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Extraction of DNA from Difficult Samples with an Automatable Portable System

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ABSTRACT

Many difficult DNA sources cause problems in clinical diagnostics, food analytics, environmental analysis and molecular forensics [1]. Fecal DNA has diagnostic relevance; analysis might, therefore, allow conclusions on the presence of (intestinal) diseases at early stages in a quick and non-invasive manner [5]. DNA extracted from soil allows conclusions on the composition of the microflora and the purification and analysis of DNA from specific bacteria. Unfortunately, all currently available methods for extraction of DNA from difficult samples 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. First, we searched for a suitable system as a base for further modification. This system was then scaled down and several modifications to the original protocol were made. 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, we used soil and stool for further evaluations. In order to make extraction possible inside a chip without the use of a centrifuge, we used sedimentation. 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.

INTRODUCTION:

Many sources from which extraction of DNA is problematic cause problems in clinical diagnostics, food analytics, environmental analysis and molecular forensics[1].Examples are body fluids (stool, urine), fatty foods or soil. DNA extracted in conventional ways from these sources is unstable and difficult to analyze because of the presence of compounds which are mutagenic and destructive against DNA and of inhibitors that influence the further processing of DNA by interference with analytical enzymes (DNA polymerases, restriction enzymes). Examples of such substances are DNases, bile acids, salts, metal ions and bacterial carbohydrates [2][3].

Fecal DNA has diagnostic relevance, as it contains DNA from various sources, like microbiome or intestinal mucosa. Analysis of fecal DNA might, therefore, allow conclusions on the presence of (intestinal) diseases at early stages in a quick and non-invasive manner [5].DNA extracted from soil allows conclusions on the composition of the microflora and the purification and analysis of DNA from specific bacteria.

Extraction of DNA from difficult sources is commonly done by adsorption of DNA to an immobilized matrix while unwanted substances are precipitated or simply washed away. We and others developed methods and reagent kits for extraction of DNA from stool and other difficult samples [4]. Unfortunately, all available methods are neither quick nor automated nor easy to use. In all cases, laboratory equipment is needed, as well as skilled personnel. In addition, methods used today are not cost-effective yet [6]. Thus, the development of an easy-to-use, automatable and portable system for extraction of DNA from difficult samples would be beneficial. It could be used on-site, e.g. bedside in hospitals, in medical practices or in the field.

As the first step of this project, we searched for a suitable system base for further modification. After comparing several different systems we decided to use the geneMAG-RNA/DNA kit (Chemicell, Berlin, Germany) based on magnetic beads coated with a silica matrix. It was scaled down in order to fit it inside a microchip system. Several modifications to the original protocol were made to optimize the extraction process, as this kit was originally developed for other sample types like bacteria culture. 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, we used soil and stool for further

evaluations. In order to make extraction possible inside a chip without the use of a centrifuge, we used sedimentation. Finally, complete DNA extraction was performed inside a chip, from sample addition to final elution. As an advantage, the majority of parts used for an extraction are reusable, except for a small fraction of tubes. This is achieved by using air pressure for pumping so that fluids run mostly directly on the chip. The chip itself might be reused as well.

MATERIALS AND METHODS:

Collection of soil samples

Soil samples were collected from a lawn in Zweibrücken, Germany. Samples were collected in 50mL falcon tubes using a small shovel and soil was acquired from a hole with a depth of approximately 10 – 15cm, stones and other solid matter (e.g. roots, worms) were avoided upon collection where possible. Samples were stored at 4°C directly after the acquisition and were used the same day, if not stated otherwise. Date, temperature, weather conditions (rain, snow etc.) and sample conditions (moisture, color) were noticed, as these factors may influence the microflora, bacteria count[7][8] and the soil sample itself (moisture, salinity, nutrients) [9].

Bacteria culture

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

Collection of stool samples

Human stool samples were collected in 50 mL falcon tubes with as little fluid as possible 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 [10].

PCR

Two primer pairs were used for analysis: 8F[11] + 926R [12] and EGFP fwd. + EGFP rev [13]. The first pair codes for the prokaryotic 16S rRNA gene[14], whereas the latter one codes for the EGFP gene[13].Primer sequences are shown in Table 1 below.

Table 1: 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

The PCR mix consisted of 10µL MyTaq™ Red Mix (Bioline, Luckenwalde, Germany), 1µL primer (10mM), 7µL H_2O and 1µL DNA sample, resulting in a total reaction volume of 20µL per sample. PCR was performed with 20, 25 or 30 cycles, if not stated otherwise, depending on the amount of DNA expected in the sample. The PCR protocol is shown in Table 2.

Table 2: PCR protocol

Step		Temp. [°C]	Time [sec]	Cycles
1	Initial Step	94	120	
2	Denaturation	94	60	20-30
3	Annealing	49 / 58	45	
4	Elongation	72	60	
5	Final Elongation	72	240	
6	Cooling	4	Pause	

For the 8F & 926R primer pair an annealing temperature of 49°C was applied, for the EGFP primer pair 58°C. Samples were analyzed by gel electrophoresis in 1% agarose gels laced with 4 µL per 100 mL Midori Green Advance DNA stain (Biozym Scientific GmbH, HessischOldendorf, Germany). Gels were run for 10 – 15 min at 86 Vand visualized in UV light. As positive controls, PCR reactions of high yield DNA extracts with the same primers as the samples were performed and loaded on the corresponding gels next to the samples.

DNA extraction

DNA was extracted using the geneMAG-RNA/DNA kit (chemicell, Berlin, Germany). The principle is shown in Figure 1 below.

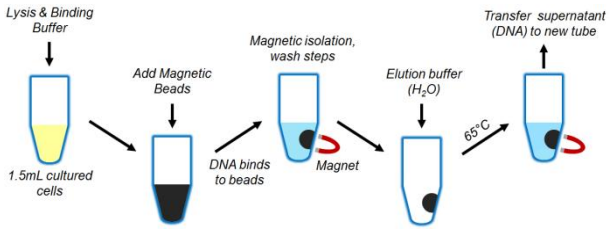

Figure 1: DNA extraction

DNA extraction steps: 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.

As the kit was initially developed for cultured bacteria and for extraction in reaction tubes, the extraction protocol was altered to suit a microchip system: First, most of the volumes were scaled down to one-fourth of the original volumes. Secondly, centrifugation steps were replaced by sedimentation: Lysis buffer was added to the sample, homogenized well and left to rest for 1 – 15min. After this, 250µL of the clear supernatant was transferred to a new reaction tube or microchip chamber for further extraction steps, when magnetic beads were added.

Microchip system

The microchips, made of cyclic olefin copolymer (COC), contained seven chambers ranging from 0.5mL to 2.0mL volume and custom channel designs for interconnection of the chambers. The chip was connected to a syringe pump (LA-100, LandgrafLaborsysteme HLL GmbH, Langenhagen, Germany) via Versilic®silicone tubes (Ø=1.0mm; Carl Roth GmbH + Co. KG, Karlsruhe, Germany). These tubes were interconnected via Y-connectors with pinch clamps for fluid direction control. Pumping was performed by air pressure (pump rate: 0.5 – 2.0mL/min), therefore inlet tubes were never in contact with a fluid. Thus, inlet tubes were reusable. The microchip was sealed with Peqlab qPCR seal (VWR International GmbH,

Darmstadt, Germany), an adhesive film activated by pressure, which is removable without residue.

The microchip system including the corresponding protocols is patent pending.

RESULTS AND DISCUSSION:

RESULTS:

Scale down

Volumes given by the manufacturer of the kit were scaled down to one half or one-fourth of the original volumes. Five hundred milligram of the same soil sample were extracted individually, followed by PCR. As shown in Figure 2 below, sufficient yields were visible on an agarose gel.

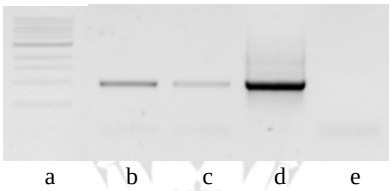

Figure 2: Scale down

Gel electrophoresis with PCR products (16S rRNA gene, 30 cycles) of DNA extracts, 0.5g of the same soil sample, different volumes (compared to original protocol); **a**: Ladder 1kb; **b**: Half volume; **c:** One-fourth volume; **d:** Positive control; **e:** Negative control

Comparison to a regular extraction method

DNA was extracted from a bacteria overnight culture in a 1.5mL reaction tube as suggested by the kit manufacturer's protocol and in a microchip for comparison. Five hundred microliter of the same *E.Coli* culture was used. Afterwards, a PCR with 8F + 926R primer pair and 25 cycles were performed. As shown in Figure 3, no significant differences were observed between the extraction in a reaction tube and a microchip, suggesting that extraction in a chip provides similar yields as extraction according to the manufacturer's protocol.

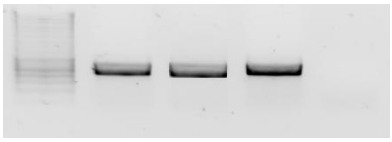

Figure 3: Comparison to regular extraction method

Gel electrophoresis with PCR products (16S rRNA gene, 25 cycles) of DNA extracts, 0.5mL of *E.Coli* overnight culture; **a:** Ladder 1kb; **b:** Reaction tube; **c:** Chip*;* **d:** Positive control; **e:** Negative control

Sedimentation instead of centrifugation

In order to make extraction possible completely inside the microchip, the centrifugation step given by the manufacturer's protocol had to be replaced. Sedimentation was applied instead with different sedimentation times ranging from 1min to 15min. Five hundred milligrams soil of the same sample were used as DNA source. After extraction, a PCR with 8F + 926R primer pair and 30 cycles were performed. As shown in Figure 4, 15min sedimentation time proved to be sufficient to replace centrifugation.

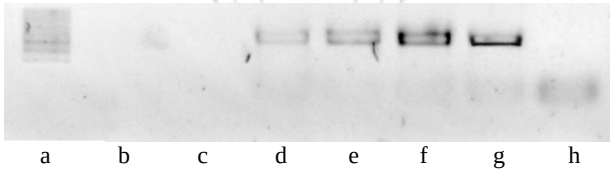

Figure 4: Sedimentation test

Gel electrophoresis with PCR products (16S rRNA gene, 30 cycles) of DNA extracts,0.5g of same soil sample, different sedimentation times; **a:** Ladder 1kb; **b:** 1min; **c:** 2min; **d:** 5min; **e:** 10min; **f:** 15min;**g:** Positive control; **h:** Negative control

Spiking of soil samples with *E.Coli* (EGFP)

In order to test the potential influence of soil on DNA quality and on the outcome of the subsequent PCR, soil samples (0.5g) were spiked with *E.Coli* that had previously been transfected with a vector containing an EGFP gene and ampicillin resistance. DNA was extracted from pure *E.Coli* culture alone, from the spiked sample and from pure soil alone. As clearly visible in Figure 5, there is no difference in yield whether soil is present or not suggesting that soil does not inhibit DNA extraction or PCR in any kind of way.

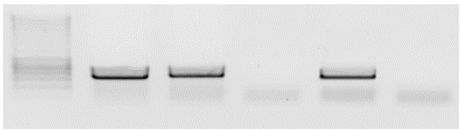

Figure 5: Spiking of soil samples

Spiking with *E.Coli* (transfected with EGFP gene), gel electrophoresis with PCR products (EGFP gene, 30 cycles) of DNA extracts; a: Ladder 1kb; b: Pure *E.Coli*; c: Spiked (soil + *E.Coli*); d: Pure soil; e: Positive control; f: Negative control

Lower detection limit

For the determination of the lower detection limit, 0.5g of soil was spiked with defined amounts of *E.Coli* (EGFP) culture with an optical density of OD_{600} = 1.6. This optical density corresponds to a concentration of approximately $1.28 \cdot 10^9$ CFU/mL[19]. Between 0.5 and 5µL of this bacteria, culture was added to the soil sample prior to DNA extraction followed by PCR with the EGFP primer pair. An agarose gel with these PCR products after 30 cycles is shown in

Figure 6. Even with the lowest amount of bacteria added (0.5µL), a clear band can be seen on the agarose gel meaning that the extraction system is very sensitive. For better contrast a PCR with 20 cycles was performed, the agarose gel is shown in

Figure 7. Here an increase in band strength can be seen with increasing bacteria culture volume.

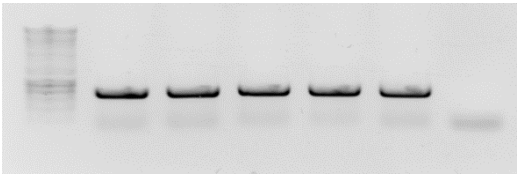

Figure 6: Lower detection limit, 30 cycles PCR

Gel electrophoresis with PCR products (EGFP gene, 30 cycles) of DNA extracts from defined amount of *E.Coli* overnight culture (OD_{600} = 1.6) in soil; a: Ladder 1kb; b: 0.5µL; c: 1.0µL; d: 2.5µL; e: 5.0µL; f: Positive control; g: Negative control

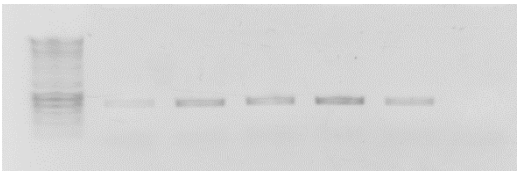

Figure 7: Lower detection limit, 20 cycles PCR

Gel electrophoresis with PCR products (EGFP gene, 20 cycles) of DNA extracts from defined amount of *E.Coli* overnight culture (OD_{600} = 1.6); a: Ladder 1kb; b: 0.5µL; c: 1.0µL; d: 2.5µL; e: 5.0µL; f: Positive control; g: Negative control

Extraction of stool DNA

DNA was extracted out of 500 mg and 250 mg human stool, followed by PCR with 8F + 926R primer pair and 30 cycles. The corresponding agarose gel is shown in

Figure 8, where clear bands are visible for both amounts of stool.

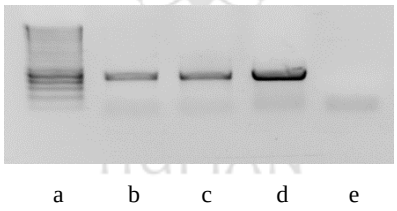

Figure 8: Extraction of stool DNA

Gel electrophoresis with PCR products (16S rRNA gene, 30 cycles) of DNA extracts from various amounts of stool; a: Ladder 1kb; b: 500mg; c: 250mg; d: Positive control; e: Negative control

Spiking of stool samples with *E.Coli* (EGFP)

Similar to the experiment with soil mentioned above, stool samples (250 mg) were spiked with *E.Coli* previously transfected with an EGFP gene and Ampicillin resistance. Between 2.5 and 40 microliters of *E.Coli* culture with an optical density of OD_{600} = 1.6 were added. After extraction, a PCR with the EGFP primer pair and 30 cycles were performed. The agarose gel is shown in

Figure 9, where clear bands with a decrease in band strength can be seen with decreasing *E.Coli* volume.

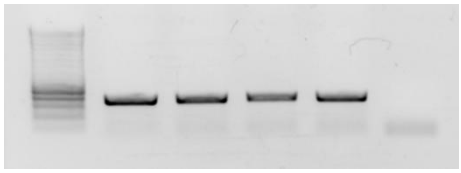

Figure 9: Spiking of stool samples

Gel electrophoresis with PCR products (EGFP gene, 30 cycles) of DNA extracts from defined amount of *E.Coli* overnight culture (OD_{600} = 1.6) in stool; a: Ladder 1kb; b: 40µL; c: 10µL; d: 2.5µL; e: Positive Control; f: Negative Control

DISCUSSION:

In this study, we showed that even with the scaled down geneMAG RNA/DNA kiton down to one-fourth of the original volume sufficient yield was achieved. DNA was extracted out of only 0.5g of soil, which is much less than in other studies and currently used methods [15][16]. In order to perform a complete DNA extraction inside a microchip, the centrifugation step was successfully replaced by sedimentation. Comparison between extraction in a chip and in a reaction tube (manufacturer's protocol) showed that there is no significant difference between both methods. With the new method DNA extraction from soil is much easier and faster than other methods previously published[17][18].

As soil is no homogenous sample and its composition changes depending on several factors (e.g. season, site, weather) [7], soil samples were spiked with a specific amount of. *Coli* bacteria transfected with an EGFP gene for better comparability. As native bacteria do not have an EGFP gene, the spiked bacteria could be identified with a PCR using specific EGFP primers[13]. Comparison to the extraction of DNA from the same amount of bacteria culture without the presence of soil showed that soil does not influence the extraction, which stands in contrast to the findings of Lakay et al.[17], who found that only a combination of several extraction methods together led to sufficient DNA yield. A possible explanation for this discrepancy might be the different soil samples or higher sensitivity of the new extraction system compared to those used by Lakay et al.

In order to show the sensitivity of the extraction system, soil samples were spiked with low amounts of bacteria. Even with as little as 0.5µL of bacteria solution with an OD_{600} of 1.6 added to 0.5g of soil, which equivalents to 10^6 bacteria per gram of soil [19], yield was still sufficient for PCR. This corresponds to the findings of Kuske et al. [20], who performed a similar experiment with another bacteria strain. The amount of bacteria added is very low, as soil usually contains $9 – 11 \cdot 10^9$ microorganisms per gram [21][22].

DNA was extracted out of human stool samples. By use of the 8F + 926R primer pair for PCR, bacteria DNA was successfully amplified, showing that extraction of DNA was possible with the system. With this method, extraction is significantly easier and faster than with current methods, like the one described by Hosomi et al. [24].

As stool is, similar to soil, a heterogeneous sample material (depending on diet [23] and sample treatment/preparation method [24], for example) various amounts of *E.Coli* bacteria culture transfected with an EGFP gene were added. DNA was extracted successfully from all samples, even with the lowest amount of *E.Coli* added, suggesting that stool does not interfere in DNA extraction and that sensitivity of the extraction system is very high.

The next steps in this topic are an evaluation of the sedimentation step and further automation of the system. Secondly, we will test pre-filled chips in order to provide a ready-to-use system. Additionally, the extraction of human DNA from stool samples will be evaluated as well as analysis of the complete fecal genome.

CONCLUSION:

This work might open whole new possibilities in several research fields, where difficult DNA sources have to be analyzed. As mentioned above, stool DNA, for example, is of great importance, as it can be used as a marker for diseases [5]. Although there are already approaches to a small scale system, as Fu et al.[25] and Birch et al.[26] have shown, these are not suitable for difficult samples as described in this study. Thus, the system developed within this work is a promising starting point for a multi-purpose DNA extraction system with many various applications.

ACKNOWLEDGMENTS:

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