is added, incubated at 65°C, magnet applied, supernatant containing DNA extract is transferred to a new tube.

According to the kit's manufacturer, 1.5 mL cultured cell suspension is transferred to a 1.5 mL reaction tube, centrifuged for 2 min at 11,000 x g and supernatant is discarded subsequently. 1 mL lysis buffer is then added as well as 100 µL of magnetic bead solution, the mixture is vortexed and incubated for 2 – 5 min. A neodymium magnet is applied for 1 min and supernatant is discarded afterwards. 1 mL wash buffer I is added and the solution is mixed gently by inverting the tube 6 – 8 times. A Magnet is then applied for 1 min and supernatant is discarded subsequently. This washing step is repeated once. For the second washing step 1 mL wash buffer II (70% isopropanol or ethanol) is added and mixed gently by inverting the tube 6 – 8 times. Again, a magnet is applied for 1 min and supernatant is discarded. This second washing step is repeated once. Then, while a magnet is applied, 1 mL H_2O is added and removed subsequently without mixing or resuspending the beads. Subsequently, 100 µL elution buffer (H_2O) is added, solution is vortexed and incubated for 10 min at 65°C in a thermo-mixer with vortexing in-between from time to time. Afterwards, a magnet is applied and the supernatant containing the eluted DNA is transferred to a new tube.

Several alterations to the manufacturer's original protocol were performed throughout this work. Fist, all volumes except for the elution buffer were reduced in order to fit into a microchip. Thus, lysis buffer volume was reduced from 1,000 µL to 750 µL. All other volumes were reduced to one fourth of the original volume: Bead solution volume was

reduced from 100 µL to 25 µL, while wash buffer I, II and H_2O volumes were reduced from 1,000 µL to 250 µL. Only the volume of the elution buffer was left unchanged because of the microchip design (loss of liquid in channels, chambers etc.) and to increase the absolute amount of DNA in order to compensate for the DNA loss due to the lowered volumes. Additionally, a third washing step with wash buffer II (70% isopropanol / ethanol) was introduced to increase DNA pureness. Final elution was performed with the reaction tube lid opened in order to let any alcohol residues evaporate.

As a centrifugation step is hard to realize inside a microchip system, the centrifugation step demanded by the manufacturer's protocol mentioned earlier was replaced with a sedimentation step. For this, the sample is mixed with 750 µL lysis buffer without the addition of bead solution. After 1 – 15 min sedimentation time 250 – 500 µL of the supernatant are transferred to a new reaction tube, where 25 µL bead solution are added. From here, the regular protocol continues with the 2 – 5 min incubation time.

2.3.7 Microchip system

The microchip must be prepared before DNA extraction. For this, tube connectors are attached to the inlets / outlets located at the rear of the chip. Silicone tubes are attached to these connectors and are fixed permanently with hot glue using a hot glue gun, as shown in Figure 3a. A considerably large amount of glue is needed to ensure air tightness, but only small amounts of hot glue should be applied at once and the chip should be cooled at 4°C immediately after addition glue to prevent deformation of the chip due to the heat of the glue, as shown in Figure 3b. The permanently attached tubes are connected to the rest of the tubing using tube connectors, which are removable.

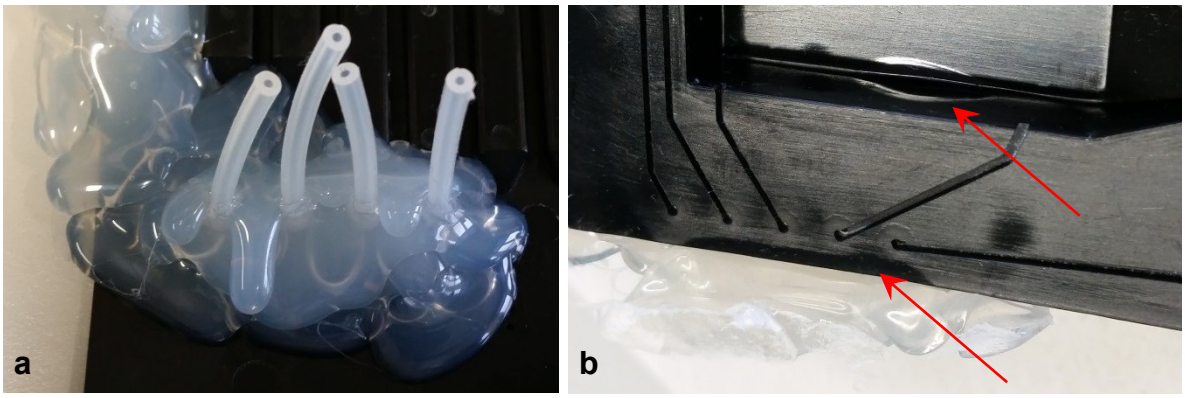

Figure 3: Tubes attached to microchip
a) Silicone tubes attached to fluid connectors which are attached to microchip, fixed with hot glue; b) Heat deformations due to excess heat exposure (marked with red arrows)

The DNA extraction protocol mentioned in the previous chapter 2.3.5 was adapted to a microchip system. First, up to 500 mg (solid) sample are added into the sedimentation chamber of the microchip, which itself is then sealed with a self-adhesive PCR seal. In case of liquid samples, samples are added after sealing using the ventilation opening located at the rear of the chip. Now silicon tubes are connected to the chip as shown schematically in Figure 4, but not to the syringe pump yet for pressure release during filling of the chip. All the liquids needed for extraction of DNA (2x H_2O, wash buffer I and II) except for the lysis buffer are then filled into the appropriate storage chambers on the chip using the rear ventilation openings. Subsequently, a syringe pump is connected to the tubes and all ventilation openings are closed with sticky tape to ensure proper pressure build up during pumping.

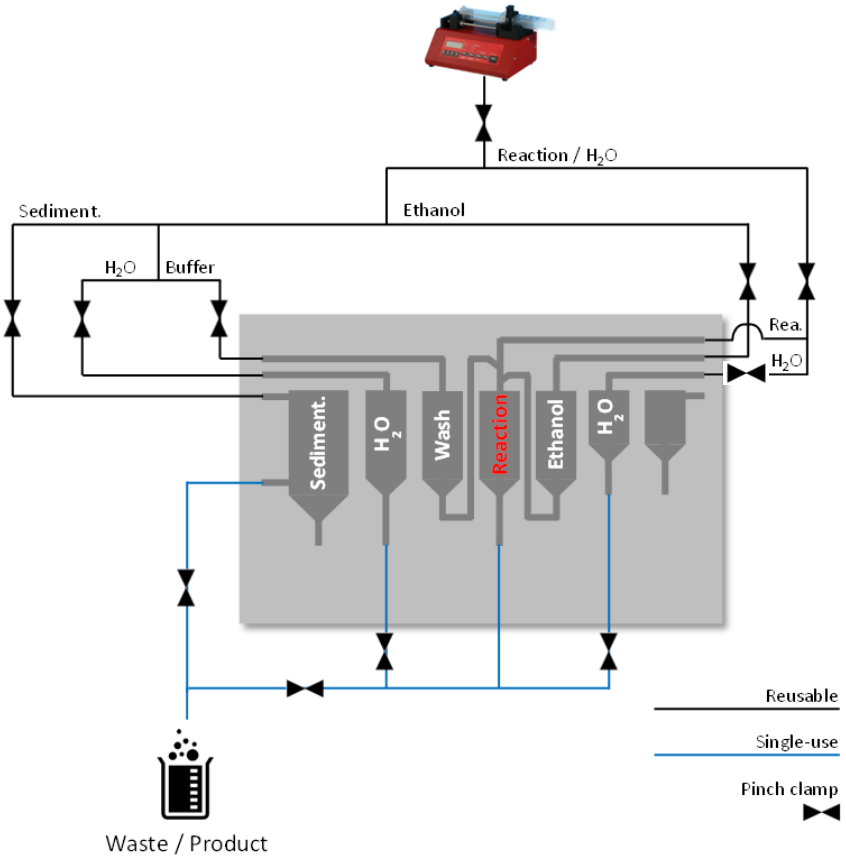

Figure 4: Microchip connection scheme
Schematic drawing of microchip (version 4) connected to a syringe pump with tubes; microchip has several chambers: Sediment. (sedimentation), H_2O (water), Wash (wash buffer), Reaction (reaction chamber), Ethanol (wash buffer II: 70% Isopropanol / ethanol); Tubes drawn in black are not in contact with liquid and can, thus, be reused; tubes drawn in blue are in contact with liquid at some point of experiment and are therefore intended for single use

After this, lysis buffer is added to the sedimentation chamber and tubes are connected to a syringe pump. Now the DNA extraction protocol is performed inside the chip with a few modifications to be considered: Mixing is performed by rocking of the chip or whacking of the chip against the table surface. A magnet is applied to the back of the chamber. Pumping is performed by applying pressure to the chamber to be emptied and opening of the pinch clamp(s) for the destination chambers in parallel. Heating is performed by fixing of the microchip onto a thermoblock with an increased elution temperature of 75°C due to the lack of direct contact to the heating surface. A magnet is fixed to the elution tube in order to hold back magnetic beads that were not held back by the magnet on the chip.

For liquid pumping pump rates between 1.0 mL/min and 2.0 mL/min were used, while for emptying of chambers (waste) a pump rate between 10.0 mL/min and 20.0 mL/min was applied for rapid evacuation.

3 RESULTS

3.1 Extraction of DNA from soil & *E. coli* bacteria

At first, DNA was extracted out of soil samples collected from a lawn in Zweibrücken using the Chemicell GeneMAG-RNA/DNA extraction kit (see chapter 2.3.1). Several changes to the manufacturer's original protocol were made. These changes and the corresponding results are shown in the following subchapters.

3.1.1 Manufacturer's protocol with light changes

The original extraction protocol provided by the kit's manufacturer was slightly changed. 0.482 g of soil sample 1 (a; stored 6 days at 4°C prior to extraction), 0.492 g of soil sample 2 (b; collected on the same day as the experiment) and 0.473 g of soil sample 3 (c; collected the day before the experiment and stored at 4°C) were transferred into 1.5 mL reaction tubes each and 1.0 mL lysis and binding buffer was added. After centrifugation supernatant was transferred to a new tube and 100 µL magnetic bead solution was added. The next steps were all performed according to the manufacturer's protocol except for the last washing step with H_2O which was repeated once before final elution of DNA in order to improve DNA purity. After extraction, DNA yield was measured with a spectrophotometer, results are shown in Table 5.

Table 5: DNA yield

Sample	DNA concentration [ng/µL]	260/280 ratio	260/230 ratio
a	16.3	1.44	0.07
b	10.0	1.59	0.06
c	34.6	1.22	0.13

The photometric results clearly show that DNA extraction was successful, although the 260/280 ratios were not ideal (ranging from 1.22 to 1.59) and the 260/230 ratios were very poor (ranging from 0.06 to 0.13), indicating that there were significant amounts of contaminants present absorbing at 230 nm (e.g. guanidinium thiocyanate, part of lysis- & binding buffer and wash buffer I) and 280 nm (e.g. protein) in the final eluate. The sample with the highest DNA yield, sample c, had a significantly lower 260/280 ratio (1.22) than the other samples (1.44 and 1.59), but the 260/230 ratio was almost doubled (0.13 compared to 0.06 and 0.07).

Subsequently, a 30 cycle PCR was performed using the 8F + 926R primer pair coding for the prokaryotic 16S rRNA gene. A gel electrophoresis picture with the PCR products is shown in the following Figure 5.

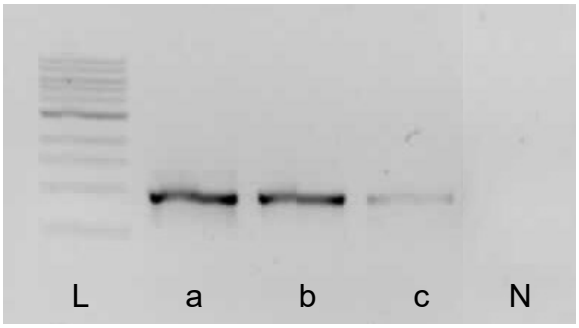

Figure 5: PCR products of soil DNA extracted with altered extraction protocol
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts; **L:** 100bp Ladder; **a:** 0.482g soil sample 1; **b:** 0.492g soil sample 2; **c:** 0.473g soil sample 3; **N:** Negative control

Figure 5 clearly shows that DNA extraction was successful, although the absolute amount of DNA was not equal. While bands a and b (soil samples 1 and 2) were of similar strength, band c (soil sample 3) was significantly weaker. The reason for this might be the different soil samples that DNA was extracted from, which may have contained varying amounts of bacteria / DNA, which stands in contrast to the photometric results, where sample c had the highest yield by far (29.8 ng/µL), followed by samples a (16.3 ng/µL) and b (10.0 ng/µL) (see Table 5).

Using the same protocol as in the previous experiments, DNA was extracted again from 0.470 g of a fresh soil sample (soil sample 4) collected the same day as the experiment. Photometric analysis of the DNA eluate leads to the results shown in Table 6.

Table 6: Experiment 1: DNA yield, continued

Sample	DNA concentration [ng/µL]	260/280 ratio	260/230 ratio
d	15.4	1.54	0.11

As shown in Table 6, extraction of DNA was, again, possible with a yield of 15.4 ng/µL, which is close to the yields of the previous extractions (samples a and b), while the 260/280 and 260/230 ratios were similarly bad as with the previous extracts. Subsequently, a PCR (30 cycles) with 16S rRNA primers (8F + 926R) was performed. The results are shown in Figure 6.

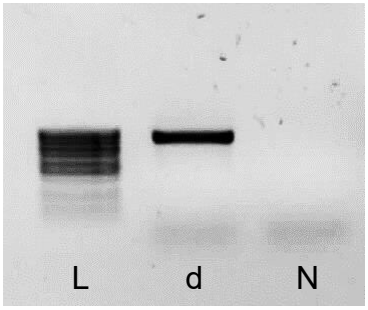

Figure 6: PCR products of DNA extracted from fresh soil sample
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extract from soil; **L**: 100bp Ladder; **d:** 0.470g soil sample 4; **N:** Negative control

As visible in Figure 6, a clear band can be seen for the DNA extract from sample d, proving that extraction of DNA out of fresh soil was successful. No extract was visible for the negative control at all.

3.1.2 Scale down

In order to fit the extraction protocol into a microchip, volumes had to be reduced. Thus, sample size and all volumes were changed, while the rest of the protocol was left unchanged as in the previous experiment. First, all volumes and sample weight were halved, and then reduced to one fourth of the original volumes / weight. 0.263 g of soil sample 5 and 0.129 g of soil sample 6, collected the same day as the extractions, were used, respectively. After extraction, DNA yield was measured photometrically, results are shown in Table 7.

Table 7: Experiment 2: DNA yield with lowered weights & volumes

Sample	DNA concentration [ng/µL]	260/280 ratio	260/230 ratio
½	9.4	1.86	0.11
¼	4.7	2.33	0.03

Photometric results show that DNA extraction was still possible with reduced sample weight and reduced working volumes. As predicted, DNA yield was lower with lowered weight / volume. As expected, with half volume comes half DNA yield (9.4 ng/µL with half volume, 4.7 ng/µL with quarter volume). The 260/280 ratios were very good with 1.86 and 2.33, while the 260/230 ratios were very poor (0.03 and 0.11). A 30 cycle PCR with 8F + 926R primer pair was performed afterwards, the corresponding gel electrophoresis result is shown in Figure 7.

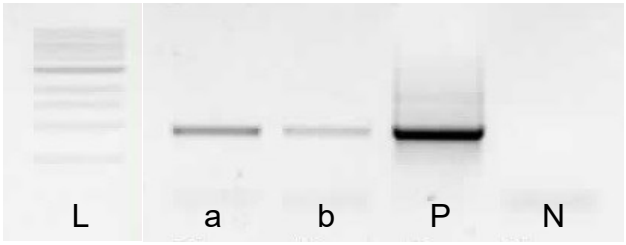

Figure 7: PCR products of DNA extraction with lowered weights / volumes
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts; **L:** 100bp Ladder; **a:** Half volume; **b:** Quarter volume; **P:** Positive control; **N:** Negative control

Figure 7 verifies the photometric results found earlier: Reduction of weight and volumes by half led to weaker bands while still giving enough yield for PCR. At half volume (a), a clear band can be seen, at quarter volume (b) there is still a band visible, although significantly weaker than band a. Positive control (P) shows a strong band, while in the negative control (N) no band is visible at all.

In order to increase DNA yield, sample weight was reduced to half of the original size while all other volumes were reduced to a quarter of the original size using 0.247 g (a) and 0.253 g (b) of soil sample 7, collected the same day as the experiment. Sample a was handled as previously, while with sample b bead solution volume was doubled to half of the original protocol's volume. Photometric analysis was performed with the extracts, the results are shown in Table 8.

Table 8: Experiment 2: DNA yield with lowered weights & volumes, continued

Sample	DNA concentration [ng/µL]	260/280 ratio	260/230 ratio
¼ II	6.0	2.10	0.03
¼ III	42.0	1.07	0.07

Photometric results indicate that DNA extraction was still possible. While the lower bead volume (sample ¼ II) lead to a yield of only 6.0 ng/µL, the higher bead volume (sample ¼ III) gave a yield of 42.0 ng/µL. The 260/280 ratio of the lower bead volume extract was great (2.10), the ratio of the higher bead volume extract was poor being only 1.07. 260/230 ratios were all very poor, ranging from 0.03 to 0.07. To verify these findings, a PCR with 30 cycles using the prokaryotic 16S rRNA primers was performed, an electrophoresis gel with the PCR products is shown in Figure 8.

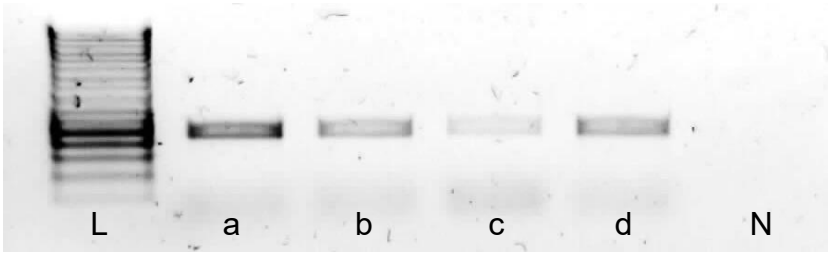

Figure 8: PCR products of DNA extraction with lowered weights / volumes, continued
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts; **L:** 100bp Ladder; **a:** Half volume (previous experiment); **b:** Quarter volume (previous experiment); **c:** Quarter volume, half weight (1/4 II); **d:** Quarter volume, half weight, half bead solution volume (1/4 III); **N:** Negative control

Again, Figure 8 shows that extraction of DNA is possible with the GeneMAG-RNA/DNA extraction kit, even with reduced volumes and sample size. Compared to the findings of the previous experiment (lanes a and b), the extraction showed a lower yield this time (lane c). By increasing bead solution volume to 50 µL (half of original volume given by the manufacturer) band d on the gel was similarly strong as in the experiment before (lane b). There was no band for the negative control (N) visible at all.

3.1.3 Variation of extraction protocol

In order to optimize the extraction kit's performance, magnetic bead volume and elution time were altered. At first, both variables were halved and doubled; later bead volume was divided by three and tripled, and divided by five and quintupled. DNA was extracted from the same soil sample for each test series. The photometric results of the variation of elution time are shown in Table 9.

Table 9: Variation of elution time

Sample	Factor	Time [min]	DNA yield [ng/µL]	Relative to standard
E1	Half	5	4.1	66%
E2	**Standard**	**10**	**6.2**	**100%**
E3	Double	20	3.7	60%
E4	Half	5	5.7	100%
E5	**Standard**	**10**	**5.7**	**100%**
E6	Double	20	8.9	158%

These results are shown graphically in Figure 9.

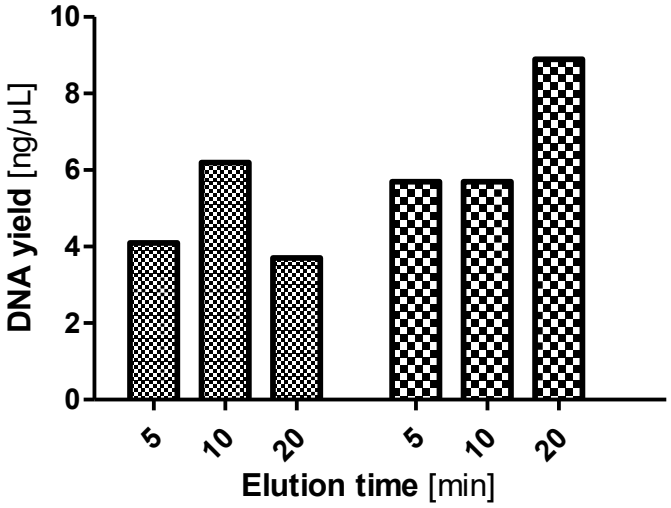

Figure 9: Variation of elution time
Photometrically measured yield after extraction of DNA from soil with different elution times (5, 10 and 20min)

The results from the variation tests are not as expected. In the first test series (E1 – E3) the standard elution time gave the best DNA yield, while both half and double elution time resulted in lower DNA yield (66% and 60%, respectively). In the second test series (E4 – E6) the doubled elution time achieved the highest DNA yield (158%), while half and standard elution time resulted in exactly the same yield (5.7 ng/µL). For verification a PCR was performed with 8F + 926R primer pair and 30 cycles, followed by gel electrophoresis. The gel is shown in Figure 10.

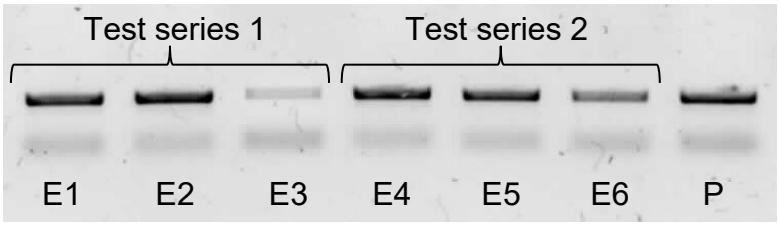

Figure 10: PCR products of DNA extracted from soil with different elution times
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts from soil using different magnetic bead volumes; **E1:** 5min; **E2:** 10min; **E3:** 20min; **E4:** 5min; **E5:** 10min; **E6:** 20min; **P:** Positive control

The PCR results are not consistent with the photometric findings. In test series one the bands E1 (5 min) and E2 (10 min) are of equal strength, while E3 (20 min) is significantly weaker. In test series 2, band E4 (5 min) is the strongest band, together with band E5 (10 min), followed by E6 (20min), which is significantly weaker, just as in test series 1. A clear band is visible for the positive control (P).

An overview of the bead volume variation experiments as well as the photometric results from those are shown in Table 10.

Table 10: Variation of magnetic bead volume

Sample	Factor	Volume [µL]	DNA yield [ng/µL]	Relative to standard
B1	Half	12.5	4.9	58%
B2	**Standard**	**25**	**8.4**	**100%**
B3	Double	50	6.9	82%
B4	One fifth	5	3.4	55%
B5	**Standard**	**25**	**6.1**	**100%**
B6	Quintuple	125	14.1	232%
B7	One third	8.3	50.5	770%
B8	**Standard**	**25**	**6.6**	**100%**
B9	Triple	75	21.4	326%

These values are shown graphically in Figure 11.

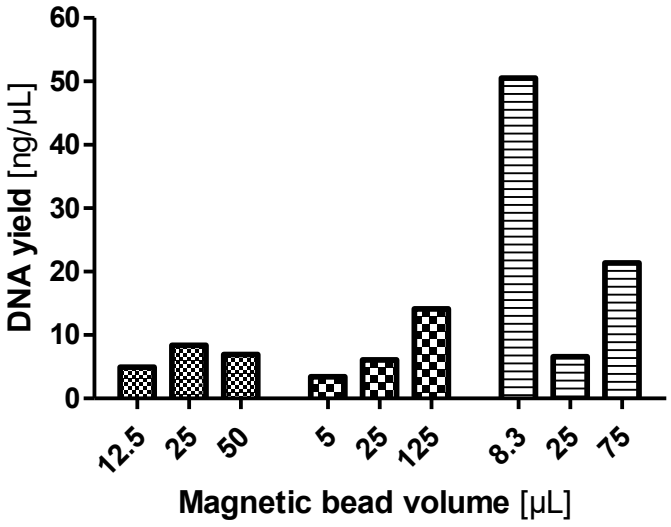

Figure 11: Variation of magnetic bead volume
Photometrically measured yield after extraction of DNA from soil with different magnetic bead volumes used; three series: 12.5µL, 25µL and 50µL; 5µl, 25µL and 125µL; 8.3µL, 25µL and 75µL

The variations of the magnetic bead volume lead to diverse results. In the first test series (B1 – B3), where bead volume was halved and doubled, the standard bead volume resulted in the highest DNA yield (8.4 ng/µL), while half of the bead volume resulted in 58% of standard yield and double volume in only 82% of the standard yield. In test series 2 (B4 – B6) results were nearly as expected: One fifth of the original volume lead to the lowest yield (55%), while the quintupled volume resulted in the highest yield (232%). Things changed in test series 3 (B7 – B9), where one third of the bead volume resulted in the highest amount of DNA by far (770%), although tripled volume still resulted in a significantly higher DNA yield (326%).

For verification of these results a 30 cycle PCR was performed with the 16S primer pair, followed by gel electrophoresis. A picture of the gel is shown in Figure 12.

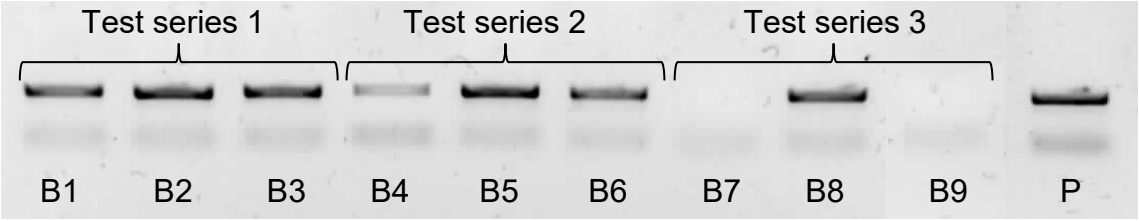

Figure 12: PCR products of DNA extracted from soil with different magnetic bead volumes
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts from soil using different magnetic bead volumes; **B1:** 12.5 µL; **B2:** 25µL; **B3:** 50µL; **B4:** 5µL; **B5:** 25µL; **B6:** 125µL; **B7:** 8.3 µL; **B8:** 25 µL; **B9:** 75µL; **P:** Positive control

As before in the experiment with the elution times, the PCR results do not always correlate with the photometric findings. While in test series 1 the PCR results match the photometric findings, where all samples show a similar yield with sample B2 (25 µL) being the strongest, followed directly by B3 (50 µL) and B1 (12.5 µL), in test series 2 the standard band (B5, 25 µL) is the strongest, followed by B6 (125 µL) and B4 (5 µL). In test series 3 only the standard band B8 (25 µL) is visible, whereas bands B7 (8.3 µL) and B9 (75 µL) show no PCR product at all. These two samples are the ones with the extremely high DNA yield in photometric measurement. Positive control (P) yielded a strong, clearly visible band.

3.2 Microchip

3.2.1 Basic Microchip design

In order to make the extraction of DNA automatable and portable, microchips were designed to perform the extraction in. The basic idea was to have a reaction chamber, where the actual extraction takes place and the magnetic beads are applied, and several storage chambers filled with the liquids needed for extraction (wash buffer, 70% isopropanol / ethanol, H_2O). Liquids would be transferred via channels milled onto the chip surface that connect the chambers to the reaction chamber. Several chips have been designed, which are shown in the following subchapters. All chips have several properties in common:

- Due to the modular system of the chip, every model has 7 chambers.
- The biggest chamber has a total volume of 2 mL, the four middle-sized ones have a volume of 1 mL and the two small ones have a total volume of 0.5 mL.
- Every chamber has a small opening for ventilation on the rear side.
- Every chamber is connected at the top to an inlet located on the side of the chip.

Silicone tubes are used to build up pressure for pumping and – partially – to transport liquids. The basic design of the chip is shown schematically in Figure 13.

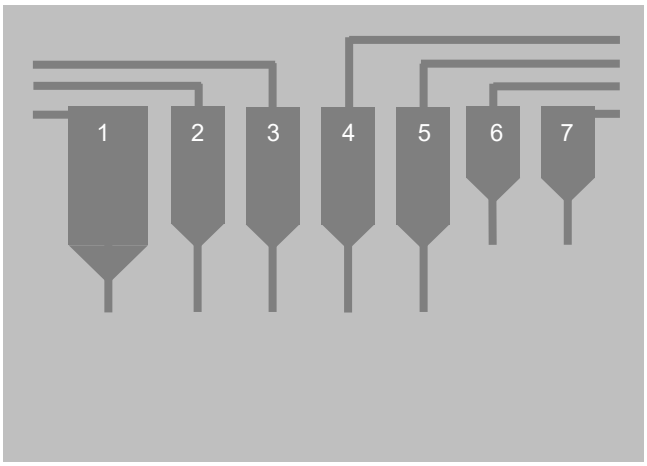

Figure 13: Basic microchip design
Schematic drawing of microchip with 7 chambers: Chamber 1 with 2 mL volume, chambers 2-4 with 1 mL volume, chambers 6-7 with 0.5 mL volume. Chambers are connected at the top with inlet openings at the side of the chip. Bottoms of chambers are connected to outlet openings located below chambers.

This basic microchip design was altered to optimize DNA extraction (chapter 3.2.3).

3.2.2 Sealing foils

As the chambers and the chip are not closed at the surface, a way to seal the chip with its channels and chambers after dry sample addition had to be found. For this purpose, PCR sealing foils used for sealing of well plates for PCR applications are ideal. These foils are self-adhesive (either directly adhesive or by pressure) and heat-resistant, as during PCR temperatures way above the ones used for DNA extraction are applied, and do not interact with DNA in any kind of way. Several foils were tested for sticking and removal, results are shown in Table 11.

Table 11: Sealing foil test

Foil	Manufacturer	Cat. No.	Type	Sticking	Removal
a	Peqlab	82-1170-99	Pressure-adhesive	Good	Good
b	Greiner Bio One	676070	Pressure-adhesive	Good	Good
c	Greiner Bio One	676040	Self-adhesive	Poor	Poor
d	Greiner Bio One	676001	Self-adhesive	Very poor	Poor
e	Peqlab	82-0558-99	Self-adhesive	Good	Very poor

It became clear that foils behave differently when it comes to sticking and removal of foil. Foils a, b and e had good sticking capabilities, while foil c and especially foil d were not sticking as well as the others. When it comes to removal, foils a and b were good, too: A lot of force was needed to remove the foil from the chip, but the foil was removed without residues and the chip surface was not sticky afterwards. Foils c, d and e left the chip with a sticky surface, while foil d was, in addition, extremely difficult to remove.

Additionally, self-adhesive foils may be problematic during extractions as solid particles from the samples and the magnetic beads may stick to the foil, especially after application of a magnet. Due to this fact, DNA extractions from *E. coli* bacteria were performed inside a microchip with each foil, followed by a 30 cycle PCR with 16S rRNA primers. The corresponding agarose gel after gel electrophoresis is shown in Figure 14.

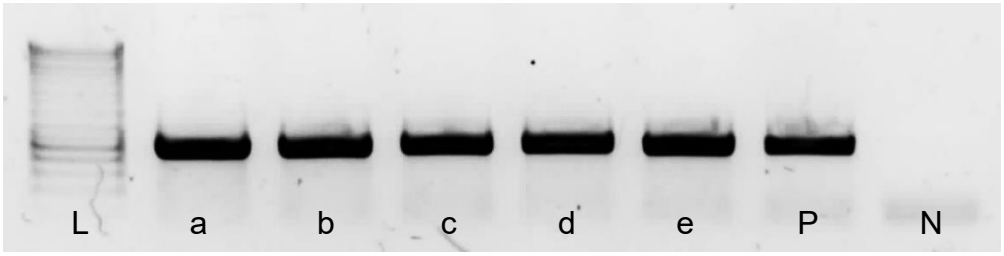

Figure 14: PCR product of extracted DNA with different sealing foils, 30 cycles
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts with different sealing foils used; **L:** 1kb Ladder; **a:** Foil a; **b:** Foil b; **c:** Foil c; **d:** Foil d; **e:** Foil e; **P:** Positive control; **N:** Negative control

On the agarose gel in Figure 14 no difference in band strength can be seen between the different samples. All bands including the positive control are very strong, suggesting that DNA yield after extraction was very high. Only negative control (N) showed no band at all, as expected.

In order to make differences visible, a PCR with only 20 cycles was performed using the same primers as before, followed by gel electrophoresis. The results are shown in Figure 15.

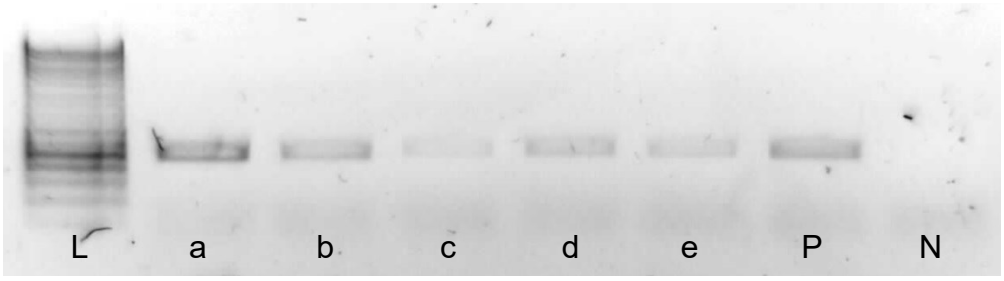

Figure 15: PCR product of extracted DNA with different sealing foils, 20 cycles
Gel electrophoresis with PCR products (8F + 926R primer pair, 20 cycles) of DNA extracts with different sealing foils used; **L:** 1kb Ladder; **a:** Foil a; **b:** Foil b; **c:** Foil c; **d:** Foil d; **e:** Foil e; **P:** Positive control; **N:** Negative control

In Figure 15 differences between the bands can finally be seen. The strongest band, in this case even stronger than the positive control, was achieved with foil a, followed by foils b, d, e and c in descending band strength order. As foil a also had good sticking and removal properties (see Table 11 for reference), all future experiments were performed using foil a. Negative control (N) yielded no visible band, as expected, while a band was clearly visible for positive control (P).

3.2.3 Chip version 1 (V1)

In the first version of the chip (V1) chambers were interconnected pairwise as shown in Figure 16.

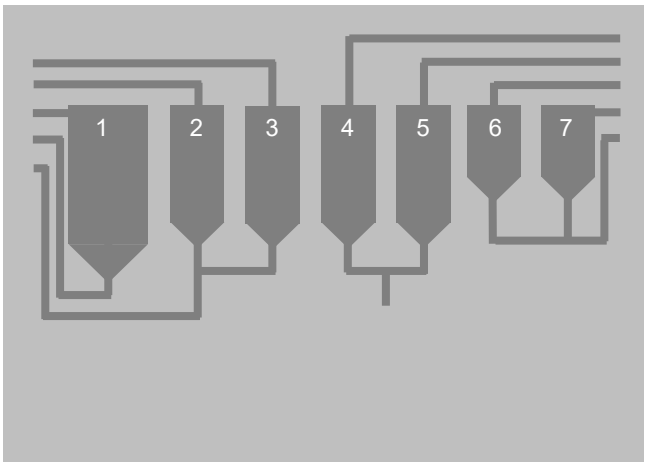

Figure 16: Microchip design V1
Schematic drawing of microchip design V1. Chamber 1 with 2 mL volume, chambers 2-4 with 1 mL volume, chambers 6-7 with 0.5 mL volume. Chambers are connected at the top with inlet openings at the side of the chip. Bottoms of chambers are connected to outlet openings located below chambers. Chambers of the same size are interconnected pairwise with each other at the bottom.

Figure 16 shows that chamber 1 was intended for use as a reaction chamber due to the higher capacity. As every liquid used for extraction is used twice according to the kit manufacturer's protocol, chambers were interconnected pairwise to reduce the amount of tubing needed. Liquid would be transferred via tubes to the reaction chamber (1).

There are several disadvantages with this design. Lots of tubing is needed to connect the tubes to each other, as liquids leave the chip and immediately re-enter the chip after a short tube passage. Thus, in future versions of the chip the chambers will be interconnected directly using channels milled onto the chip surface. Additionally, the pairwise interconnection of chambers is impractical, as liquids from one chamber tend to enter the other chamber when pressure is applied making the volumes transferred inaccurate.

3.2.4 Chip version 2 (V2)

With the disadvantages of chip version 1 in mind a new version was designed, shown in Figure 17.

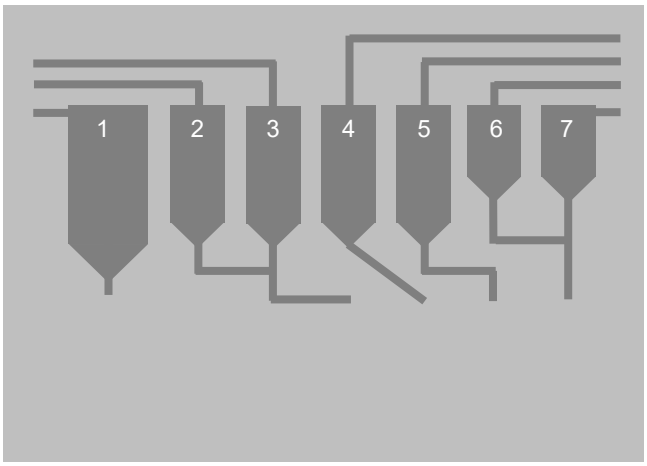

Figure 17: Microchip design V2
Schematic drawing of microchip design V2. Chamber 1 with 2 mL volume, chambers 2-4 with 1 mL volume, chambers 6-7 with 0.5 mL volume. Chambers are connected at the top with inlet openings at the side of the chip. Bottoms of chambers are connected to outlet openings located below chambers. Chambers 2 & 3 and 6 & 7 are interconnected at the bottom. Two different channel interconnection designs were used.

In Figure 17 it can be seen that in chip V2 the reaction chamber was now chamber 4, which is not connected to another chamber directly on the chip. Interconnection of chambers 2 & 3 and 6 & 7 was realized in two different ways to test fluid pumping properties. After several tests with this chip version it became clear that none of the two versions was any better than the previous design from chip V1 (see chapter 3.2.3 for reference). Thus, a new improved chip design had to be developed (chapter 3.2.8).

3.2.5 Sedimentation

As a centrifugation step is difficult to realize in a portable small-scale microchip, centrifugation was replaced by sedimentation. In this experiment 750 µL lysis buffer was added directly to a soil sample in a 1.5 mL reaction tube, homogenized by vortexing and let to sediment for a specific time, ranging from 1 to 15 min. After sedimentation, the supernatant was transferred into a new tube, where the regular extraction protocol was continued. The different sedimentation times and photometrically measured DNA yields are shown in Table 12.

Table 12: Sedimentation times

Sample	Weight [g]	Sedimentation time [min]	DNA concentration [ng/µL]	260/280 ratio	260/230 ratio
a	0.255	1	12.3	3.17	0.02
b	0.248	2	17.1	1.52	0.11
c	0.259	5	7.9	1.69	0.07
d	0.252	10	5.1	1.50	0.06
e	0.250	15	2.7	3.38	0.02

Photometric results suggest that the highest DNA yield was achieved after 2 min sedimentation, followed by 1 min and then in descending order with longer times, which is surprising. 260/280 ratios were not good, some were low, ranging between 1.50 and 1.69, and some were even above 3. 260/230 ratios were very bad with values between 0.02 and 0.11. To verify these findings, a PCR with the 16S rRNA primer pair and 30 cycles was performed. An agarose gel with the PCR products is shown in Figure 18.

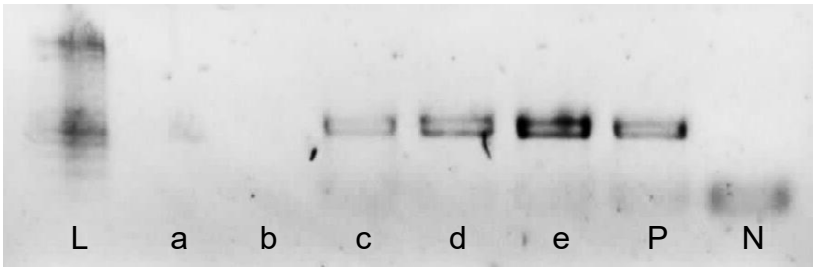

Figure 18: Sedimentation times
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts after different sedimentation times; L: 1kb Ladder; **a:** 1min; **b:** 2min; **c:** 5min; **d:** 10min; **e:** 15min; **P:** Positive control; **N:** Negative control

The results after PCR shown in Figure 18 are contradictory to the previous photometric results. Here, DNA yield was higher with longer sedimentation time, as expected, while photometric results tell a different story (Table 12). A sedimentation time of 15 min seems to be sufficient, as DNA yield after PCR was even higher than in the positive control (P). There was no band visible at all for the negative control (N), as expected.

3.2.6 Sedimentation: Height of solid phase

In order to make sedimentation possible inside a microchip, a sedimentation chamber had to be introduced. This chamber would be the biggest chamber (1) on the chip. A channel was needed for transfer of supernatant to the reaction chamber. This channel would be milled in a specific height above the solid phase that develops during sedimentation. In order to define this specific height, sedimentation tests with 750 µL lysis buffer and soil were performed inside microchip version V2 (chapter 3.2.4) and the height of the solid phase was measured. The results are shown in Table 13.

Table 13: Sedimentation: Solid phase height

Weight [g]	**Solid phase height** [cm]
0.250	1.1
0.500	1.4

As solid phase height may vary with every sample, the supernatant channel was defined at 1.65 cm, leaving a safety distance to the highest measured liquid phase (1.4 cm).

3.2.7 Sedimentation: Location of DNA in liquid phase

As it is unclear if DNA settles down inside the liquid phase, which would mean losing large amounts of DNA upon transfer from the top of the liquid phase after sedimentation, especially in microchips with a sedimentation chamber, where the outlet for the supernatant is not directly above the solid phase (see previous chapter), this fact had to be spotlighted. Two test series were performed in reaction tubes, test series 1 with 0.504 g soil and test series 2 with 0.501 g soil. 1,000 µL lysis and binding buffer was added to each soil sample and let to settle. After 15 min, 250 µL of supernatant from the top of the liquid phase and 250 µL from the bottom of the liquid phase were transferred to new tubes, were regular extraction of DNA was performed. After extraction, DNA yield was measured photometrically, the results are shown in Table 14.

Table 14: Extraction of DNA from bottom and top of liquid phase after sedimentation

Test series	Sample	Location	DNA yield [ng/µL]
1	B1	Bottom	6.1
	T1	Top	7.3
2	B2	Bottom	5.0
	T2	Top	4.9

As Table 14 shows, in test series one the difference in DNA yield was 1.2 ng/µL, meaning that DNA yield in the bottom of the liquid phase was 16% lower than in the top phase. Things changed in test series two, where the difference was extremely small (0.1 ng/µL) with the bottom part having the slightly higher yield. To verify these findings, a 30 cycle PCR with 16S primer pair was performed, followed by gel electrophoresis. A picture of the gel is shown in Figure 19.

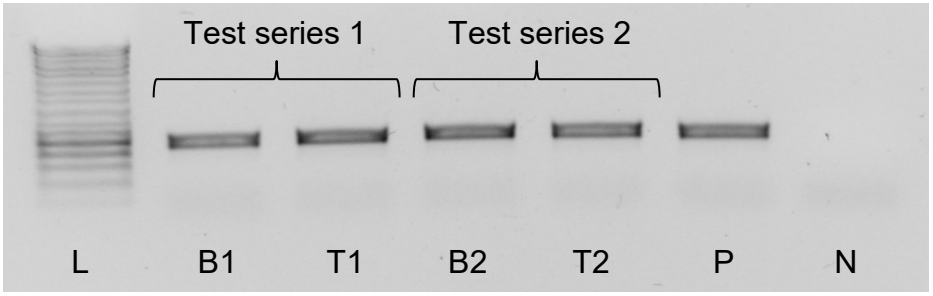

Figure 19: Extraction of DNA from bottom and top of liquid phase after sedimentation
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts taken either from top or from bottom of liquid phase after sedimentation; **L:** 1kb Ladder; **B1:** Test series 1, bottom of liquid phase; **T1:** Test series 1, top of liquid phase; **B2:** Test series 2, bottom of liquid phase; **T2:** Test series 2, top of liquid phase; **P:** Positive control; **N:** Negative control

In Figure 19 there are, in contrast to the photometric findings, no significant differences in band strength of the PCR products visible, suggesting that DNA does not settle down during the sedimentation step and that the amount of DNA is equal anywhere in the sedimentation supernatant phase. Positive control (P) yielded a strong band as well, while negative control (N) showed, as expected, no band at all.

3.2.8 Chip version 3 (V3)

After the problems with chamber interconnections in the previous chip versions (chapters 3.2.3 and 3.2.4) a new version V3, shown in Figure 20, was designed.

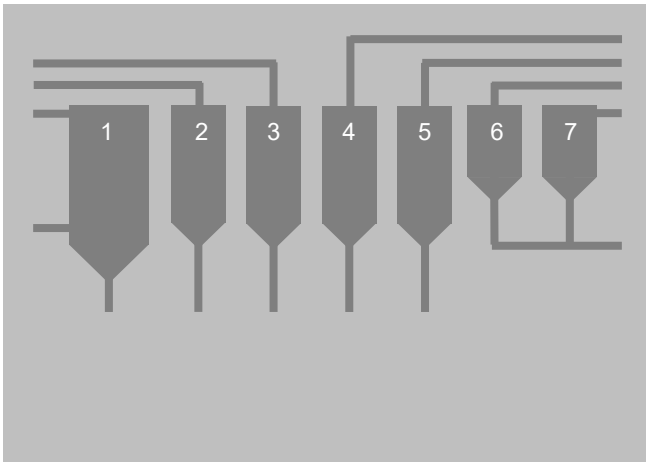

Figure 20: Microchip design V3
Schematic drawing of microchip design V3. Chamber 1 with 2 mL volume, chambers 2-4 with 1 mL volume, chambers 6-7 with 0.5 mL volume. Chambers are connected at the top with inlet openings at the side of the chip. Bottoms of chambers are connected to outlet openings located below chambers. Chambers 6 & 7 are interconnected at the bottom. Chamber 1 is used as sedimentation chamber with additional outlet above solid phase height (see chapter 3.2.6).

The most important innovation in this version, pictured in Figure 20, is the sedimentation chamber (1) which has an outlet channel milled onto the chip surface at 1.65 cm height for transfer of supernatant after sedimentation (see chapter 3.2.6 for reference). All other chambers except for the smaller chambers 5 and 6, used for water, are not interconnected with each other. With this configuration, fluid transfer between

chambers is possible without problems, except for chamber 6 and 7, where liquids tend to enter the interconnected chamber instead of the outlet tube leading to the reaction chamber. A major drawback with this configuration is the immense amount of tubing and pinch clamps needed to transfer fluids without replugging, which leads to fluid loss.

3.2.9 Sedimentation yield

With the new sedimentation chamber established, the supernatant yield was analyzed. For this, approximately 0.5 g of soil was filled into the sedimentation chamber and 750 µL lysis & binding buffer was added. After 15 min of sedimentation the supernatant was pumped into a 1.5 mL reaction tube. The tube was weighed before and after supernatant addition. The difference between both values was the weight of the supernatant. Results are shown in Table 15 below.

Table 15: Sedimentation Yield

Sample	**Weight** [g]			**Yield** [g]		
	Soil	**Tube empty**	**Tube full**	**Sample**	**Mean**	**Standard Deviation**
a	0.498	0.514	0.792	0.278	0.266	0.0432
b	0.502	0.513	0.776	0.263		
c	0.508	0.516	0.799	0.283		
d	0.507	0.508	0.747	0.239		
e	0.505	0.509	0.858	0.349		
f	0.504	0.513	0.741	0.228		
g	0.509	0.509	0.734	0.225		

These results are shown graphically in Figure 21.

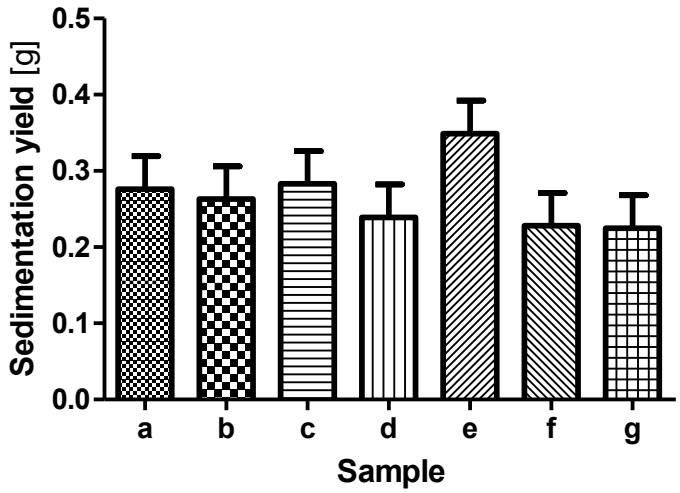

Figure 21: Sedimentation Yield
Supernatant yield after 15min of sedimentation from soil samples, columns with standard deviation

It becomes clear that supernatant yield is constant throughout this experiment, ranging from 0.225 g to 0.349 g with a very low standard deviation of only 0.0432. There was only one sample (e) where yield was much higher than the others with 0.349 g.

3.2.10 Chip version 4 (V4)

With the results of the sedimentation tests using the sedimentation chamber of chip version 3, a new design V4 was developed with milled channels connecting the supply chambers with the reaction chamber, as shown in the following schematic drawing (Figure 22).

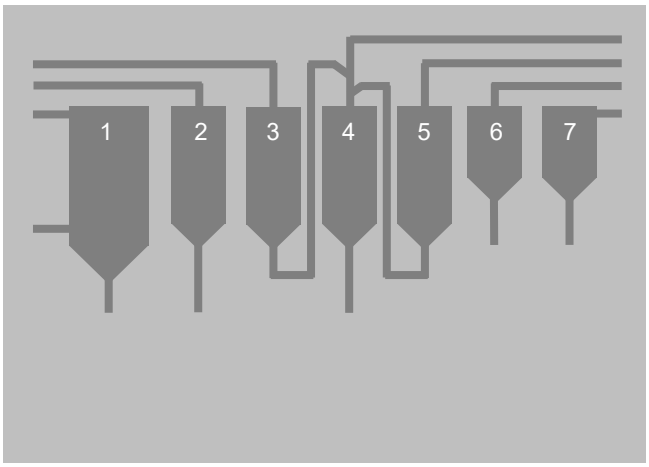

Figure 22: Microchip design V4
Schematic drawing of microchip design V4. Chamber 1 with 2 mL volume, chambers 2-4 with 1 mL volume, chambers 6-7 with 0.5 mL volume. Chambers are connected at the top with inlet openings at the side of the chip. Chamber 1 is used as sedimentation chamber with additional outlet above solid phase height (see chapter 3.2.6). Chambers 3 and 5 are connected at the bottom to the inlet channel of the reaction chamber (4). Bottoms of the other chambers are connected to rear outlet openings located below chambers.

In chip version V4 (Figure 22) the outlets of supply chambers 3 (wash buffer I) and 5 (70% isopropanol / ethanol) are connected directly to the inlet of reaction chamber 4 via channels milled onto the chip surface. There is no direct cross section between these, as chamber 3 connects above the connection of chamber 5 to avoid fluids travelling between chambers 3 and 5 instead of chamber 4. With this direct connection of chambers less tubing is needed. The interconnection between chambers 6 and 7, as present in the previous chip version V3 (chapter 3.2.8), was discarded.

After testing of this new chip design, it became clear that the connection of chambers 3, 4 and 5 was problematic. Although chamber 3 connects above channel 5 to channel 4, fluids often tend to “skip” reaction chamber 4 and travel directly into chamber 5 and vice versa. This problem was solved partly by applying back pressure to the other chamber upon pumping into the reaction chamber.

3.2.11 Comparison between extraction in reaction tube and in chip

As it is not known if the chip and the other materials used (tubing, foil) interact with DNA or interfere with the extraction process itself, the difference in yield between extraction in a reaction tube (T), as suggested by the kit’s manufacturer, and in a microchip (C) was evaluated. Several extractions were performed from the same soil sample each with every method. Sample weights and photometrically measured DNA yield are shown in Table 16.

Table 16: Comparison between DNA extraction in tube and in chip from soil samples

Soil	Sample	Weight [g]	DNA yield [ng/µL]	260/280 ratio	260/230 ratio
1	T1	0.502	4.9	1.27	0.07
	C1	0.503	5.9	1.31	0.03
2	T2	0.505	47.4	1.37	0.36
	C2	0.503	189.2	1.31	0.49
3	T3	0.502	5.9	1.88	0.10
	C3	0.506	7.4	1.51	0.05

As shown in Table 16, for soil one and soil three the extraction in a chip resulted in slightly better yields (4.9 versus 5.9 and 5.9 versus 7.4). With soil two very high DNA yields were achieved: 47.4 ng/µL with the tube and 189.2 ng/µL with the chip. In this case, extraction in a chip resulted in a fourfold increased yield. All 260/280 ratios were bad, ranging between 1.27 and 1.37, except for the extractions from soil three, where with the chip a ratio of 1.51 and with the tube a very good ratio of 1.88 were achieved. 260/230 ratios were all very poor with values between 0.03 and 0.36.

To verify these findings, a PCR with 30 cycles and 16S primer pair was performed, followed by gel electrophoresis. The PCR products on the agarose gel are shown in Figure 23.

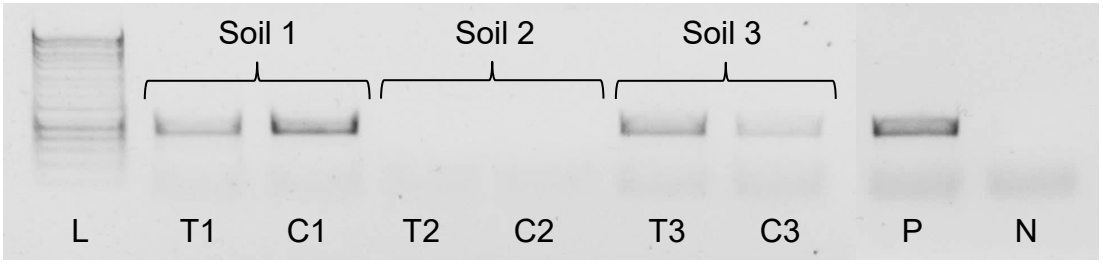

Figure 23: Comparison between extraction of DNA in tube and in chip from soil
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts from 3 different soil samples (1-3), performed inside reaction tube (T) or chip (C); **L:** 1kb Ladder; **T1:** Soil 1, reaction tube; **C1:** Soil 1, chip; **T2:** Soil 2, reaction tube; **C2:** Soil 2, chip; **T3:** Soil 3, reaction tube; **C3:** Soil 3, chip; **P:** Positive control; **N:** Negative control

As Figure 23 shows, the band strengths of the PCR products do not match all results of the photometrical DNA yield measurement (Table 16). With soil one the band of the chip extraction (C1) is stronger than the one from the reaction tube extraction (T1), which matches the photometrical findings, although the difference in the photometrically measured yield is rather small. The PCR results of soil two do not match the photometric results that showed a very high DNA yield, as there are no PCR products visible at all on the gel (bands T2 and C2). With soil three photometric results are even contradictory to the PCR findings: Photometrically measured DNA yield of

the chip extraction is higher than the one extracted in a reaction tube, while on the agarose gel the band for the reaction tube extraction (T3) is stronger than the one for the chip extraction (C3). Positive control (P) was successful with a very strong band as well as negative control (N), where no band was visible. Generally, it can be said that if PCR is possible after an extraction of DNA in a reaction tube or in a chip, a PCR with the extract from the other method is successful as well.

To verify these findings and to eliminate the influence of soil on DNA yield, DNA was extracted out of pure *E. coli* bacteria overnight liquid culture, once in a tube (T4) and once in a chip (C4). A reduced amount of lysis and binding buffer (500 µL) was applied; otherwise the protocol was standard with quarter volumes, 15 min sedimentation time etc. 250 µL of bacteria culture in TB medium were used for each extraction. Due to the reduced lysis buffer volume sedimentation was performed in tubes for both extractions. After sedimentation, extraction was continued in tube or chip, respectively. DNA yield was measured photometrically; results are shown in Table 17.

Table 17: Comparison between DNA extraction in tube and in chip from *E. coli* overnight culture

Sample	DNA yield [ng/µL]	260/280 ratio	260/230 ratio
T4	15.2	1.65	0.34
C4	21.0	2.28	0.04

The photometric findings match the previous photometric results (Table 16), where extractions in chip always lead to a higher DNA yield. In this experiment extraction in a tube yielded 15.2 ng/µL DNA, whereas extraction in a microchip resulted in 21.0 ng/µL DNA. The 260/280 ratio from the tube extraction was acceptable (1.65), whereas the ratio for the chip extraction was superior (2.28). The 260/230 ratios were both very poor, with the tube reaction having a slightly better ratio (0.34) than the chip extraction (0.04). To verify these findings, three PCRs with the 16S primer pair were performed with 30, 25 and 20 cycles to make the difference in yield visible. After gel electrophoresis, the following pictures (Figure 24) were taken.

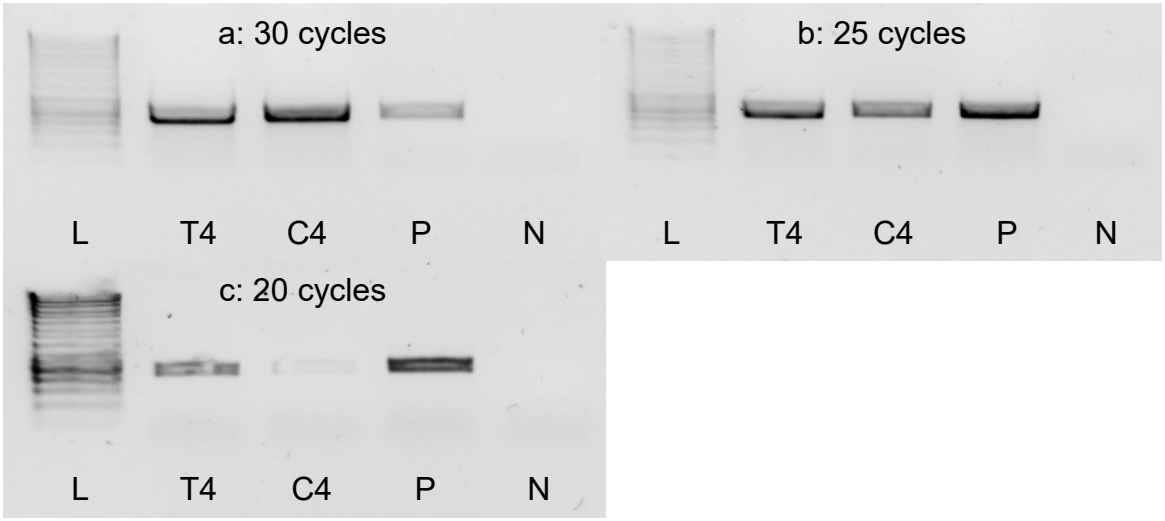

Figure 24: Comparison between extraction of DNA in tube and in chip from *E. coli* culture
Gel electrophoresis with PCR products (8F + 926R primer pair; **a:** 30 cycles; **b:** 25 cycles; **c:** 20 cycles) of DNA extracts from *E. coli* overnight culture, performed inside reaction tube (T) or chip (C); **L:** 1kb Ladder; **T4:** Reaction tube extraction; **C4:** Chip extraction; **P:** Positive control; **N:** Negative control

In Figure 24a, it can be seen that after 30 PCR cycles the band from the chip extraction (C4) is stronger than the one from tube extraction (T4). This matched the photometric findings mentioned earlier (Table 17). After 25 cycles, the results changed: Here, the tube extraction (T4) resulted in a stronger band than the one from the microchip extraction (C4). The same appeals to the results after 20 PCR cycles: The tube reaction's band (T4) is clearly visible, while the one from chip extraction (C4) is barely visible. All positive controls (P) show strong bands, except for the one from the 30 cycle PCR, which is significantly weaker. All negative controls (N) showed no bands at all, as expected.

3.3 Normalizing of soil samples

As soil is no homogenous sample material and changes its microflora and bacteria count depending on several factors like season, site and weather [78] [82] and also changes its properties (moisture, salinity, nutrients) [84], a way to equalize DNA samples had to be found.

3.3.1 Creation of aliquots from soil

The first idea was to acquire a soil sample, mix it with lysis buffer, homogenize it and create smaller aliquots which would then be stored at -80°C. With this, the lysis step would already be performed within the soil sample and DNA would be preserved by freezing. Theoretically, the amount of DNA should then be constant in every aliquot making it easier to compare DNA extraction results.

For this experiment, 10.012 g soil was mixed with 15 mL lysis & binding buffer and homogenized afterwards. The mixture was allowed to settle for 15 min, after which the supernatant was transferred into freezing tubes in aliquots of 1 mL each. Ten aliquots were obtained out of the soil sample and were subsequently frozen at -80°C for long-term storage.

After multiple days of freezing, three samples were thawed and DNA was extracted in a reaction tube. DNA yield was measured photometrically, results are shown in Table 18.

Table 18: DNA yield from extraction of soil aliquots in reaction tube

Sample	Aliquot	DNA yield [ng/µL]	Standard deviation	260/280 ratio	260/230 ratio
a	2	5.7	0.92	1.61	0.16
b	6	3.9		1.28	0.05
c	10	5.3		1.85	0.04

Table 18 shows that two aliquots, 2 and 10, showed similar yields after extraction (5.7 ng/µL and 5.3 ng/µL), while the third extraction, where aliquot 6 was used, resulted in a slightly lower yield of 3.9 ng/µL. With these values, a standard deviation of 0.92 was calculated. The 260/280 ratios were bad (sample b, 1.28), good (sample a, 1.61) and very good (sample c, 1.85). 260/230 ratios were all very bad, ranging between 0.04 and 0.16.

To verify these findings, a 30 cycle PCR with 8F + 926R primer pair was performed. A gel electrophoresis picture with the PCR products is shown in Figure 25.

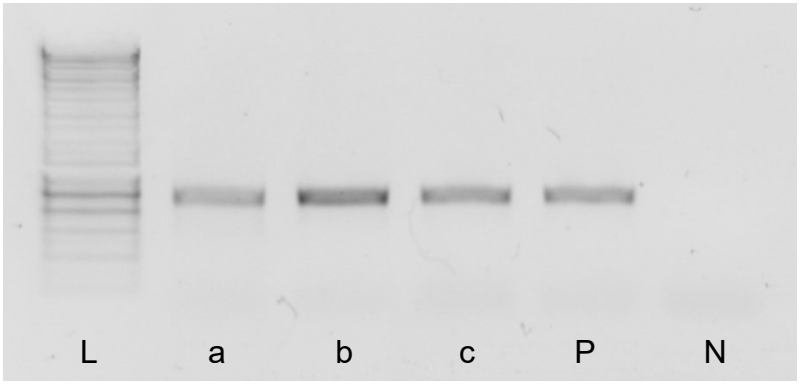

Figure 25: DNA extraction from soil aliquots in reaction tube
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts from 3 different soil aliquots; **L:** 1kb Ladder; **a:** aliquot 2; **b:** aliquot 6; **c:** aliquot 10; **P:** Positive control; **N:** Negative control

The band strengths on the agarose gel are, again, contradictory to the photometrical findings: With both analysis methods DNA yield was measurable / visible and samples a and c show similar yields, but photometrically measured DNA yield of sample b was lower than the others, while on the gel the band strength of sample b was stronger, suggesting that DNA yield upon extraction was higher. Nevertheless, DNA yield is mostly constant with a standard deviation of 0.92. Positive control (P) showed a clear band and negative control (N) showed no band at all, both as predicted.

The extractions were repeated with different aliquots, performed in a chip. The photometrically measured DNA yields are shown in Table 19.

Table 19: DNA yield from extraction of soil aliquots in chip

Sample	Aliquot	DNA yield [ng/µL]	Standard deviation	260/280 ratio	260/230 ratio
a	5	4.8	1.7	1.39	0.04
b		6.5		1.87	0.01
c	1	2.3		1.28	0.05
d		4.0		1.76	0.05
e		2.6		1.84	0.12

Photometric Results from Table 19 are worse than the previous results (Table 18). Standard deviation is almost twice as high. This time extractions were repeated from the same aliquot, but even then, there were differences: DNA yields from aliquot 5 were 4.8 ng/µL and 6.5 ng/µL, from aliquot 1 yields were 2.3 ng/µL, 4.0 ng/µL and 2.6 ng/µL. Some 260/280 ratios were very good with values between 1.76 and 1.84 (samples b, d and e), while others were poor (sample a: 1.39 and sample c: 1.28). All 260/230 ratios were very poor, ranging between 0.01 and 0.12.

For verification of these results a PCR was performed with 30 cycles and 16S primers. The gel electrophoresis results are shown in Figure 26.

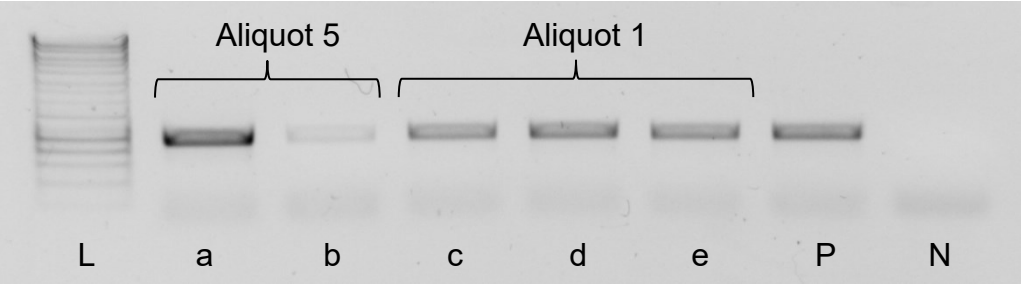

Figure 26: DNA extraction from soil aliquots in chip
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts from 2 different soil aliquots; **L:** 1kb Ladder; **a-b:** aliquot 5; **c-e:** aliquot 1; **P:** Positive control; **N:** Negative control

As in the previous experiments, the photometric results do not match the PCR results. While PCR products from aliquot 1 (samples c – e) show a similar band strength, photometric results suggest a slightly stronger band for sample d. Sample a shows the strongest band strength and sample b the lowest, hardly visible, although they were both from the same aliquot. Photometric results for these samples are vice versa: Sample b had a slightly higher yield than sample a, although the difference was not as high as on the gel. Positive control (P) yielded a strong band, while negative control (N) showed no band.

As results were not consistent at all, new soil aliquots were created. For this, 29,996 g of fresh soil was mixed with 45 mL lysis and binding buffer, homogenized and let to settle for 15 min. Afterwards supernatant was transferred into freezing tubes yielding 25 aliquots. In contrast to the previous experiment, DNA was extracted from the same aliquot (2). The photometrically measured DNA yield is shown in Table 20.

Table 20: DNA yield from extraction of soil aliquots in chip, continued

Sample	Aliquot	DNA yield [ng/µL]	Standard deviation	260/280 ratio	260/230 ratio
a	2	5.8	1.8	1.46	0.10
b		4.5		1.20	0.07
c		2.2		1.30	0.09

Photometric results in Table 20 show that DNA yield varied. From the same aliquot three different yields were achieved: 2.2 ng/µL, 4.5 ng/µL and 5.8 ng/µL, resulting in a standard deviation of 1.8. 260/280 ratios were poor, being 1.20, 1.30 and 1.46, while 260/230 ratios were all very poor with values between 0.07 and 0.10. For verification a PCR with 16S primers and 30 cycles was performed. A gel electrophoresis with the PCR products followed, shown in Figure 27.

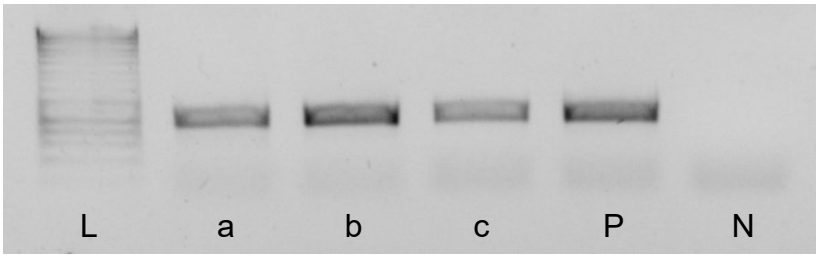

Figure 27: DNA extraction from soil aliquots in chip, continued
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts from 3 different soil aliquots; **L:** 1kb Ladder; **a-c:** aliquot 2; **P:** Positive control; **N:** Negative control

In Figure 27 it can be clearly seen that DNA yield was quite high in all three extracts. But there are still differences: Sample b shows the strongest band, followed by sample a and sample c, which has the weakest band, although still clearly visible. This stands in contrast to the photometric findings, were sample a had the strongest yield, followed by b and c. Positive control (P) had a very strong band, while negative control (N) showed no band at all.

3.3.2 EGFP *E. coli* bacteria

Another approach to normalized soil samples was to spike a soil sample with a defined amount of genetically modified *E. coli* bacteria. After extraction, the DNA of these bacteria could easily be identified by PCR with the right primers. For these experiments bacteria were obtained from a member of our group who transfected *E. coli* bacteria with a genetically engineered pDisplay vector containing a gene for the enhanced green fluorescent protein (EGFP), which can later be identified by PCR, and an ampicillin resistance for selection upon cultivation. The EGFP gene codes for a 238 amino acids long protein (26.9 kDa) emitting fluorescence when exposed to light in the right range. This protein originates from *Aequorea Victoria*, a jellyfish also known as crystal jelly.

The vector used here is shown in Figure 28 below.

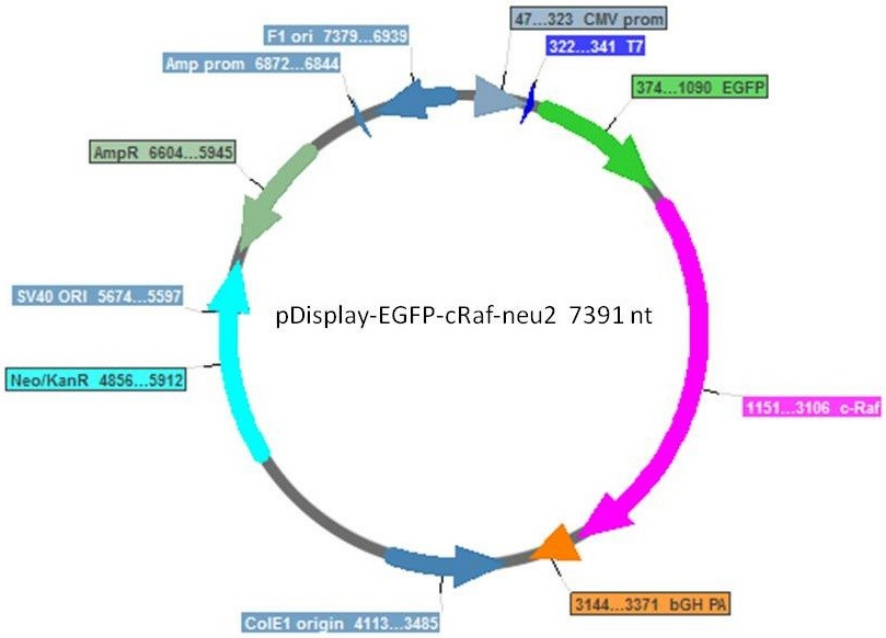

Figure 28: pDisplay vector with EGFP gene and ampicillin resistance
Schematic drawing of genetically engineered pDisplay vector "pDisplay-EGFP-cRaf-neu2 7391 nt"; **T7:** T7 promoter system for EGFP gene; **EGFP:** EGFP gene; **AmpR:** Ampicillin resistance; **Amp prom:** Ampicillin resistance promoter; **c-Raf:** RAF proto-oncogene serine/threonine-protein kinase gene (not used); **bGH PA:** Bovine growth hormone polyadenylation signal (termination sequence; not used) **CMV prom:** Cytomegalovirus promoter sequence; **Neo/KanR:** Neomycin / kanamycin resistance (not used); **ColE1 origin:** ColE1 plasmid origin of replication; **SV40 ORI:** SV40 plasmid origin of replication; **F1 ori:** F1 plasmid origin of replication

At first, EGFP *E. coli* bacteria were cultivated and DNA was extracted from these. In order to test the new EGFP primers (see Table 2 for reference), two PCRs with 30 cycles were performed with the extracts, one with the EGFP primers and one with the 16S rRNA primers used in previous experiments. Subsequently a gel electrophoresis was performed; the results are shown in Figure 29.

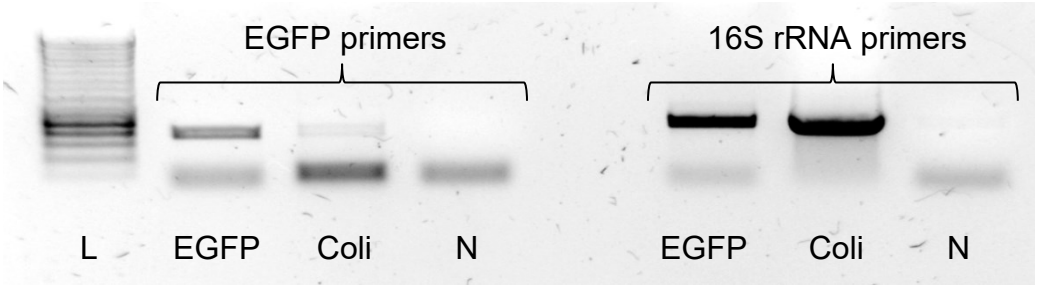

Figure 29: PCR with EGFP primers and 16S rRNA primers
Gel electrophoresis with PCR products (EGFP and 16S rRNA primer pairs, 30 cycles) of DNA extracts from EGFP *E. coli* bacteria; **L:** 1kb Ladder; **EGFP:** Extract from EGFP *E. coli* bacteria; **Coli:** *E. coli* bacteria without EGFP gene; **N:** Negative control

Figure 29 clearly shows that extraction and amplification of the EGFP gene was possible. With the EGFP primer pair a clear band is visible for the EGFP *E. coli* extract. However, there is also a very weak band in the positive control visible, which should not be, as the bacteria DNA was extracted from did not contain an EGFP gene. On the other side, with the 16S rRNA primers, results are as expected: Strong bands for both

extracts. Thus, the EGFP primers can be used for identification of EGFP *E. coli* bacteria in the following experiments. All negative controls (N) yielded no visible bands.

3.3.3 Spiking of soil samples with EGFP *E. coli* bacteria

With the EGFP *E. coli* bacteria and EGFP primer pair established, the first spiking experiment was conducted. Three DNA extractions were performed: From pure EGFP *E. coli* overnight culture (R), from a spiked sample (S) and from soil without bacteria addition (E). For all three samples 15 min sedimentation instead of centrifugation was used, even for the bacteria culture. For spiking, 0.506 g of soil were covered with 1 mL of EGFP *E. coli* overnight culture and homogenized well. The soil sample consisted of 0.500 g soil. DNA was extracted using the established DNA extraction protocol. DNA yield was measured photometrically and is shown in the following Table 21.

Table 21: Spiking of soil samples with EGFP *E. coli* bacteria

Sample	Description	DNA yield [ng/µL]	260/280 ratio	260/230 ratio
R	Pure *E. coli* overnight culture	19.2	2.02	0.08
S	Soil spiked with EGFP *E. coli*	12.7	1.73	0.21
E	Pure soil	8.0	1.84	0.04

Photometric results turned out as expected: DNA yield from pure *E. coli* overnight culture was the highest with 19.2 ng/µL, followed by the spiked soil sample with 12.7 ng/µL and pure soil with only 8.0 ng/µL. As the photometer measures all DNA present in the extract and not only EGFP DNA, a 30 cycle PCR was performed with the EGFP primer pair, followed by gel electrophoresis. The results are shown in Figure 30.

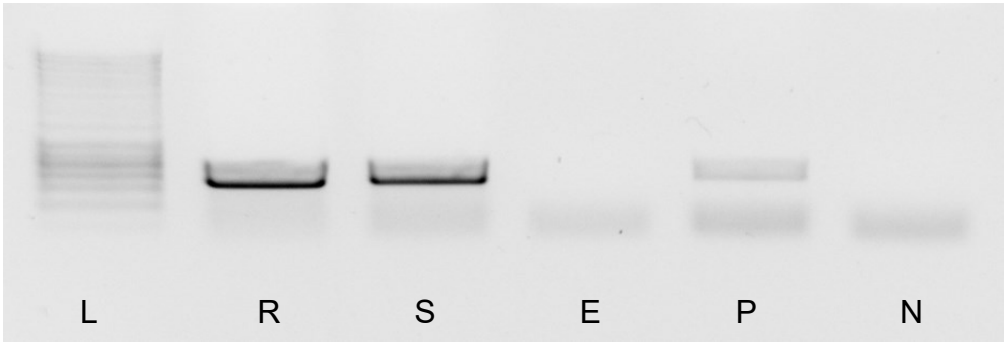

Figure 30: Spiking of soil with EGFP *E. coli* bacteria
Gel electrophoresis with PCR products (EGFP primer pair, 30 cycles) of DNA extracts from soil spiked with EGFP *E. coli* bacteria; **L:** 1kb Ladder; **R:** Pure EGFP *E. coli* bacteria; **S:** Soil spiked with EGFP *E. coli* bacteria; **E:** Pure soil without bacteria addition; **P:** Positive control; **N:** Negative control

In Figure 30 it can clearly be seen that spiking of soil samples with bacteria is possible. The band of the spiked sample (S) is of equal strength as the band from the pure bacteria culture (R). In the soil sample (E) no band was visible at all, as there were no EGFP *E. coli* added to this sample. Positive control (P) worked as well, although the band was surprisingly weak, but still visible. Negative control (N) was not visible at all, as expected.

3.3.4 Evaluation of sensitivity

In order to evaluate the sensitivity of the extraction system, several dilution series of *E. coli* bacteria cultures were created and DNA was extracted from these. First, an EGFP *E. coli* bacteria stock solution was created with an optical density of OD_{600} = 1.6 to produce comparable amounts of bacteria for every experiment. For reference, 100 µL of bacteria culture were seeded in 1:10,000,000 dilution onto four petri dishes with LB agar and colonies were counted after one day incubation at 37°C. The counting results, converted to cells per mL, are shown in Table 22 and graphically in Figure 31.

Table 22: Cell counting at OD_{600} = 1.6

Plate	Colony count	Dilution	Cells per mL	Mean	Standard deviation
a	103	1:10,000,000	$1.03 \cdot 10^{10}$	$1.17 \cdot 10^{10}$	$1.30 \cdot 10^{9}$
b	133		$1.33 \cdot 10^{10}$		
c	120		$1.20 \cdot 10^{10}$		
d	110		$1.10 \cdot 10^{10}$		

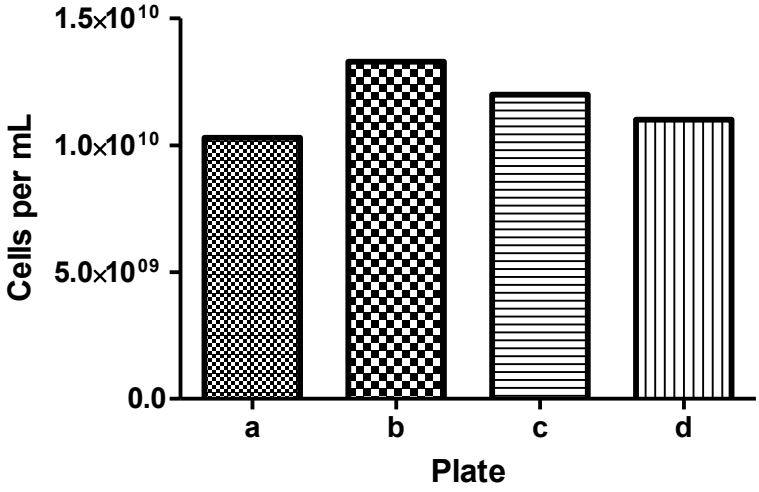

Figure 31: Cell counting at OD_{600} = 1.6
Four plates (a – d) were seeded with 100µL of 1:10,000,000 diluted bacteria culture and incubated over night at 37°C. Counting results were converted into cells per mL.

Cell counting resulted in an average cell count of $1.30 \cdot 10^{10}$ cells per mL with a standard deviation of $1.30 \cdot 10^{9}$ cells per mL. With these results the first dilution series was prepared with the following volumes: 1,000 µL, 800 µL, 600 µL, 400 µL, 200 µL, 100 µL and 50 µL. These volumes were added directly to 750 µL lysis and binding buffer. Centrifugation was used instead of sedimentation due to the low number of particles and ease of handling. Extractions were all performed in reaction tubes, followed by photometric measurement of DNA yield. The results are shown in Table 23.

Table 23: Dilution series 1

Sample	Volume [µL]	DNA yield [ng/µL]	260/280 ratio	260/230 ratio
a	1,000	10.9	1.95	0.29
b	800	11.0	2.01	0.21
c	600	6.5	3.62	0.19
d	400	7.4	1.97	0.17
e	200	4.5	2.17	0.22
f	100	5.6	1.13	0.33
g	50	3.7	0.94	0.35

The photometric results from dilution series 1 (Table 23) show a trend: DNA yield decreases with decreasing *E. coli* culture volume. However, DNA yield from the 1,000 µL and the 800 µL samples are nearly identical with 10.9 ng/µL and 11.0 ng/µL, respectively. Additionally, the DNA yield from the 400 µL sample (7.4 ng/µL) is significantly higher than yield from the 600 µL sample (6.5 ng/µL). The same appeals to the 100 µL sample which showed a higher yield (5.6 ng/µL) than the

200 µL sample (4.5 ng/µL). The 50 µL sample showed the lowest yield of them all (50 ng/µL). It also has to be said that the 260/280 ratios were superior for all samples, ranging from 1.95 to 3.62, except for the 100 µL and 50 µL samples, which were poor (1.13 and 0.94). The 260/230 ratios were, again, very poor, ranging from 0.17 to 0.35. To verify these findings, a PCR was performed with EGFP primers and 30 cycles, followed by gel electrophoresis. The corresponding gel picture is shown in Figure 32.

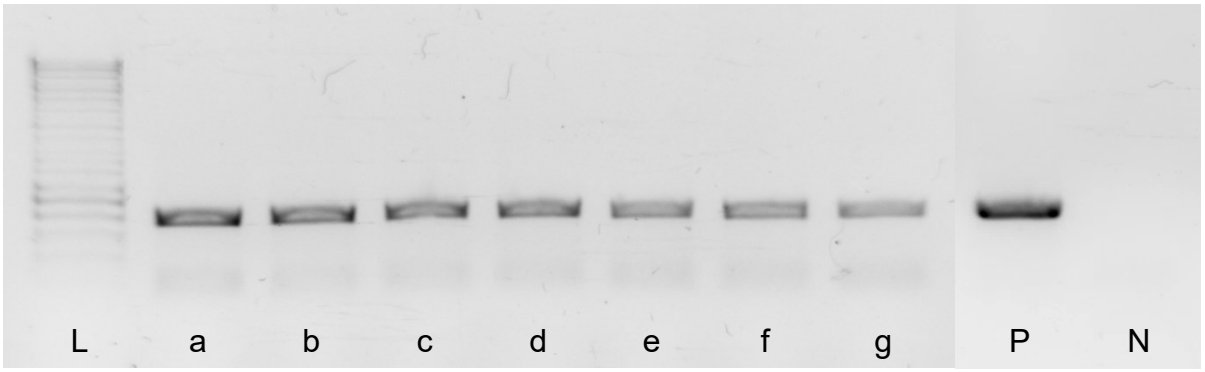

Figure 32: Gel electrophoresis picture of PCR products from dilution series 1
Gel electrophoresis with PCR products (EGFP primer pair, 30 cycles) of DNA extracts from different volumes of *E. coli* culture; **L:** 1kb Ladder; **a:** 1,000µL; **b:** 800µL, **c:** 600µL; **d:** 400µL; **e:** 200µL; **f:** 100µL; **g:** 50µL; **P:** Positive control; **N:** Negative control

The gel electrophoresis picture in Figure 32 mainly matches the photometric findings (Table 23), results were as expected: The band strength was directly proportional to the *E. coli* volume added, with the highest volume (1,000 µL) having the strongest band (a) and the lowest volume (50 µL) having the least strong band (g). However, as band g was still clearly visible, higher dilutions should be used for the next dilution series. The positive control (P) showed a very strong band, while in the negative control (N) there was, as expected, no band at all.

For dilution series 2 the following volumes of EGFP *E. coli* overnight culture with an optical density of OD_{600} = 1.6 were used: 20 µL, 10 µL, 5.0 µL, 2.5 µL, 1.0 µL and 0.5 µL. The extraction was performed in reaction tubes using the same protocol as with the previous dilution series. After extraction DNA yield was measured photometrically and results are shown in Table 24.

Table 24: Dilution series 2

Sample	Volume [µL]	DNA yield [ng/µL]	260/280 ratio	260/230 ratio
a	25	4.6	1.36	0.31
b	10	3.5	1.20	0.06
c	5.0	3.3	1.47	0.08
d	2.5	2.0	1.21	0.02
e	1.0	1.6	1.18	0.09
f	0.5	1.5	1.36	0.07

Photometric measurements, shown in Table 24, show a trend towards higher yield with increased bacteria volume. Sample a, where 25 µL bacteria solution were added, had a yield of 4.6 ng/µL, followed by sample b with 3.5 ng/µL, sample c with 3.3 ng/µL, sample d with 2.0 ng/µL, sample e with 1.6 ng/µL and sample f, where the lowest bacteria volume of only 0.5 µL was added, with 0.5 ng/µL. 260/280 ratios were average with values between 1.18 (sample e) and 1.47 (sample c), whereas 260/230 ratios were all very poor, ranging between 0.02 and 0.31.

To verify these findings, a 30 cycle PCR using EGFP primers was performed with the extracts from dilution series 2, followed by gel electrophoresis. A picture of the gel is shown in Figure 33.

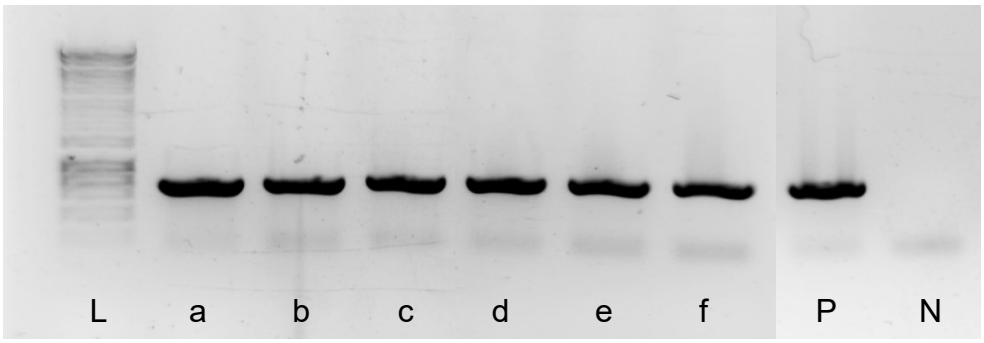

Figure 33: Gel electrophoresis picture of PCR products from dilution series 2
Gel electrophoresis with PCR products (EGFP primer pair, 30 cycles) of DNA extracts from different volumes of *E. coli* culture; **L:** 1kb Ladder; **a:** 25µL; **b:** 10µL, **c:** 5.0µL; **d:** 2.5µL; **e:** 1.0µL; **f:** 0.5µL; **P:** Positive control; **N:** Negative control

Figure 33 shows that extraction of DNA was possible, even from amounts of as little as 0.5 µL of EGFP *E. coli* liquid culture (f). All bands were very strong with no visible difference in band strength, except for band the 25 µL band (a), which was broader than the others. Positive control (P) showed a strong band, while negative control (N) showed no band at all, just as expected.

As DNA yield from extraction of DNA from pure EGFP *E. coli* culture was highly successful, even with culture volumes as low as 0.5 µL, the dilution series was

repeated with the addition of soil. Approximately 0.5 g of soil were transferred into reaction tubes and 0.0 µL, 0.5 µL, 1.0 µL, 2.5 µL, 5.0 µL and 10 µL of liquid EGFP *E. coli* culture with an optical density of OD_{600} = 1.6 were added. Extraction was performed in reaction tubes with 15 min sedimentation time. After sedimentation 250 µL of supernatant were used for extraction. Photometric measurement results of DNA yield and weight of added soil are shown in Table 25.

Table 25: Dilution series 3 with added soil

Sample	Volume [µL]	Soil weight [g]	DNA yield [ng/µL]	260/280 ratio	260/230 ratio
a	0.0	0.497	6.9	1.16	0.27
b	0.5	0.500	7.0	1.36	0.35
c	1.0	0.500	3.4	1.23	0.23
d	2.5	0.498	7.3	1.40	0.26
e	5.0	0.500	6.0	2.04	0.34
f	10	0.502	8.6	1.34	0.25

Photometrically measured DNA yield, shown in Table 25, shows no clear trend, as the differences in DNA yield are small, except for the 1.0 µL sample (c), which was significantly lower. DNA yield from the other samples was within a range between 6.0 ng/µL and 8.6 ng/µL. However, it has to be said that DNA yield includes also DNA extracted from the soil sample itself and not only from the added EGFP *E. coli* bacteria. Thus, a PCR with 30 cycles using the EGFP primers was performed to differentiate between soil DNA and added EGFP *E. coli* DNA, followed by gel electrophoresis. The corresponding gel is pictured in Figure 34.

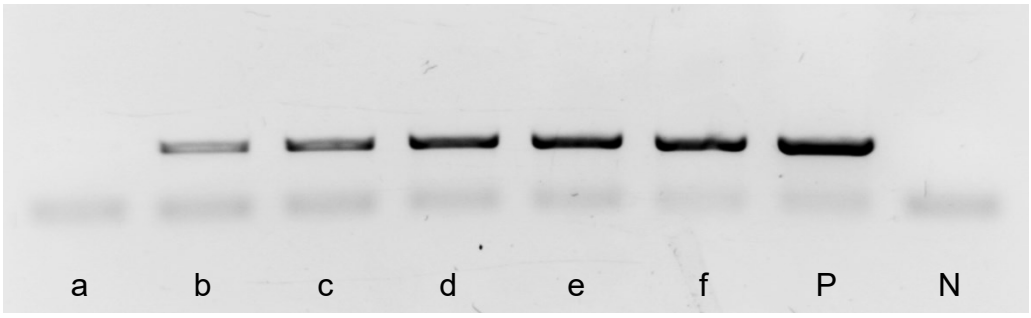

Figure 34: Gel electrophoresis picture of PCR products from dilution series 3 with added soil
Gel electrophoresis with PCR products (EGFP primer pair, 30 cycles) of DNA extracts from different volumes of *E. coli* culture with 0.5g soil added; **a:** 0.0µL; **b:** 0.5µL, **c:** 1.0µL; **d:** 2.5µL; **e:** 5.0µL; **f:** 10µL; **P:** Positive control; **N:** Negative control

Figure 34 clearly shows a trend: The higher the volume of EGFP *E. coli* culture added, the stronger the corresponding band is. With no bacteria added, no band is visible at all (a). Even with as little as 0.5 µL added, a band is clearly visible (b), but weaker than the others. Band c (1.0 µL) is stronger than band b (0.5 µL), but weaker than band d

(2.5 µL). The other bands are of similar strength. Positive control (P) yielded in a very strong band, while negative control (N) showed no band at all, as expected.

In order to make differences between the stronger bands visible, a PCR with 20 cycles was performed using the same primers. After gel electrophoresis, the following gel picture (Figure 35) was taken.

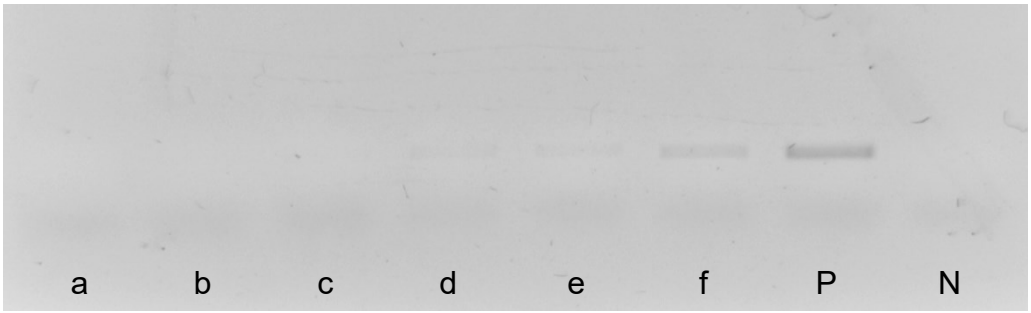

Figure 35: Gel electrophoresis picture of PCR products from dilution series 3 with added soil, 20 cycles
Gel electrophoresis with PCR products (EGFP primer pair, 20 cycles) of DNA extracts from different volumes of *E. coli* culture with 0.5g soil added; **a:** 0.0µL; **b:** 0.5µL, **c:** 1.0µL; **d:** 2.5µL; **e:** 5.0µL; **f:** 10µL; **P:** Positive control; **N:** Negative control

Figure 35 shows that after only 20 cycles the bands are significantly weaker than after a 30 cycle PCR (Figure 34). For the 0.0 µL (a), 0.5 µL (b) and 1.0 µL (c) extracts there is no band visible at all. The first visible band can be seen for the 2.5 µL extract (d), although very weak and hardly visible. The 5.0 µL extract (e) yielded a band that is a little bit stronger than the previous one, but still very weak. The 10 µL extract (f) showed a clear, but still weak band. The strongest band was the one from the positive control (P), while the negative control (N) showed no band at all. In general, in Figure 35 a trend can be seen where DNA yield is better the higher the initial EGFP *E. coli* culture volume is and that extraction of DNA is possible in the presence of soil, even when only low amounts of DNA are available (in this experiment: 2.5 µL of EGFP *E. coli* culture).

3.4 Extraction of DNA from stool

3.4.1 Evaluation of volumes and handling

In this experiment DNA was extracted out of a stool sample that was frozen overnight (see chapter 2.3.3 for details). For the first extraction, 0.500 g of stool was transferred into a reaction tube and 750 µL lysis and binding buffer was added. The mixture was very difficult to homogenize, even vortexing on maximum speed for several minutes was not enough. After sedimentation, the solution still retained its brown color and high turbidity, transfer of supernatant was not possible. Thus, the sample was centrifuged for 3 min at 13,000 rpm afterwards, which resulted in significantly better handling. Pictures of the sample after sedimentation (a) and after centrifugation (b) are shown in Figure 36.

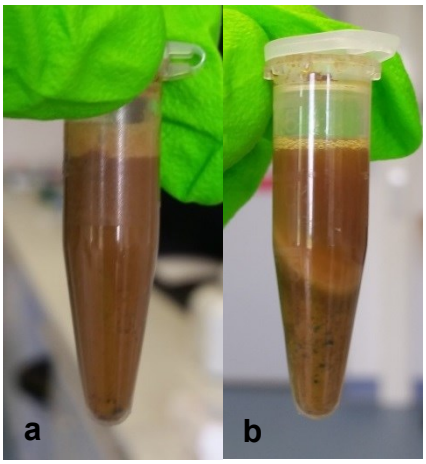

Figure 36: Stool sample with lysis and binding buffer
0.500g stool sample homogenized with 750µL lysis and binding buffer; **a:** After 15min sedimentation; **b:** After 15min sedimentation and 3min centrifugation at 13,000rpm

For the second extraction only 0.250 g stool were used, but 1,000 µL lysis and binding buffer was added. With these amounts, homogenization was significantly easier and quicker to perform, sedimentation was also successful. The regular extraction protocol with quarter volumes was performed. After extraction, DNA yield was measured photometrically, the results are shown in Table 26.

Table 26: Extraction of DNA from stool

Sample	Stool weight [g]	Lysis and binding buffer volume [µL]	DNA yield [ng/µL]	260/280 ratio	260/230 ratio
a	0.500	750	9.6	0.95	0.19
b	0.250	1,000	19.1	1.22	0.29

Interestingly, extraction of DNA from half amount of stool (sample b: 0.250 g) resulted in doubled DNA yield (19.1 ng/µL), while the bigger initial stool sample (sample a: 0.500 g) yielded only 9.6 ng/µL DNA. The 260/280 ratios were very poor being 0.95 (a) and 1.22 (b), as well as the 260/230 ratios with only 0.19 and 0.29. For verification of these findings a PCR was performed with 16S primers (8F & 926R) and 30 cycles. After gel electrophoresis, the following gel picture was taken (Figure 37).

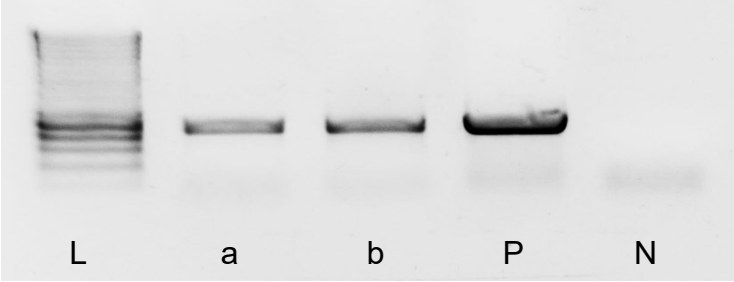

Figure 37: Extraction of DNA from stool
Gel electrophoresis with PCR products (8F + 926R primer pair, 30 cycles) of DNA extracts from stool samples; **a:** 0.500g stool + 750µL lysis and binding buffer; **b:** 0.250g stool + 1,000 µL lysis and binding buffer; **P:** Positive control; **N:** Negative control

The gel picture in Figure 37 clearly shows that extraction of DNA was possible from both stool samples. The 0.250 g band (b) is a little bit stronger than the one from the 0.500 g band (a), which matches the photometric results, although the difference is not that high (the difference in photometrically measured yield was double). Positive control (P) resulted in a strong band, even stronger than both samples, and negative control (N) showed no band at all.

3.4.2 Spiking of stool samples with EGFP *E. coli* bacteria

In order to evaluate the influence of stool on DNA extraction, stool samples were spiked with EGFP *E. coli* bacteria, analog to the previous spiking experiments in chapter 3.3.3. Bacteria were diluted to an optical density of OD_{600} = 1.6. Three stool samples were prepared with 0.257 g (a), 0.247 g (b) and 0.252 g (c) of stool and 40 µL (a), 10 µL (b) and 2.5 µL (c) of bacteria solution, respectively.

Table 27: Spiking of stool samples with EGFP *E. coli* bacteria

Sample	Stool weight [g]	*E. coli* volume [µL]	DNA yield [ng/µL]	260/280 ratio	260/230 ratio
a	0.257	40	29.7	1.34	0.26
b	0.247	10	26.1	1.27	0.28
c	0.252	2.5	18.9	1.29	0.27

Photometric results (Table 27), which include all DNA present in the sample, not only EGFP *E. coli* DNA, show that extraction of DNA was possible. With the highest volume

of bacteria added (40 µL), sample a showed the highest yield (29.7 ng/µL), followed directly by sample b with only 10 µL *E. coli* added and 26.1 ng/µL yield. With the addition of 2.5 µL bacteria solution, a yield of 18.9 ng/µL was achieved. The 260/280 ratios were not good, ranging between 1.27 and 1.34, and the 260/230 ratios were very poor, ranging from 0.26 to 0.28. In order to differentiate between the complete genome and EGFP vector DNA, a PCR with EGFP primer pair and 30 cycles was performed, followed by gel electrophoresis. The results are shown in Figure 38.

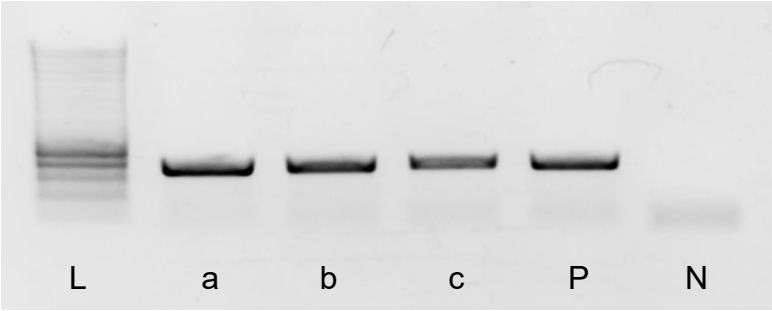

Figure 38: Spiking of stool samples with EGFP *E. coli* bacteria
Gel electrophoresis with PCR products (EGFP primer pair, 30 cycles) of DNA extracts from stool samples spiked with EGFP *E. coli* bacteria culture; **a:** 0.257g stool + 40µL bacteria culture; **b:** 0.247g stool + 10 µL bacteria culture; **c:** 0.252g stool + 2.5µL bacteria culture; **P:** Positive control; **N:** Negative control

Looking at Figure 38, it can be seen that extraction of EGFP vector DNA was possible with strong bands in all cases. Sample a (0.257 g stool + 40 µL *E. coli* solution) and sample b (0.247 g stool + 10 µL *E. coli* solution) yielded in bands of similar strength, while the band from sample c (0.252 g stool + 2.5 µL *E. coli* solution) was a little bit weaker. This means that stool does not inhibit extraction of DNA, even when very low amounts of DNA are present (in this case only 2.5 µL of *E. coli* liquid culture with an optical density of OD_{600} = 1.6). The positive control (P) showed a strong band, while the negative control (N) had no band at all, as expected.

3.4.3 Detection of human DNA in DNA extracts from stool

The next step was to detect human DNA in stool samples. For this experiment DNA extracts from chapter 3.4.1 were used and 30 cycle PCRs were performed with both human GAPDH and both beta-actin primer pairs (see Table 2 and chapter 2.2.3 for reference). For positive control DNA was extracted out of human blood. PCR was followed by gel electrophoresis. Pictures of the gels are shown in Figure 39 (GAPDH) and Figure 40 (beta-actin).

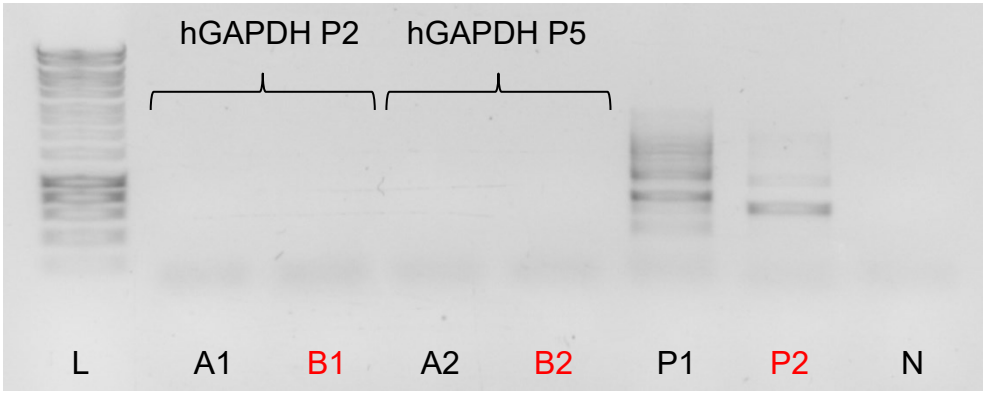

Figure 39: Detection of hGAPDH gene in DNA from stool samples
Gel electrophoresis with PCR products (hGAPDH primer pairs P2 and P5, 30 cycles) of DNA extracts from stool samples; **L:** Ladder 1kb; **A:** 0.500g stool + 750µL lysis and binding buffer; **B:** 0.250g stool + 1,000µL lysis and binding buffer; **1:** hGAPDH P2; **2:** hGAPDH P5; **P1:** Positive control hGAPDH P2; **P2:** Positive control hGAPDH P5; **N:** Negative control

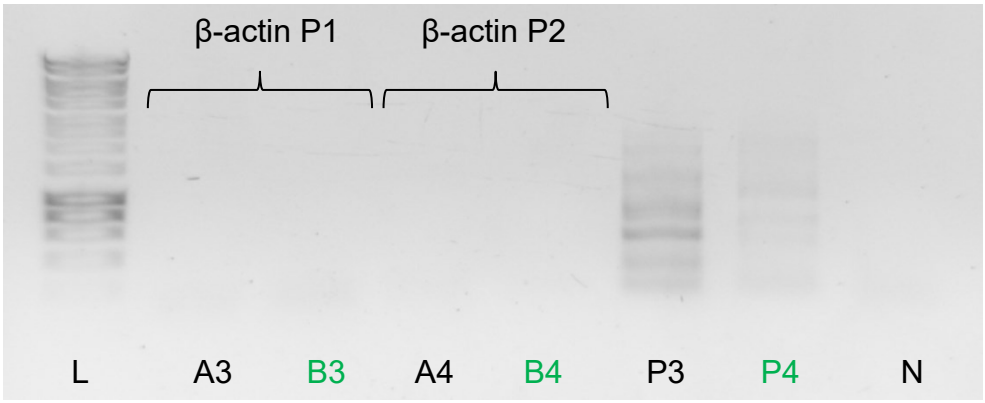

Figure 40: Detection of beta-actin gene in DNA from stool samples
Gel electrophoresis with PCR products (β-actin primer pairs P1 and P2, 30 cycles) of DNA extracts from stool samples; **L:** Ladder 1kb; **A:** 0.500g stool + 750µL lysis and binding buffer; **B:** 0.250g stool + 1,000µL lysis and binding buffer; **3:** β-actin P1; **4:** β-actin P2; **P3:** Positive control β-actin P1; **P4:** Positive control β-actin P2; **N:** Negative control

In Figure 39 and Figure 40 no bands are visible for stool samples at all (lanes A and B), suggesting that no human DNA was present in the extract at all or the amount of DNA was too low for PCR. The new primers used here all worked, as positive controls (P) showed strong bands, although the positive controls for the human GAPDH primer 5 (band P2) and beta-actin Primer 2 (band P4) were significantly weaker than the others. Negative controls (N) showed no bands as all, just as expected.

4 DISCUSSION

4.1 Sample materials

During this work several different sample materials were used to extract DNA from. In most experiments soil was used as a difficult sample. In other experiments liquid *E. coli* bacteria culture was used as a source for easy to extract DNA, e.g. for spiking experiments (chapters 3.3.3 and 3.4.2). At the end of this work stool was introduced as difficult sample material.

4.1.1 *E. coli* bacteria culture

Liquid bacteria culture was used as an easy to extract sample material. Extraction of DNA, especially from bacteria, is commonly used and has become a basic procedure in many laboratories [1]. It is easy to perform, as cell lysis is not difficult (in contrast to plant cells, for example), samples are readily available, the number of bacteria respective the amount of DNA in a sample can be measured prior to extraction and, thus, samples with a defined amount of DNA / number of bacteria can be prepared. Defined amounts were needed for spiking (chapters 3.3.3 and 3.4.2) and sensitivity experiments (chapter 3.3.4).

All experiments within this work involving extraction of DNA from bacteria were successful, demonstrating how easy and reliable extraction from bacteria is by using the system developed here.

4.1.2 Soil

Soil is considered a difficult sample, which was, again, proved during this work. In many experiments variation in DNA yield was very high, for example in chapter 3.1.1.

One explanation for this is the composition of the soil sample itself, as it contains compounds that are mutagenic and destructive against DNA and interfere with downstream processing of DNA. DNases, bile acids, salts, metal ions and bacterial carbohydrates [24] [25] are examples for such compounds. Especially fulvic and humic acids, which inhibit the activity of DNA polymerase, are of great importance here, as other studies have previously shown [83] [84]. These substances are often purified together with the DNA during the extraction process, causing variations in DNA yield and / or analysis. These problems are explained in detail in chapters 1.2 and 1.3.1.

Another important fact is that soil and its composition are prone to changing. Outside conditions like weather (rain, snow), temperature and the condition of the soil sample itself (moisture, color) may influence the number of bacteria found in the sample [78] [82] and the properties of the sample itself (moisture, salinity, nutrients) [71].

Additionally, soil is a very inhomogeneous sample material, as it consists of a wide variety of components. Every sample and often even aliquots are unique in their own way, in some cases two aliquots of the same soil sample can vary heavily, for example in color or particle size. The latter is very important for small-scale applications like the microchip system used here. This key point is discussed in detail in chapter 4.4.3.

In order to achieve comparable results and to avoid the factors mentioned above, soil samples were spiked with defined amounts of EGFP *E. coli* bacteria (chapter 3.3.3).

4.1.3 Stool

Stool is another example for DNA sources where DNA is difficult to extract from. It is, similar to soil, prone to changing upon environmental factors, handling methods and storage conditions, as other researchers have previously shown [88] [89] [90]. Stool does also have an inhomogeneous character, meaning that variation in results is expected to be high. It may, too, contain bigger particles, influencing extraction of DNA, especially in a microchip system (see chapter 4.4.3 for more details).

With these facts in mind, stool samples were spiked with EGFP *E. coli* bacteria in order to receive comparable results with defined amounts of bacteria / DNA (chapter 3.4.2).

4.2 Photometric results

As photometric results were often not concurrent to the PCR / gel electrophoresis findings, several aspects are discussed in the following subchapters.

4.2.1 DNA yield

When comparing photometric results with gel electrophoresis results after PCR, in many experiments within this work there was little to no correlation. In some cases, results were even contradictory, for example in chapter 3.2.5, where DNA yield after different sedimentation times was evaluated. While photometric results (Table 12) showed a trend to higher yield with lower sedimentation time, gel electrophoresis of PCR products (Figure 18) resulted in the opposite: DNA yield was higher the higher

the sedimentation time was. A reason for this discrepancy could be the complex composition of samples DNA was extracted from. Soil and stool contain many substances which are difficult to separate from DNA [1] [68] and affect photometric measurement due to their absorption maxima near 260 nm.

4.2.2 DNA Purity

For examination of DNA purity in photometric measurement 260/280 and 260/230 ratios are commonly used. The 260/280 ratio is used for determination of contamination by organic compounds, e.g. phenols [91]. With values between 1.80 (DNA) and 2.00 (RNA) a sample is relatively free from contaminants [86]. On the other hand, the 260/230 ratio describes impurities like carbohydrates, peptides, buffer salts and other aromatic compounds [87].

The 260/280 ratio varied throughout the experiments conducted within this work. In some experiments, for example in chapter 3.3.3 (Table 21), ratios were very good, closely to the range between 1.80 and 2.00, to be precise. In contrast, in many other experiments 260/280 ratios were worse, mostly in a region between 1.00 and 1.50, for example in chapter 3.3.4 (Table 25). These values indicate a contamination with organic contaminants from the initial sample that have not been washed away upon extraction of DNA. Another explanation can be the acidity of the samples, which affects the 260/280 ratio to some extent. According to Wilfinger et al. [92], acidic solutions lower the 260/280 ratio, while basic solutions increase them. Additionally, the nucleotide mix itself has an influence on the ratio [93]. As downstream processing of DNA was not affected by these factors, the 260/280 ratios were not considered any further, as suggested by Thermo Scientific [91].

The 260/230 ratios, on the other hand, were very poor throughout this work. Values did never exceed a ratio of 0.49 (Table 16 in chapter 3.2.11), in many cases the ratio was lower than 0.10 (for example in Table 12, chapter 3.2.5). This indicates a strong contamination with carbohydrates, peptides, buffer salts and / or other aromatic compounds [15], which may also be present in the extraction kit. According to Chemicell customer support (Mr. Cengiz Ozturk), lysis and binding buffer and wash buffer I both contain guanidinium thiocyanate and wash buffer I, additionally, contains Triton™ X-100. Both substances have an absorption maximum near 260 nm [91] [94]. An idea to improve 260/230 ratios could be the use of a diluted lysis and binding buffer / wash buffer I solution for blank measurement in order to eliminate the effect of the

contaminants present in these buffers. However, as downstream processing of DNA (e.g. PCR) was possible in presence of these contaminants, these poor 260/230 ratios were not regarded any further, as suggested by Thermo Scientific [91].

4.3 Microchip system

In the following subchapters the individual aspects of DNA extraction using a microchip are discussed.

4.3.1 Microchip design

With evolution of chip design from version V1 to version V4 the microchip system became better and better. After testing more and more improvements were made with each design. Until the final version V4 for the time being (chapter 3.2.10), the number of tubes was significantly reduced by interconnection of chambers directly on the chip surface, a sedimentation chamber was introduced (V3, chapter 3.2.8) and channels were optimized for easy and quick extraction of DNA inside the chip.

Several problems were encountered during development, some of which could already be solved. So was the loss of liquid that occurred while switching tubes. This problem was eliminated by the use of Y-connectors and pinch clamps for fluid control, so that switching was no longer necessary, which also reduced the number of tubes needed as a positive side effect.

The sedimentation chamber (chapter 3.2.8) was probably the most important improvement made to the microchip. With this chamber extraction was possible inside the chip completely, from sample addition to final elution of extracted DNA. However, it has to be considered that the outlet is positioned in a fixed height. Depending on the sample composition (especially amount of solid matter) and volume, the amount of supernatant that is transferred to the reaction chamber and DNA is extracted from in the next step is prone to variation. With varying supernatant volume DNA yield is affected as well, leading to poor comparability, depending on the sample type. This would not be the case with extraction in a reaction tube, were supernatant is transferred using a micropipette with a defined volume. Liquid samples without many solid particles (liquid bacteria culture, for example) are not affected by this effect, as sample height in the chamber should be nearly the same for all liquid samples when added in an exact volume (with a micropipette, for example).

With the latest version of the chip, version V4 (chapter 3.2.10), a cross section was introduced. Here, the outlets of chambers 3 (wash buffer I) and 5 (70% ethanol / isopropanol) connect to the inlet channel of chamber 4, the reaction chamber (see Figure 22 for reference), promoting direct transfer of buffers without the need for excessive tubing. As already described briefly in chapter 3.2.10, this cross section was troublesome, as fluids from chamber 3 tended to travel to chamber 5 and vice versa instead of entering the reaction chamber, as they were supposed to. When this effect occurred, usually air bubbles could be observed in the chamber the liquid travelled to. Thus, possible reasons for this effect could be poor air tightness of the sealing foil, the tube connector(s) or the sealing of the tube connector itself, consisting of glue dispensed by a hot glue gun. An increased amount of glue around the tube connectors, as pictured in Figure 3a, was applied, yet the effect was limited. A light improvement of the initial problem was observed, but further investigation into this problem has to be done. Still, extraction of DNA was possible inside the chip with attention on liquid flow. Occasional application of back pressure relieved the problem to a certain extent.

4.3.2 Sealing foils

For sealing of the microchip several sealing foils initially developed for sealing of multiwall-plates for PCR applications were tested for sticking and removal properties and for interaction with the DNA extraction itself. As these foils have initially been designed for use with DNA (no interaction with DNA) and under high temperatures, they are the ideal solution for sealing of the microchip. There were two groups of foils, one with pressure-sensitive glue and one with a self-adhesive side. Differences in sticking and removal properties were surprisingly huge (Table 11). In general, foils with pressure-sensitive glue showed overall good properties, sticking and removal, while self-adhesive foils seemed to have stronger glue resulting in overall bad removal properties. Some were even sticking too well requiring a lot of force to remove, others disintegrated into pieces upon removal, leaving small residues on the surface of the chip. Interestingly, the strong glue was difficult to utilize, as two of three foils had poor or even very poor sticking properties. Additionally, some foils left the surface of the chip sticky, resulting in the need of additional cleaning steps after extraction of DNA.

PCR and subsequent gel electrophoresis showed that interaction with DNA and the extraction process itself was low. While after a 30 cycle PCR (Figure 14) no differences were visible, a 20 cycle PCR (Figure 15) shows differences: In general, self-adhesive

foils seem to result in lower DNA yields, with the pressure-sensitive foils showing stronger bands. However, there were also differences in band strength with the latter ones. Maybe the glue on the self-adhesive foils interacts with the magnetic beads, immobilizing them, resulting in lower DNA yield. Additionally, the glue could possibly cause problems during sedimentation, as soil may stick to the glue. This has not been tested, as to the time of the foil tests sedimentation has not yet been introduced to the extraction system.

With these facts in mind, the foil with the overall best capabilities – sticking, removal and DNA yield – was selected: Foil a, Peqlab qPCR seal, Cat. No. 82-1170-99. With this foil all following experiments involving a microchip were performed.

4.4 Extraction of DNA

4.4.1 Extraction from soil and *E. coli* bacteria

As already mentioned in chapter 3.2, extraction of soil is possible in a microchip, even with very low reagent volumes (chapter 3.1.2) and low initial sample size (chapter 3.3.4). However, comparison between extraction in a reaction tube, as suggested by the kit manufacturer, and extraction in a chip (chapter 3.2.11) revealed diverse results.

In the first experiment, conducted with soil as sample material, three comparisons were made. On the gel (Figure 23) in one case extraction in a chip gave higher yield, in one case the reaction tube showed higher yield, while in another case no yield was achieved at all with both extraction methods. An explanation for this could be the inhomogeneous sample material. Depending from where in the sample tube the individual samples were taken, the composition can be very different (solid matter, parts of plants, worms, beetles etc.). Additionally, temperature, weather conditions at the time of sample acquisition (rain and temperatures below 0°C, for example) and the sample condition itself (moisture, color) may have an influence on the soil sample and its individual DNA content. These factors may affect microflora and bacteria count [69] [70] and the properties of the soil sample itself (moisture, salinity, nutrients) [71].

In the second experiment (chapter 3.2.11) DNA was extracted from liquid *E. coli* overnight culture in order to eliminate the influence of soil and its inhomogeneous character on DNA yield. The gel electrophoresis (Figure 24), performed directly after PCR with DNA extracts, shows striking results. After 30 cycles, the extraction in chip seems to show a higher yield, whereas PCR with 25 and 20 cycles tells a completely

different story: Here, extraction in a chip resulted in lower yield, especially after 20 cycles, where the band is hardly visible. As this happened twice, it is assumed that the 30 cycles PCR was faulty, probably due to pipetting errors. However, there was still enough DNA in the microchip extract, so that it can be said that extraction of DNA in a microchip gives enough yield for downstream processing of DNA, like PCR.

4.4.2 Variation of extraction protocol

In chapter 3.1.3 the parameters elution time and magnetic bead volume were altered in order to optimize the extraction process. In the first test, elution time was halved and doubled and the test was conducted twice. Results were not consistent: After PCR and gel electrophoresis (Figure 10) it became clear, that a prolonged elution time lead to lower DNA yields in both test series. In contrast, a shorter elution time seemed not to affect DNA yield. Interestingly, while the 5 min and 10 min bands were of equal strength in both test series, the band for the doubled elution time was significantly stronger in test series 2. Again, a possible explanation for this may be the inhomogeneity of soil as sample material (see chapter 4.2.1 for details).

In the second part of this experiment three test series were performed with altered magnetic bead volume (factors two, three and five). PCR with the extracts and subsequent gel electrophoresis (Figure 12) revealed that the initial volume used (25 µL) achieved the best results. In all test series strong bands were visible for this volume. While alteration in volume with the factor two (test series 1) lead to strong bands for all three volumes, an alteration with the factor five lead to a significantly weaker band for the lowered volume (5 µL) and, surprisingly, a slightly weaker band for the increased volume (125 µL). In test series 3 only the standard volume yielded in a visible band at all. Again, DNA yield may be affected by the inhomogeneous character of soil as sample material (see chapter 4.2.1 for details). As the standard bead volume (25 µL) gave strong bands in all three test series, this volume was used for all future experiments.

4.4.3 Sedimentation

Sedimentation was introduced as replacement for centrifugation in the extraction process, as centrifugation is hard to realize in microchip size. In the first sedimentation experiment (chapter 3.2.5), various sedimentation times were tested during extractions of DNA from soil in reaction tubes. As expected, DNA yield was higher the longer the

sedimentation time was (Figure 18). Thus, the longest of all tested sedimentation times (15 min) was selected for all future experiments incorporating sedimentation.

After examination of solid phase height past sedimentation in a chip (chapter 3.2.6), which was 1.1 cm for 0.250g soil and 1.4 cm for 0.500 g soil, and investigation if DNA settles during sedimentation (chapter 3.2.7), which was not the case, a sedimentation chamber was built into chip version V3 (chapter 3.2.8). With this chip version the amount of supernatant gained from the sedimentation chamber after sedimentation was investigated. Results were very constant, ranging between 0.734 g and 0.858 g (Table 15 and Figure 21). Variations in these values are most probably due to the inhomogeneous character of soil as sample (see chapter 4.1.2 for details). Maybe there were bigger solid particles in the sample (e.g. stones or parts of plants), leading to a different height of the solid phase. As the sedimentation chamber's outlet is fixed in its height and cannot be changed, a bigger height of the solid phase in the sedimentation chamber leads to higher supernatant yield, as the space between the supernatant outlet and the top of the solid phase, where supernatant cannot be obtained and is therefore discarded, becomes smaller. This space is shown schematically in Figure 41.

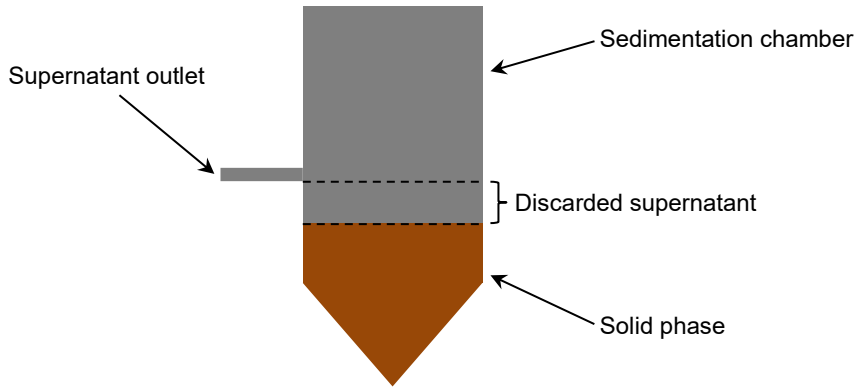

Figure 41: Discarded supernatant after sedimentation
Schematic drawing of sedimentation chamber with discarded supernatant marked; Solid phase & supernatant outlet are shown.

With the sedimentation yield proved to be constant and reproducible, the sedimentation step was fully evaluated and applied for all following experiments, if not stated otherwise.

4.4.4 Extraction from stool

At the end of this work DNA was extracted from stool samples. In a first experiment two different sample weights were tested together with two different lysis and binding

buffer volumes, as volume is limited, especially in microchip-scale (chapter 3.4.1). This experiment lead to two conclusions: Firstly, it showed that DNA was successfully extracted from a stool sample, as a PCR with universal 16S primers and subsequent gel electrophoresis showed (Figure 37): Strong bands were visible for all extracts.
Secondly, the approach with lower sample weight and higher buffer volume lead to an increased DNA yield compared to the other approach (Figure 37). Thus, 0.250 g sample weight and 1,000 µL lysis and binding buffer volume were chosen for all future experiments involving stool as sample to extract DNA from.
After successful proof of bacterial DNA in the stool extracts, human DNA was to be analyzed in the stool sample. For this, human primers were designed (chapter 2.2.3, Table 2) coding for the GAPDH and beta-actin genes. After PCR and gel electrophoresis no bands were visible for the stool extracts. As positive controls for the primers, consisting of the author's own blood samples, were positive for all primers (β-actin P2 showed low yield), it is assumed that the primers were functional and that the PCR protocol worked for these primer combinations. Thus, the reason for the missing bands must be the samples or the extraction system. As the amount of host DNA in stool samples is very low and, thus, hard to amplify [95], it is assumed that the concentration of human DNA in the sample was not sufficient for PCR. Further tests with increased samples sizes or pooling of samples could possibly lead to better results.

4.5 Spiking

4.5.1 Sensitivity

For evaluation of sensitivity DNA was extracted from extremely low amounts of EGFP bacteria. Liquid bacteria culture was diluted to an optical density of OD_{600} = 1.6 as stock solution. All following dilutions were made from this stock solution. In order to examine bacteria count in a liquid bacteria culture with an OD_{600} of 1.6, bacteria were seeded onto agar plates, resulting in a mean cell count of $1.17 \cdot 10^{10}$ cells per mL with a relatively low standard deviation of only $1.30 \cdot 10^{9}$ cells per mL. The resulting cell count was significantly higher than the calculated one according to literature [96], which should be $1.28 \cdot 10^{9}$ cells per mL. However, as size and shape of the cells have an influence on optical density [97], this calculated / estimated number can only be regarded as a rough estimate of the actual cell density. Other possible explanations for this deviation could be pipetting errors or dilution errors (wrong dilution factor

calculation, for example). Other reasons could be bad spreading of liquid culture on the agar plates or too long incubation time at 37°C resulting in huge colonies that were probably counted as separate colonies leading to higher cell count.

In dilution series 1 bacteria solutions (OD_{600} = 1.6) in volumes as low as 50 µL were used for extraction (Table 23). 50 µL should, theoretically, contain the following number of bacteria:

$$1.17 \cdot 10^{10} \frac{\text{cells}}{\text{mL}} \cdot 0.05\,\text{mL} = 5.85 \cdot 10^{8}\,\text{cells}$$

With only $5.85 \cdot 10^8$ bacteria diluted in 800 µL (50 µL bacteria solution + 750 µL lysis buffer), the lowest initial concentration was as low as $7.31 \cdot 10^8$ cells per mL. However, extraction of DNA was still possible with this very low concentration, as the PCR result shown in Figure 32 shows. Thus, in dilution series 2 even lower volumes were used (Table 24), with the lowest one being only 0.5 µL of OD_{600} = 1.6 bacteria stock solution. This results in a bacteria count of:

$$1.17 \cdot 10^{10} \frac{\text{cells}}{\text{mL}} \cdot 0.0005\,\text{mL} = 2.92 \cdot 10^{5}\,\text{cells}$$

Diluted in 755 µL total volume, this resulted in a final concentration as low as $3.87 \cdot 10^5$ cells per mL, while extraction was still successful, as Figure 33 shows. This shows that the extraction system developed within this work performs with very high sensitivity when it comes to extraction of liquid bacteria culture.

As this system was intended for use with difficult samples, EGFP bacteria solution with the same optical density as before (OD_{600} = 1.6) were added to soil samples in similarly low volumes (Table 25), with the lowest volume being the same as in dilution series 2. Even with such low volumes extraction of DNA was successfully performed, as the PCR products in Figure 34 show. Even after only 25 PCR cycles (Figure 35), some of the bands are still visible, showing how powerful and sensitive the extraction system performs, even in the presence of difficult sample material, soil in this case.

For further evaluation of the inhibitory effect of difficult sample materials on the extraction of bacteria DNA, soil and stool samples were spiked with EGFP bacteria in the following subchapters 4.5.2 (soil) and 4.5.3 (stool).

4.5.2 Soil

Because of the inhomogeneous character of soil DNA yields after extraction tend to vary, even from the same sample or even aliquot (this fact has been discussed already in chapter 4.1.1). Thus, in chapter 3.3.3 a soil sample was laced with 1 mL of liquid

E. coli overnight culture. Bacteria transfected with a vector containing an EGFP gene were used to distinguish the added bacteria DNA from the DNA already present in the soil sample after extraction. By using specific primers this DNA can be visualized on a gel. Photometric analysis came out as expected, with the raw *E. coli* culture sample having the highest yield (19.2 ng/µL), as there were no inhibitors or contaminants present in the sample, making extraction very easy. However, a yield in this range is very low for pure concentrated bacteria culture. The reasons for this might be short incubation time and low initial number of bacteria. In contrast, the raw soil without bacteria addition achieved the lowest DNA yield, being only 8.0 ng/µL. The spiked soil returned a higher yield than the raw soil sample, but still a lower yield (12.7 ng/µL) than the raw *E. coli* sample, just as expected. Although one might expect a yield of circa 21 ng/µL (8.0 ng/µL soil + 13 ng/µL *E. coli*), it is assumed that the contaminants present in the soil and the inhibiting effect of soil itself most probably lead to a lowered DNA yield.

On a gel after PCR (Figure 30), results looked as predicted: The raw *E. coli* band was almost of the same strength as the spiked sample band, which was slightly weaker, suggesting that soil itself does not interfere much with the extraction of DNA from bacteria. Raw soil delivered no band at all, as there were no transfected bacteria containing an EGFP gene present in this sample.

4.5.3 Stool

As stool is an inhomogeneous sample material, similar to soil when it comes to extraction of DNA, variations in yield upon extraction of DNA were expected. Thus, in chapter 3.4.2 stool samples were spiked with EGFP *E. coli* bacteria in order to achieve comparable results, similar to the experiments performed with soil (chapter 3.3.3). In this experiment, three extractions were made with different volumes of bacteria culture added. Photometric measurements after extraction revealed high DNA yields (Table 26): 29.7 ng/µL with addition of 40 µL bacteria culture, 26.1 ng/µL with 10 µL and 16.9 ng/µL with 2.5 µL. Surprisingly, a fourfold higher addition of bacteria lead to an increase in yield of only 3.6 ng/µL or 13.8%, respectively, while a quartered bacteria volume resulted in a yield only 7.2 ng/µL or 25.6%, respectively, smaller. A possible explanation for this phenomenon may be the inhomogeneity of the soil sample, which lead to a higher / lower inhibition of the DNA extraction or a higher / lower overall bacteria count in the sample itself. Another reason could be the extraction itself, as the

final extracts were not absolutely clear. This means that there were probably contaminants left in the final eluate that may influence photometric measurement.
Results looked different after PCR and gel electrophoresis (Figure 38): All bands were of similar strength, with the biggest bacteria addition being the strongest band, followed directly by the medium addition and the lowest addition. Differences in band strength were very marginal, especially between the highest and the medium bacteria addition, where a difference can hardly be seen. These results stand in contrast to the photometric findings, but it also has to be considered that PCR only detects added bacteria with EGFP gene, while photometrically measured yield includes all DNA present in the sample. In general, a difference in band strength was expected, with the highest number of bacteria added resulting in the strongest band, which was not the case here. Possible explanations for this unexpected outcome may be the inhomogeneity of the stool sample, contaminants that have not been washed away upon extraction and an inhibition of the extraction by the stool sample itself, as mentioned earlier.

4.6 Conclusion

With the microsystem developed here extraction of DNA from difficult samples is possible. After modification of the extraction process (chapter 3.1) and microchip design (chapter 3.2), DNA can be extracted in an easy and quick manner. Sample material can be loaded onto the chip, the chip is sealed and extraction process is performed. Extraction of DNA from liquid bacteria culture resulted in enough yield for subsequent PCRs with 30 cycles or less. For difficult samples like soil or stool sedimentation was introduced as replacement for the sedimentation step in the kit manufacturer’s extraction protocol, which was successful (chapter 3.2.5). A sedimentation chamber was designed and introduced to the chip and, thus, extraction with sedimentation was possible inside the chip, making extraction of DNA possible completely inside the chip from sample addition to final elution. This makes time-intensive sample pre-treatment, as required by many extraction methods for difficult samples [30] [81], unnecessary for this extraction system.
After all these modifications the extraction system was tested in different situations, which were successful in nearly all cases. DNA was extracted from highly diluted liquid bacteria solutions (chapter 3.3.4) while subsequent PCR was still possible. Extractions of DNA from soil (chapter 3.1) and stool (chapter 3.4), two examples for difficult sample

materials to extract DNA from, were successful as well (chapter 3.1). Afterwards, EGFP-transfected *E. coli* bacteria were added to soil and stool samples (chapters 3.3.3 and 3.4.2), DNA was extracted and subsequently bacterial DNA was successfully amplified. These results prove the extremely high sensitivity (low sample volumes / low initial DNA concentration) and resistance to inhibitory effects of difficult sample types (soil, stool) of the extraction system.

Extraction with the microsystem developed within this work is easy to perform and quick. Compared to column-based systems, significantly less time is required (approx. 35 min) and manual handling steps are reduced to an absolute minimum. With a few modifications to the system extraction can be automated (see the following chapter 4.7 for a more detailed outlook), making the extraction even easier and quicker. Additionally, this system is cheaper than comparable extraction systems [98].

The only drawback within this work was the impossibility to extract or detect human DNA out of stool samples (chapter 3.4.3), which was probably caused by too low DNA concentration in the small sample sizes used (0.250 – 0.500 g). It is assumed that a bigger sample size or pooling of stool samples may lead to positive results, as discussed earlier in chapter 4.4.4.

In general, it can be said that this work was highly successful: A microsystem for rapid extraction of DNA from difficult samples was developed and was tested successfully with various sample types. Tests have proved the superb sensitivity and excellent resistance to inhibitory factors present in difficult samples.

4.7 Outlook

The next steps of this work lead to further automation of the system. By introduction of automatable valves, either directly on the chip or by magnetic pinch clamps, a heating unit, for example a peltier element, and an electromagnet the extraction process itself can be fully automated. Additionally, chip sealing can further be simplified, for example with liquid storage on the chip, so that chips could be pre-filled and only the sedimentation chamber would have to be sealed manually or with an external sedimentation chamber connected directly to the chip. With this concept filling of the chip with all the liquids needed (buffers etc.) would no longer be necessary upon extraction which would save a significant amount of time. Additionally, PCR could be performed directly on the chip, probably with all required liquids already pre-stored on the chip. Similar approaches have already been done for other applications by other

researchers [99]. This would be very beneficial for the system developed within this work, as PCR could be carried out automatically directly after extraction. With application of specific markers, for example for PCR, a fully automated rapid detection system could be developed, that only requires the addition of the sample and delivers a desired signal (positive / negative) within less than 60 minutes without the need of any laboratory equipment or skilled personnel. Such a system could be used on-site, e.g. bedside in hospitals, medical practices or in field (soil samples).

Chip design can be improved as well with fully customized chips that can then be mass-produced to further reduce cost instead of the modular chip system used within this work that is, compared to a mass-produced chip, significantly more expensive.

Another aim for the future would be further improvement of the extraction system with stool samples, so that human DNA can be extracted. As already mentioned in chapter 4.4.4, pooling of stool samples to increase sample size could be a possible way as well as modification of the reagents used for extraction (lysis and binding buffer, wash buffers). Maybe the introduction of an additional extraction step or improvements in sample acquisition and handling could lead to better results.

5 SUMMARY

Extraction of DNA from so-called difficult samples like feces and soil is problematic, because of the presence of compounds which are mutagenic and destructive against DNA and inhibitors that influence further processing of DNA. As fecal DNA contains DNA from various sources, like gut flora or intestinal mucosa, it has diagnostic relevance. Analysis of fecal DNA might therefore allow conclusions on the presence of (intestinal) diseases like tumors and infections at early stages in a quick and non-invasive manner. DNA extracted from soil, on the other hand, allows conclusions on the composition of the microflora and the purification and analysis of DNA from specific bacteria. Unfortunately, all methods for extraction of DNA from difficult samples currently available are neither quick nor automated nor easy to use. Thus, the development of an easy-to-use, automatable and portable system for extraction of DNA from difficult samples would be beneficial.

At first, a suitable system was searched as base for further modifications. This system was then scaled down to microchip-size and several modifications to the original protocol were made to adapt the extraction system to difficult samples. A number of microchip designs were developed, built and tested with the new extraction method and the protocol was adapted to it. As examples for difficult samples soil and, in later experiments, human feces were used for further evaluations.

In order to make extraction possible inside a chip without the use of a centrifuge, sedimentation was introduced to replace a centrifugation step in the extraction protocol. Finally, complete DNA extraction was performed inside a chip, from sample addition to final elution. As an advantage, the majority of parts are reusable, except for a small fraction of tubes. The chip itself might be reused as well.

Bacteria DNA was successfully extracted from soil and from stool samples with the microsystem, even from spiked samples with very low bacteria count. However, DNA yield from stool was lower than from soil samples. Extraction and detection of human DNA from stool was not successful. For this, further modifications to the extraction protocol and / or the system itself are needed.

The microsystem developed within this work is easy to use and the established protocol is quick to perform. It is significantly faster than current column-based methods and requires a minimal number of manual steps. The system offers options for automation, so that it might be possible to increase speed and simplicity even further.

Additionally, a PCR step could be integrated in the chip as well as specific markers, so that the extraction system may be expanded to a detection system, e.g. for diseases (stool) or specific bacteria (soil).

6 REFERECES

[1] Wink M: An Introduction to Molecular Biotechnology: Molecular Fundamentals, Methods and Application in Modern Biotechnology. Wiley-VCH, Weinheim, Germany, 2006

[2] Tan SC, Yiap BC: DNA, RNA, and protein extraction: the past and the present. J Biomed Biotechnol. 2009;2009:574398

[3] Doyle K: The Source of Discovery: Protocols and Applications Guide, PROMEGA, Madison,Wis, USA, 1996, rev. 12/09

[4] Cseke LJ, Kaufman PB, Podila GK, Tsai CJ: Handbook of Molecular and Cellular Methods in Biology and Medicine. CRC Press, Boca Raton, Fla, USA, 2nd edition, 2004

[5] Dahm R: Friedrich Miescher and the Discovery of DNA. Elsevier, Amsterdam, The Netherlands, 2004

[6] Sambrook J, Russel D: Molecular Cloning: A Laboratory Manual, vol. 3. Cold Spring Harbor Laboratory Press, New York, NY, USA, 3rd edition, 2001.

[7] Chomczynski P, Sacchi N: The single-step method of RNA isolation by acid guanidinium thiocyanate-phenolchloroform extraction: twenty-something years on. Nature Protocols, vol. 1, no. 2, pp. 581–585, 2006

[8] Courtesy of Courvoisier, Portrait Sammlung, University of Basel; Online: http://www.dnaftb.org/15/bio.html, retrieved June 2nd, 2018

[9] Buckingham L, Flaws ML: Molecular Diagnostics: Fundamentals, Methods, & Clinical Applications, F.A. Davis, Philadelphia, Pa, USA, 2007

[10] Smarason SV, Smith AV: Method for desalting nucleic acids, United State patent US 2003/0186247 A1, deCODE genetics ehf., October 2003

[11] Birnboim HC, Doly J: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research, vol. 7, no. 6, pp. 1513–1523, 1979

[12] Esser KH, Marx WH, Lisowsky T: Nucleic acidfree matrix: regeneration of DNA binding columns. BioTechniques, vol. 39, no. 2, pp. 270–271, 2005

[13] Gjerse DT, Hoang L, Hornby D: RNA Purification and Analysis: Sample Preparation, Extraction, Chromatography. Wiley-VCH, Weinheim, Germany, 1st edition, 2009

[14] Esser KH, Marx WH, Lisowsky T: MaxXbond: first regeneration system for DNA binding silica matrices. Nature Methods, vol. 3, no. 1, pp. 1–2, 2006

[15] Kojima K, Ozawa S: Method for isolating and purifying nucleic acids. United State patent US 2002/0192667 A1, December 2002

[16] Smith CE, Holmes DL, Simpson DJ, Kayzhendler J, Bitner RH, Groseh JC: Mixed-bed solid phase and its use in the isolation of nucleic acids. United State patent US 2002/0001812 A1, Promega Corporation, January 2002

[17] Arnold TE, Meyering MT, Chesterson RS: Nucleic acid binding matrix. United States patent US 6869532 B2, CUNO Incorporated, March 2005

[18] Berensmeier S: Magnetic particles for the separation and purification of nucleic acids. Applied Microbiology and Biotechnology, vol. 73, no. 3, pp. 495–504, 2006

[19] Promega Corporation: Personal Automation™ for Workflow Optimization in the Clinical Lab [pamphlet], Promega, San Luis Obispo, Calif, USA, 2008

[20] Boyd J: Robotic laboratory automation. Science, vol. 295, no. 5554, pp. 517–518, 2002

[21] Loeffler J, Schmidt KD, Hebart H, Einsele H: Automated nucleic acid extraction. Encyclopedia of Genomics and Proteomics, pp. 93–96, 2004

[22] Promega Corporation: Maxwell® 16: Personal Automation™ from Promega, Promega, San Luis Obispo, Calif, USA, 2006

[23] Olson J, Whitney DH, Durkee K, Shuber AP: DNA stabilization is critical for maximizing performance of fecal DNA-based colorectal cancer tests. Diagn Mol Pathol 2005;14(3):183-191

[24] Al-Soud WA, Ouis IS, Li DQ, Ljungh S, Wadstrom T: Characterization of the PCR inhibitory effect of bile to optimize real-time PCR detection of Helicobacter species. FEMS Immunol Med Microbiol 2005;44(2):177-182

[25] Al-Soud WA, Radstrom P: Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples. Appl Environ Microbiol 1998;64(10):3748-3753

[26] Vogelstein B, Kinzler KW: Digital PCR. Proc Natl Acad Sci U S A 1999;96(16):9236-9241

[27] Machiels BM, Ruers T, Lindhout M, Hardy K, Hlavaty T, Bang DD, Somers VA, Baeten C, von MM, Thunnissen FB: New protocol for DNA extraction of stool. Biotechniques 2000;28(2):286-290

[28] Nechvatal JM, Ram JL, Basson MD, Namprachan P, Niec SR, Badsha KZ, Matherly LH, Majumdar AP, Kato I: Fecal collection, ambient preservation, and DNA extraction for PCR amplification of bacterial and human markers from human feces. J Microbiol Methods 2008;72(2):124-132

[29] Stark D, Beebe N, Marriott D, Ellis J, Harkness J: Evaluation of three diagnostic methods, including real-time PCR, for detection of Dientamoeba fragilis in stool specimens. J Clin Microbiol 2006;44(1):232-235

[30] Deuter R, Pietsch S, Hertel S, Müller O: A method for preparation of fecal DNA suitable for PCR. Nucleic Acids Res 1995;23(18):3800-3801

[31] Müller O, Deuter R: Process for purifying, stabilising or isolating nucleic acids from biological materials. European Patent 0 851 937; International Patent PCT/EP 96/03595 1998

[32] Stark D Beebe N, Marriott D, Ellis J, Harkness J: Detection of Dientamoeba fragilis in fresh stool specimens using PCR. Int J Parasitol. 2005 Jan;35(1):57-62

[33] Gabor EM, de Vries EJ, Janssen DB: Efficient recovery of environmental DNA for expression cloning by indirect extraction methods. FEMS Microbiol Ecol. 2003 May 1;44(2):153-63

[34] Schneegurt MA, Dore SY, Kulpa CF Jr.: Direct extraction of DNA from soils for studies in microbial ecology. Curr Issues Mol Biol. 2003 Jan;5(1):1-8

[35] Robe P, Nalin R, Capellano C, Vogel TM, Simonet P: Extraction of DNA from soil. Eur J Soil Biol. 2003 Oct; 39(4):183-190

[36] Amann RI, Ludwig W, Schleifer KH: Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev. 1995 Mar;59(1):143-69

[37] Miao T, Gao S, Jiang S, Kan G, Liu P, Wu X, An Y, Yao S: A method suitable for DNA extraction from humus-rich soil. Biotechnol Lett. 2014 Nov;36(11):2223-8

[38] Feces (Faeces) – Formation, Composition, Color and Odor. Online: http://www.healthhype.com/feces-faeces-formation-composition-color-and-odor.html, retrieved May 25th, 2018

[39] Sidransky D, Tokino T, Hamilton SR, Kinzler KW, Levin B, Frost P, Vogelstein B: Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. Science. 1992 Apr 3;256(5053):102-5

[40] Beg M, Singh M, Saraswat MK, Rewari BB: Occult Gastro-intestinal Bleeding: Detection, Interpretation, and Evaluation. JIACM 2002; 3(2): 153-8

[41] Levin B, Hess K, Johnson C: Screening for colorectal cancer: a comparison of 3 fecal occult blood tests. Arch Intern Med 1997; 157: 970-6

[42] Hol L, van Leerdam ME, van Ballegooijen M, van Vuuren AJ, van Dekken H, Reijerink JC, van der Togt AC, Habbema JD, Kuipers EJ: Screening for colorectal cancer: randomised trial comparing guaiac-based and immunochemical fecal occult blood testing and flexible sigmoidoscopy. Gut 2010; 59:62-8

[43] Deuter R, Müller O: Detection of APC mutations in stool DNA of patients with colorectal cancer by HD-PCR. Hum Mutat 1998;11(1):84-89

[44] García-Bilbao A, Armañanzas R, Ispizua Z, Calvo B, Alonso-Varona A, Inza I, Larrañaga P, López-Vivanco G, Suárez-Merino B, Betanzos M: Identification of a biomarker panel for colorectal cancer diagnosis. BMC Cancer 2012;12:43

[45] Nishisho I, Nakamura Y, Miyoshi Y, Miki Y, Ando H, Horii A, Koyama K, Utsunomiya J, Baba S, Hedge P: Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science. 1991 Aug 9;253(5020):665-9

[46] Vogelstein B, Kinzler KW: The multistep nature of cancer. TIG, 1993, 9:138–142

[47] Levin B, Lieberman DA, McFarland B, Andrews KS, Brooks D, Bond J, Dash C, Giardiello FM, Glick S, Johnson D, Johnson CD, Levin TR, Pickhardt PJ, Rex DK, Smith RA, Thorson A, Winawer SJ; American Cancer Society Colorectal Cancer Advisory Group; US Multi-Society Task Force; American College of Radiology Colon Cancer Committee: Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. Gastroenterology 2008; 134:1570–95.

[48] Mandel JS, Church TR, Bond JH, Ederer F, Geisser MS, Mongin SJ, Snover DC, Schuman LM: The effect of fecal occult-blood screening on the incidence of colorectal cancer. N Engl J Med 2000; 343:1603–607.

[49] Atkin WS, Edwards R, Kralj-Hans I, Wooldrage K, Hart AR, Northover JM, Parkin DM, Wardle J, Duffy SW, Cuzick J; UK Flexible Sigmoidoscopy Trial Investigators: Once-only flexible sigmoidoscopy screening in prevention of colorectal cancer: a multicentre randomised controlled trial. Lancet 2010; 375:1624–33

[50] Kutzner N, Hoffmann I, Linke C, Thienel T, Grzegorczyk M, Urfer W, Martin D, Winde G, Traska T, Hohlbach G, Müller KM, Kuhnen C, Müller O: Non-invasive

detection of colorectal tumours by the combined application of molecular diagnosis and the faecal occult blood test. Cancer Lett. 2005 Nov 8;229(1):33-41

[51] Rengucci C, De Maio G, Menghi M, Scarpi E, Guglielmo S, Fusaroli P, Caletti G, Saragoni L, Casadei Gardini A, Zoli W, Falcini F, Amadori D, Calistri D: Improved stool DNA integrity method for early colorectal cancer diagnosis. Cancer Epidemiol Biomarkers Prev. 2014 Nov;23(11):2553-60

[52] Baier T, Hansen-Hagge TE, Gransee R, Crombé A, Schmahl S, Paulus C, Drese KS, Keegan H, Martin C, O'Leary JJ, Furuberg L, Solli L, Grønn P, Falang IM, Karlgård A, Gulliksen A, Karlsen F: Hands-free sample preparation platform for nucleic acid analysis. Lab Chip. 2009 Dec 7;9(23):3399-405

[53] Le HP: Progress and Trends in Ink-jet Printing Technology. J. Imaging Sci. Technol., 1998, 42(1), 49–62

[54] Terry SC, Jerman JH, Angell JB: A gas chromatographic air analyzer fabricated on a silicon wafer. IEEE Trans. Electron Devices, 1979, 26(12), 1880–1886

[55] Shoji S, Esashi M, Matsuo T: Prototype miniature blood gas analyser fabricated on a silicon wafer. Sens. Actuators, 1988, 14(2), 101–107

[56] Van Lintel HTG, Vandepol FCM, Bouwstra S: A piezoelectric micropump based on micromachining of silicon. Sens. Actuators, 1988, 15(2), 153–167

[57] Gass V, Vanderschoot BH, Jeanneret S, Derooij NF: Integrated flow-regulated silicon micropump. Sens. Actuators, A, 1994, 43(1–3), 335–338

[58] Weigl B, Domingo G, Labarre P, Gerlach J: Towards non- and minimally instrumented, microfluidics-based diagnostic devices. Lab Chip. 2008 Dec;8(12):1999-2014

[59] Haeberle S, Zengerle R: Microfluidic platforms for lab-on-a-chip applications. Lab Chip. 2007 Sep;7(9):1094-110

[60] Yager P, Edwards T, Fu E, Helton K, Nelson K, Tam MR, Weigl BH: Microfluidic diagnostic technologies for global public health. Nature. 2006 Jul 27;442(7101):412-8

[61] Pugia MJ, Blankenstein G, Peters RP, Profitt JA, Kadel K, Willms T, Sommer R, Kuo HH, Schulman LS: Microfluidic tool box as technology platform for hand-held diagnostics. Clin Chem. 2005 Oct;51(10):1923-32

[62] Ahn CH, Choi JW, Beaucage G, Nevin JH, Lee JB, Puntambekar A, Lee JY: Disposable Smart Lab on a Chip for Point-of-Care Clinical Diagnostics. Proceedings of the IEEE 2004;92:154

[63] Lim HJ, Choi JH, Son A: Necessity of purification during bacterial DNA extraction with environmental soils. Environ Health Toxicol. 2017 Aug 8;32:e2017013

[64] Norton A: Stool DNA test for colon cancer not yet cost-effective. Reuters Health News. 2010 Sept; retrieved May 28th, 2018

[65] Quintero E, Castells A, Bujanda L, Cubiella J, Salas D, Lanas Á, Andreu M, Carballo F, Morillas JD, Hernández C, Jover R, Montalvo I, Arenas J, Laredo E, Hernández V, Iglesias F, Cid E, Zubizarreta R, Sala T, Ponce M, Andrés M, Teruel G, Peris A, Roncales MP, Polo-Tomás M, Bessa X, Ferrer-Armengou O, Grau J, Serradesanferm A, Ono A, Cruzado J, Pérez-Riquelme F, Alonso-Abreu I, de la Vega-Prieto M, Reyes-Melian JM, Cacho G, Díaz-Tasende J, Herreros-de-Tejada A, Poves C, Santander C, González-Navarro A; COLONPREV Study Investigators: Colonoscopy versus fecal immunochemical testing in colorectal-cancer screening. N Engl J Med. 2012 Feb 23;366(8):697-706

[66] Song K, Fendrick AM, Ladabaum U: Fecal DNA testing compared with conventional colorectal cancer screening methods: a decision analysis. Gastroenterology. 2004 May;126(5):1270-9

[67] Geschke O, Klank H, Telleman P: Microsystem engineering of lab on a chip devices. Wiley-VCH, Weinheim, 2004

[68] Cheah PY, Bernstein H: Colon cancer and dietary fiber: cellulose inhibits the DNA-damaging ability of bile acids. Nutr Cancer. 1990;13(1-2):51-7

[69] Vidovic S, Block HC, Korber DR: Effect of soil composition, temperature, indigenous microflora, and environmental conditions on the survival of Escherichia coli O157:H7. Can J Microbiol. 2007 Jul;53(7):822-9

[70] Egeberg RO, Elonin AE, Egeberg MC: Effect of salinity and temperature on coccidioides immitis and three antagonistic soil saprophytes. J Bacteriol. 1964 Aug;88:473-6

[71] Han Z, Deng M, Yuan A, Wang J, Li H, Ma J: Vertical variation of a black soil's properties in response to freeze-thaw cycles and its links to shift of microbial community structure. Sci Total Environ. 2017 Dec 27;625:106-113

[72] Amann RI, Ludwig W, Schleifer KH: Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev. 1995 Mar;59(1):143-69

[73] Muyzer G, Teske A, Wirsen CO, Jannasch HW: Phylogenetic relationships of Thiomicrospira species and their identification in deep-sea hydrothermal vent

samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch Microbiol. 1995 Sep;164(3):165-72

[74] Liu WT, Marsh TL, Cheng H, Forney LJ: Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol. 1997 Nov;63(11):4516-22

[75] Hoffmann B, Depner K, Schirrmeier H, Beer M: A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J Virol Methods. 2006 Sep;136(1-2):200-9

[76] Barber RD, Harmer DW, Coleman RA, Clark BJ: GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol Genomics. 2005 May 11;21(3):389-95

[77] Raff T, van der Giet M, Endemann D, Wiederholt T, Paul M: Design and testing of beta-actin primers for RT-PCR that do not co-amplify processed pseudogenes. Biotechniques. 1997 Sep;23(3):456-60

[78] Vidovic S, Block HC, Korber DR: Effect of soil composition, temperature, indigenous microflora, and environmental conditions on the survival of Escherichia coli O157:H7. Can J Microbiol. 2007 Jul;53(7):822-9

[79] Egeberg RO, Elonin AE, Egeberg MC: Effect of salinity and temperature on coccidioides immitis and three antagonistic soil saprophytes. J Bacteriol. 1964 Aug;88:473-6

[80] Han Z, Deng M, Yuan A, Wang J, Li H, Ma J: Vertical variation of a black soil's properties in response to freeze-thaw cycles and its links to shift of microbial community structure. Sci Total Environ. 2017 Dec 27;625:106-113

[81] Gorzelak MA, Gill SK, Tasnim N, Ahmadi-Vand Z, Jay M, Gibson DL: Methods for Improving Human Gut Microbiome Data by Reducing Variability through Sample Processing and Storage of Stool. PLoS One. 2015 Aug 7;10(8):e0134802

[82] Egeberg RO, Elonin AE, Egeberg MC: Effect of salinity and temperature on coccidioides immitis and three antagonistic soil saprophytes. J Bacteriol. 1964 Aug;88:473-6

[83] Lakay FM, Botha A, Prior BA. Comparative analysis of environmentalDNA extraction and purification methods from different humic acid-rich soils. J Appl Microbiol 2007;102(1):265-273.

[84] H. Luo,H.Qi, K. Xue, andH. Zhang, “Apreliminary application of PCR-DGGE to study microbial diversity in soil,” Acta Ecologica Sinica, vol. 23, pp. 1570–1575, 2003

[85] Han Z, Deng M, Yuan A, Wang J, Li H, Ma J: Vertical variation of a black soil’s properties in response to freeze-thaw cycles and its links to shift of microbial community structure. Sci Total Environ. 2017 Dec 27;625:106-113

[86] Nicklas JA, Buel E: Quantification of DNA in forensic samples. Anal. Bioanal. Chem. 376 (2003) 1160–1167

[87] Hansen TV, Simonsen MK, Nielsen FC, Hundrup YA: Collection of blood, saliva, and buccal cell samples in a pilot study on the Danish nurse cohort: comparison of the response rate and quality of genomic DNA. Cancer Epidemiol Biomarkers Prev. 2007 Oct;16(10):2072-6

[88] Choo JM, Leong LE1, Rogers GB: Sample storage conditions significantly influence faecal microbiome profiles. Sci Rep. 2015 Nov 17;5:16350

[89] Shaw AG, Sim K, Powell E, Cornwell E, Cramer T, McClure ZE, Li MS, Kroll JS: Latitude in sample handling and storage for infant faecal microbiota studies: the elephant in the room? Microbiome. 2016 Jul 30;4(1):40

[90] Gerasimidis K, Bertz M, Quince C, Brunner K, Bruce A, Combet E, Calus S, Loman N, Ijaz UZ: The effect of DNA extraction methodology on gut microbiota research applications. BMC Res Notes. 2016 Jul 26;9:365

[91] Thermo Scientific. Assessment of Nucleic Acid Purity. Wilmington, Delaware USA: NanoDrop Spectrophotometers; [cited on April 17th, 2018]; [Technical Bulletin T042]; Available from: https://tools.thermofisher.com/content/sfs/brochures/TN52646-E-0215M-NucleicAcid.pdf

[92] Wilfinger WW, Mackey K, Chomczynski P: Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques. 1997 Mar;22(3):474-6, 478-81

[93] Leninger AL: Biochemistry, 2nd ed., Worth Publishers, New York, 1975

[94] Opitz S, Hannika F, Krüger T, Rhode H: The removal of Triton X-100 by dialysis is feasible! Anal Bioanal Chem. 2015 Feb;407(4):1107-18

[95] Abbaszadegan MR, Velayati A, Tavasoli A, Dadkhah E: Rapid DNA extraction protocol from stool, suitable for molecular genetic diagnosis of colon cancer. Iran Biomed J. 2007 Jul;11(3):203-208

[96] Agilent Trusted Answers: *E. coli* Cell Culture Concentration from OD_{600} Calculator. Online: http://www.genomics.agilent.com/biocalculators/calcODBacterial.jsp, retrieved: May 14th, 2018

[97] Janke SA, Fortnagel P, Bergmann R: Microbiological turbidimetry using standard photometers. Biospektrum, 1999, Vol. 6, 501-502

[98] Qiagen: QIAamp DNA Stool Mini Kit. Online: http://www.qiagen.com/de/shop/sample-technologies/dna/genomic-dna/qiaamp-dna-stool-mini-kit/, retrieved: May 17th, 2018

[99] Lee NY: A review on microscale polymerase chain reaction based methods in molecular diagnosis, and future prospects for the fabrication of fully integrated portable biomedical devices. Mikrochim Acta. 2018 May 8;185(6):285

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SS2011	Beginning of master studies in Biotechnology at Mannheim University of Applied Sciences focus: Bioprocess Development
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