Detektion af E.coli DNA i ledningsvand

Afrapportering for projekt støttet af VTU-Fonden

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Vandsektorens Teknologiudviklingsfond

Projekt ID nr. 7847.2015: Detektion af E.coli DNA i ledningsvand
Hovedansøger: Bluense-Diagnostics Aps
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Bilag: 2

Content

1 Perspectives

1.1 Describe business plan and go to market strategy

The project has allowed a huge step-ahead for the development of BluSense Diagnostics's (BSD's) technology for water analysis. We have been able to demonstrate all the key technological steps needed to perform a fast and sensitive molecular analysis of water samples. Clearly, although considerable progress has been made during the past 9 months' project, the product is not yet ready for market.
We still need to develop a more robust LAMP based assay for the detection of E. coli in water and also to finalize the development of the reader (bring it from feasibility stage into development stage) and work on the integration of sample preparation with current developed technology (which was not part of the present project). Please refer to part 3 for more on the technical progress and needs for further development.

For this reason, we have applied for another round of funding, this time from MUDP[1]. This funding should bring us to the development of a fully validated prototype, capable of performing drinking water analysis. From there, the next step will be commercialization.

We have however also realized that it would be a considerable advantage if we could combine our technology with a high bacteria pre-concentration system from Amphi-bac into a **single detection unit, that can be used directly in the field**, without the need to up-concentrate e.coli as a separate step. We foresee this combined solution to have great potential value on water utilities[2]. Therefore, Amphi-bac is part of the MUDP consortium that BSD has been organizing. The MUDP funding will run for 2 years, and employ 3,5 FTEs from BSDs, as well as further resources from Amphi-Bac and Kalundborg Forsyning. Through this financing we can finalize the technology development, which will consist of a complete unit capable to perform molecular analysis from sample to answer in less than 30 minutes. Next step is final validation on-site and then the product will be ready for sale.

1.2 Business opportunities

The project has made BSD come closer to developing a product to a potentially very big water analysis market. As written in the application, we foresee several market opportunities for the product, both for public water utilities and for industrial applications, i.e. wastewater analysis, both in Denmark and abroad.

In Denmark, we estimate that the market for our integrated e.coli detection reader for water utilities will be approx. 30-40 million, depending on how many units the individual utilities are installing. If the technology is developed further, with the possibility of simultaneous and rapid assessment of other bacteria e.g. campylobacter, legionella, the market will be significantly larger. The industrial market in Denmark is expected to be at least 10 -20 times greater. At the same time, the use of the sensor opens up the possibility to a much greater recycling of water in the industrial sector, and thus a greater use of the sensor.

Internationally, the primary focus will be on the industrial water use, as chlorination is widely used in water supply around the world. North America and the EU are collectively approximately 100 times larger than Denmark, so a market estimate could be 3-4 billion DKK for water utilities and an even larger industrial market alone in this part of the world. A recently published market report from December 2015, estimates that the

[1] Miljøministeriets Miljøteknologiske Udviklings- og Demonstrationsprogram
[2] Please see "Støtteerklæring fra 3Vand", appendix 1

global e.coli testing market will grow with almost 7% p.a. until 2022, where the total market is projected to be worth USD 2,1 (approx. DKK 14 bn). up from USD 1,2 (approx. DKK 8 bn) in 2013.

Even though there are many competitors in the market, it is our estimate that the solution we are aiming at developing could be extremely competitive. Many devices for water monitoring are based on enzymatic measuring principles (e.g. CALM by Colifast A/S; Coliguard BACTcon by MicroLAN; TECTA by Veolia). The biggest disadvantages of the enzymatic method are the response time, because it can take 4-12 h. Also, quite high sampling volumes are required. Biotrack has developed a device (Aquascope) which is based on filter cytometry & FISH measuring principles. This technology is very expensive compared to others in the market. There are also devices based on optical measuring principle e.g. Bacmon by Grundfos A/S, able to count the total amount of bacteria/particles present in water. These technologies are however not suitable for distinguishing between various types of bacteria. Please see competitive overview in appendix 2.

Compared to that, BSD aims at developing a unique low-cost, fast and 100% reliable molecular based e.coli detector that can be used directly in the field. Therefore, our aim is to launch a product on the market within the next three years. The potential we foresee is that this market could employ 10 FTE's in BSD within 5-6 years' time and 3 to 5 employees as well at Amphi-Bac. In addition to that, the development of BSD's technology for bacteria analysis in water can be a door-opener for several other novel application areas in the environmental monitoring field.

1.3 Communication

We are planning to send out a press release to water sector stakeholders about our progress so far. Moreover, thanks to this project, we have established a very strong synergy with the group of Prof. Mikkel F. Hansen at DTU Nanotech. Together with his group we are planning to present the results at international conferences (we will submit an abstract for MicroTas conference, November, 2016) and to write a scientific paper in the autumn gathering the key results achieved during the project. This project is also the core activity of two Master students's projects at BSD, who will write their thesis respectively about the e.coli molecular assay and cartridge development process. In addition to that, the results obtained in this project are almost weekly presented to investors and other business meeting and conferences where we present, e.g. at Zurich Venture Summit, BioVaria in Munich and newly held conference Innovex in Taipei (see below one of our slide). The project has also been mentioned in the MUDP grant that we applied for in May.

[3] The research study is from Transparency Market Research and is titled "E. coli Testing Market - Global Industry Analysis, Size, Share, Growth, Trends and Forecast 2014 - 2022

BLUSENSE

Bacteria Diagnostics for water applications

BLUSENSE ACQUA

Real-time water monitoring solutions

- Quick and affordable detection of E. coli bacteria in water
- Molecular diagnostics of E. coli strands at 10^3 CFU/mL
- Designed to be integrated in on-line systems
- Automated on-line reporting of bacteria detection events

Amphi-Bac

KALUNDBORG FORSYNING

2 Evaluation

2.1 The aim of the project

The overall aim of the project has been to develop a proof-of-concept system to cheaply and reliably detect the presence of e. coli in the drinking water within less than 30 minutes. The system consists of a handheld device (a reader) and disposable chips that make the actual analysis from pre-treated water samples.
The reader controls the chip and ensures that results are forwarded via wireless internet to the user.

To that purpose, two milestones were identified in the project application:

M1: The project leader from BSD will together with Prof. M F Hansen and his group at DTU Nanotech, develop a suitable disposable chip for the detection of amplified E. coli DNA.

M2: A suitable assay design and isothermal amplification method will be identified with our partner from SciLifeLab at the same time as the monitoring of this system will be developed in the reader.

When the project was prolonged in January, we added two additional milestones:

M3: Optomagnetic detection of E.coli bacteria in drinking and waste water using LAMP amplification

M4: First prototype of integrated LAMP amplification on cartridge (from bacteria lysate)

The status on each milestone is explained below.

2.2 Has the project reached its goals

The project has reached the main target of providing a solid technical proof of the capability of BSD's readout technology to detect and recognize water samples containing e.coli, using a molecular approach. Thanks to this project we have been able to develop the technology for water analysis application, which has opened a completely new market opportunity. In addition, the project gave us the possibility to interact (however limited) and to connect to Kalundborg Forsyning. This is of key importance, as connecting the technology provider and the user from the very early stage of development, has allowed us to design the most promising solution from the very beginning. In addition, this project has given us the resources to identify the next steps needed to bring this technology to market and has identified a novel potential user and collaborator, Amphi-bac.

M1: A strong focus of the project has been put on developing a mass-producible cartridge capable to perform molecular analysis of water. The key requirements in this sense are solid bonding which can avoid any leakages that can cause sample cross-contamination, biocompatibility of plastics components and transparency to ensure optimal optical measurements.

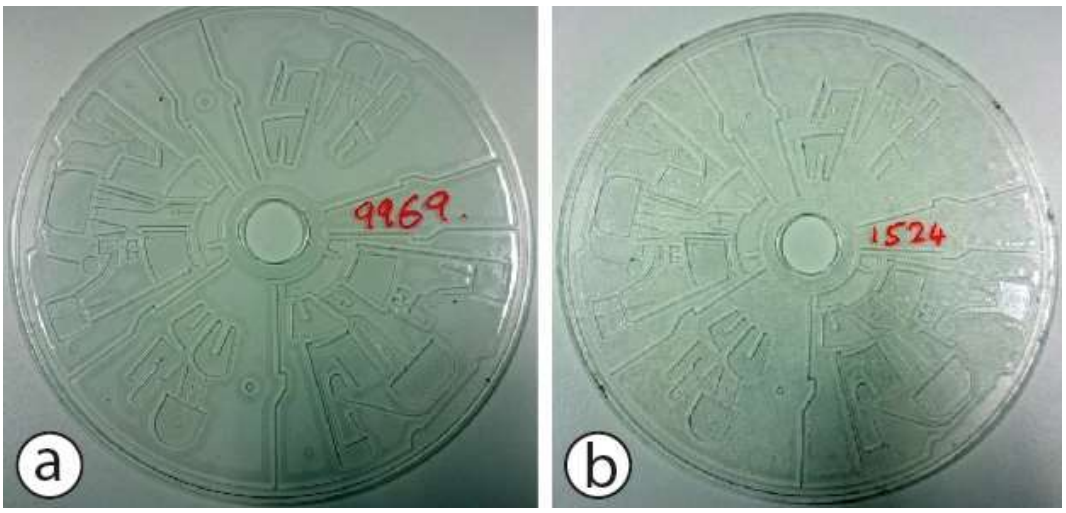

Figure 1: Injection molded discs (each containing 3 cartridge slices) bonded with different adhesives. There is a clear difference in terms of transparency between (a) and (b) as well as difference in ability to maintain stable bonding up to 67 degrees.

Finalized cartridge design has been realized using 3M pressure adhesive tape and PMMA[4] parts, which have been found to be stable enough to 67 degrees, which is the temperature used for DNA amplification prior to sample detection. Bad bonding of cartridges has caused several contamination problems during on chip DNA amplification, but has been solved in the last part of the work.
The milestone M1 has been successfully completed (100%) and we have now in control a prototyping and production process for cartridges compatible with water analysis.

M2: As mentioned in the project interim reports, we have been starting the project with the idea of developing the readout system based on Rolling Circle isothermal Amplification with SciLifeLab. In the first part of the project we however realized that such amplification method is too slow to achieve the target sensitivities (1 E.coli cell in 100mL water) in the target time. We then decided to move toward a completely novel approach, i.e. the development of LAMP amplification combined with BSD's opto-magnetic readout method.
A completely novel detection strategy based on the use of biotinylated primers and integration on-chip without any sample exchange and/or prior amplification, has been developed throughout the project. We faced significant challenges in reducing contaminants and securing stable results over time as well as ensuring batch to batch reproducibility. The main challenges have been solved, so therefore we consider the milestone M2 successfully completed, as we believe LAMP amplification is the ideal technique to be applied to DNA amplification in water. For the reader development to LAMP, please see M4.

M3: Thanks to the very positive results of LAMP amplification we focused on detection of e.coli bacteria in water. As will be explained more in detail in the next section, we achieved state of the art sensitivity, compared with real-time PCR (10^2/10^3 CFU/mL) using 20 μL sample in only 20 min amplification. We also achieved in-house coating of magnetic nanoparticle with streptavidin from Click Chemistry which enables high sensitivity detection

[4] poly-methylmethacrylate

of biotinylated amplified LAMP products. The assay has been optimized by changing particle ratio, DNA strands length, particle blocking, type of beads, and streptavidin coating.

The step of DNA extraction has not been integrated yet in the microfluidic cartridge, but we successfully tried to amplify directly a water sample with bacteria and we could clearly detect positive from negative samples, please see Figure 4. However, more work has to be invested in optimizing the sample preparation in the next stage of technology development, as the current readout module will need to be coupled with a sample pre-concentration system (as Amphi-Bac unit).

M4: We successfully developed a prototype which integrates LAMP amplification directly on the reader module. This milestone, completed together with our subsidiary BBT (Blusense Biotech Taiwan) has required substantial electronic circuit development and mechanical tooling as well as software design. The development of temperature stable rotating unit, which is able to maintain good electrical contact while rotating at high speed, has required additional resources and took longer time than initially planned, please also refer to the financial reporting. The task was achieved by developing a temperature controlled chuck which supports the cartridge as shown in Figure 2. The characterization of the system has been performed together with DNA amplification tests on the cartridge. The finalized integration of all the steps has not been achieved yet, as we need to further improve the temperature stability.

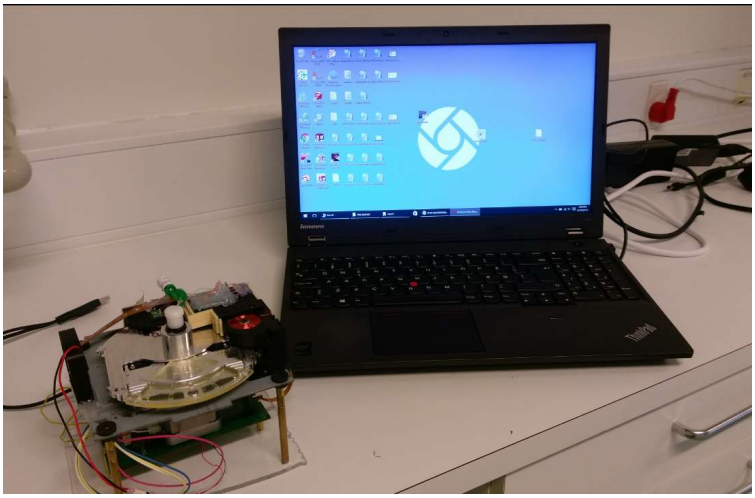

Figure 2: Picture of the final prototype which embeds temperature control and therefore possibility of performing LAMP amplification on the reader. Technical details of the reader unit are described in section 3.

3 Technical description

3.1 Experimental set-up

We have been performing several experiments aimed at calibrating our readout unit, establishing the detection limit and extracting an e.coli bacteria dose-response characterization. The core of the experimental activity has been represented by the optomagnetic detection of 16S RNA of e.coli, amplified through isothermal loop-mediated amplification (LAMP).

Samples:
We have been cultivating e.coli bacteria at DTU which then has been spiked in water and we have afterwards been performing DNA lysis as well, as we have been using synthetic targets for internal calibration. We also have been working and testing water samples from Kalundborg Forsyning potentially containing e.coli.

Targets:
Whole nucleic acid extract (100 ng/µl) containing 16S RNA; e.coli cells (10^8 bacteria/ml – stock after culturing). The stock *RNA*+DNA sample (also called the whole nucleic acid cell content) concentration, 100 ng/µl, is equal to 5·10^6 bacteria in water and was loaded onto DNA extraction unit and extracted by 250 µl of buffer (that gives an output 2·10^7 bacteria/ml).
For example, 1 µg of the whole nucleic acid cell content is equal to 2·10^5 bacteria.
The 5·10^6 cells is the max loading capacity of the columns and slightly more cells (10^7) were loaded in order to saturated the column.

Lamp Primers (ID6)
F3 5' GGGTGCAAGCGTTAATCGG
B3 5' TGAGCGTCAGTCTTCGTCC
FIP 5' AGCCCGGGGATTTCACATCTG-AATTACTGGGCGTAAAGCGC (5'-biotin F1c-F2)
BIP 5' GCTTGAGTCTCGTAGAGGGGGG-TTCGCCACCGGTATTCCT (B1c-B2)
LF 5' CTTAACAAACCGCCTGCGT
LB 5' CGGTGAAATGCGTAGAGATCTGG

Magnetic Nanoparticles
We have during the course of the project developed a novel "click-chemistry" based technique capable to coat streptavidin on magnetic nanoparticles. During the course of the project we have been comparing results using commercial streptavidin coated MNPs (from Micromod) with commercial plain nanoparticles (from Merck), coated in-house. The latter may provide us with a more reliable and scalable production process, as the key reagents coating would be performed in-house.

Technology test protocol
Samples containing bacteria, DNA lysate, or syntetic DNA are mixed with magnetic nanoparticles (streptavidin coated), biotinylated primers and different chemical components allowing for LAMP reaction directly into the plastic microfluidic cartridge. The DNA amplification is performed via on-chuck heating of the cartridge. The readout is performed using optomagnetic detection method – data are then analyzed.

3.2 Describe experimental set-up

In order to perform all the experimental work, we have been using either separate units for isothermal amplification (external temperature controlled heater) together with a separate prototype for detection while a

core part of the project has been represented by the development of an integrated unit for amplification and detection with our partner BBT (milestone M4).

The system is built so that a water sample (or a pre-concentrated sample of water) is inserted in the cartridge, the cartridge is inserted in the reader and the DNA amplification and detection is performed automatically – see figure 3 for technical details.

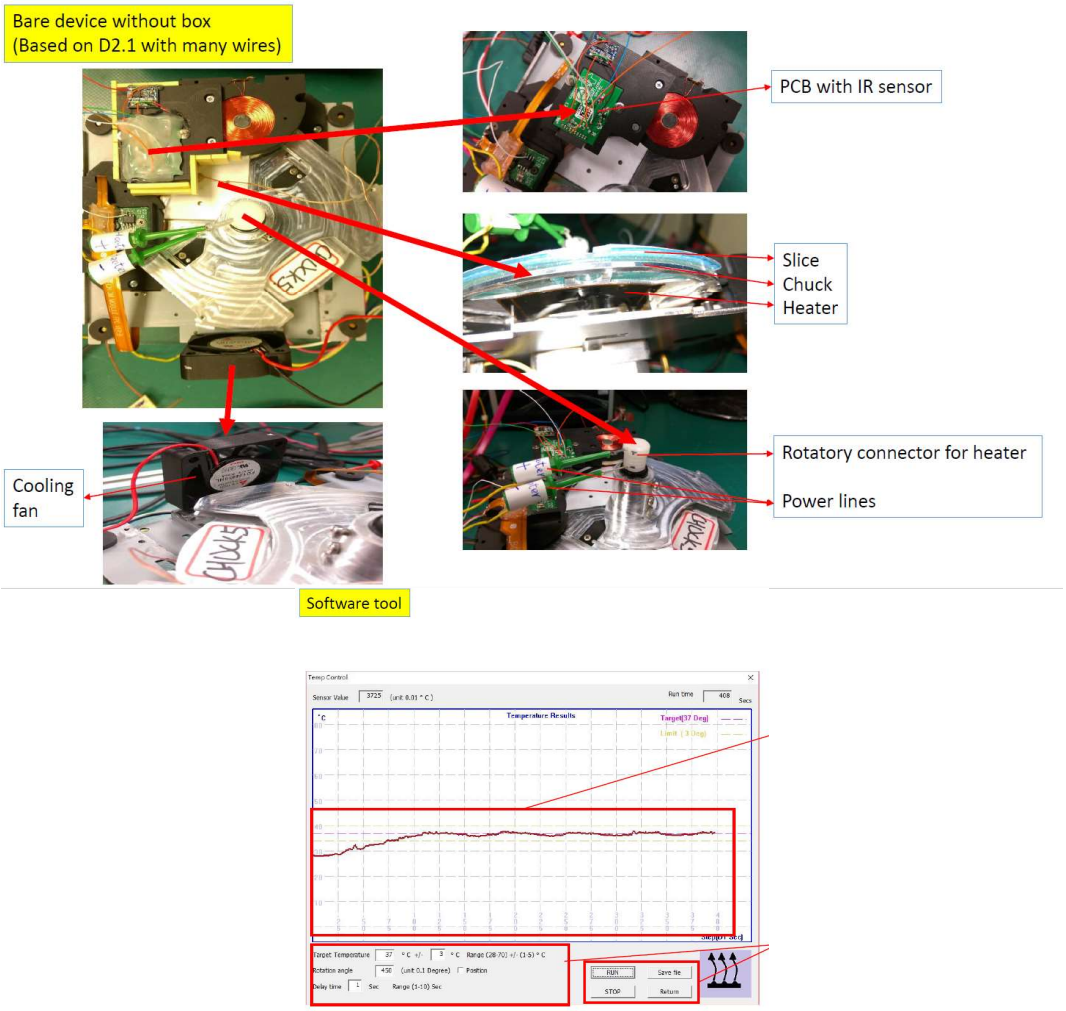

Figure 3: Detailed description of the key elements of the final readout module, which embeds a rotatory connector and an IR sensor to provide feedback loop to the reader CPU– see temperature control software on the bottom. The whole system is developed with mass-producible components.

3.3 Documentation of functionality

Detection limit in calibrants (spiked E.coli DNA in water)

We have been establishing the detection limit of our system to be between 10-100 aM of synthetic E.coli DNA in only 20min of amplification, which theoretically could signify a limit of detection well below 100 bacteria/mL.

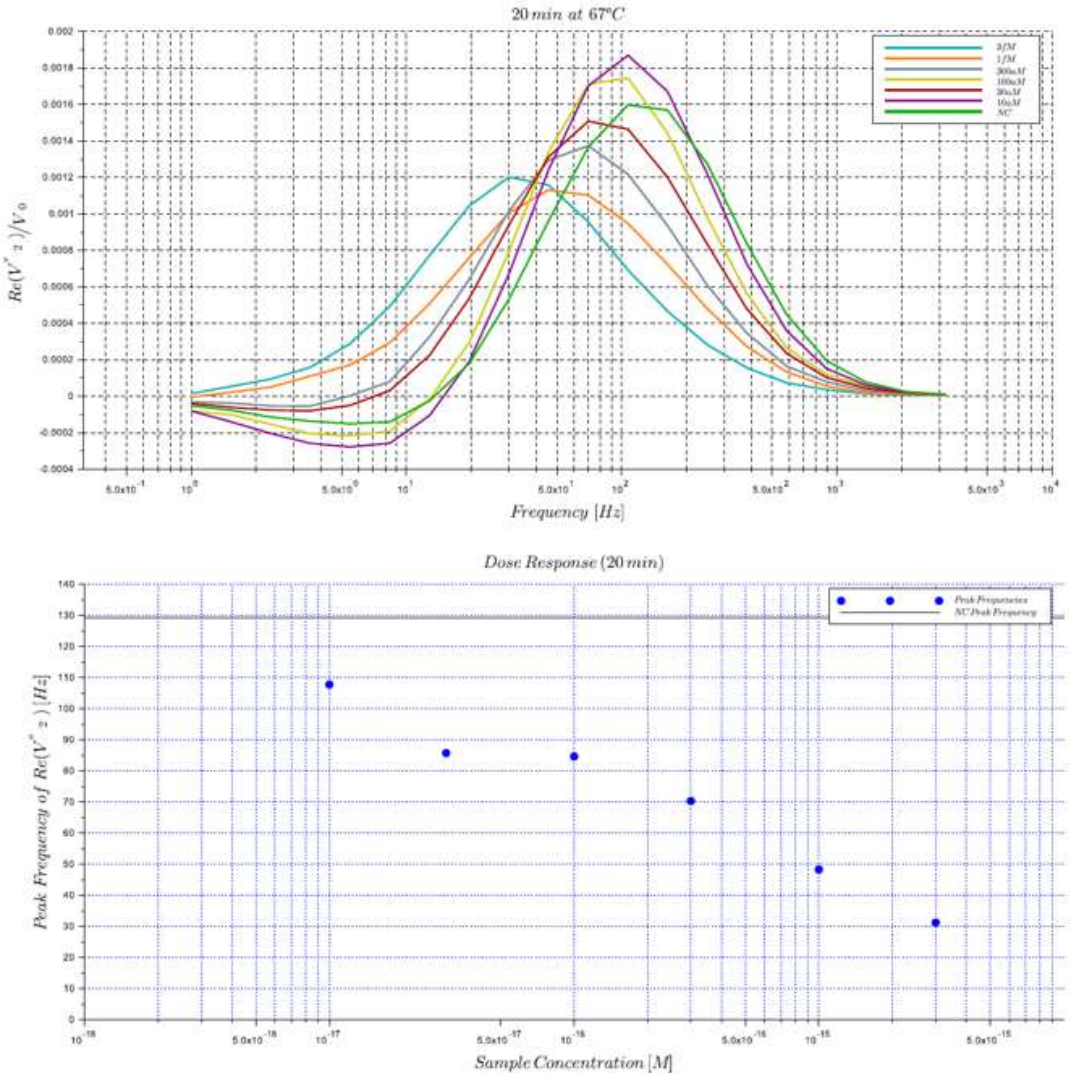

Figure 4: Top – Optomagnetic response of different samples containing a different amount of E.coli DNA. Bottom- Dose response curve, which indicates a limit of detection of around 10aM of DNA. (NC=Negative Control). Note than only 25 µL of samples have been used.

Detection limit and dose-response curve for cultured and lysed E.coli bacteria spiked in water

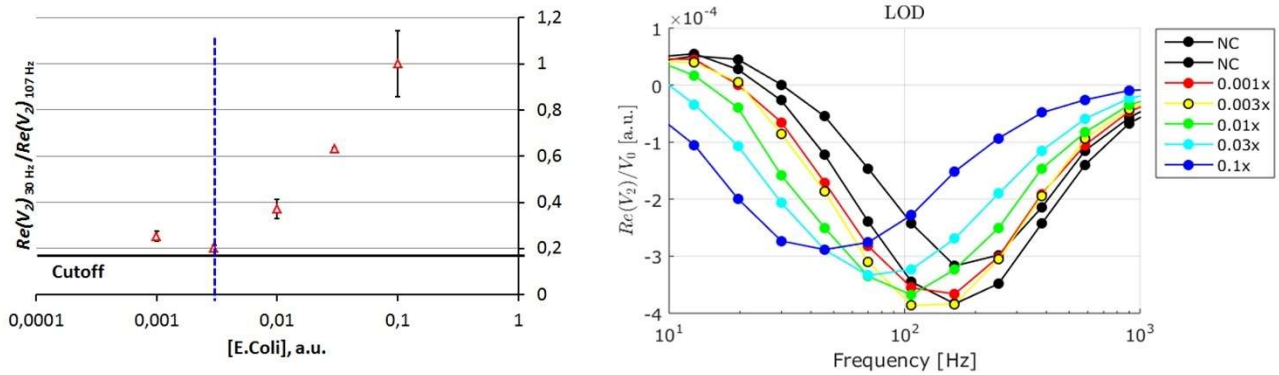

Figure 5: The LOD equivalent to ca. 10-100 CFU in 25 µl was reproduced (the yellow curve recorded for target conc. equivalent to 60 CFU of E.coli in 25 µl is different from the NC curves). Even the red curve (the lowest conc. equivalent to 10 CFU) is slightly different.

Detection limit and dose-response curve for direct amplification from E.coli bacteria in water

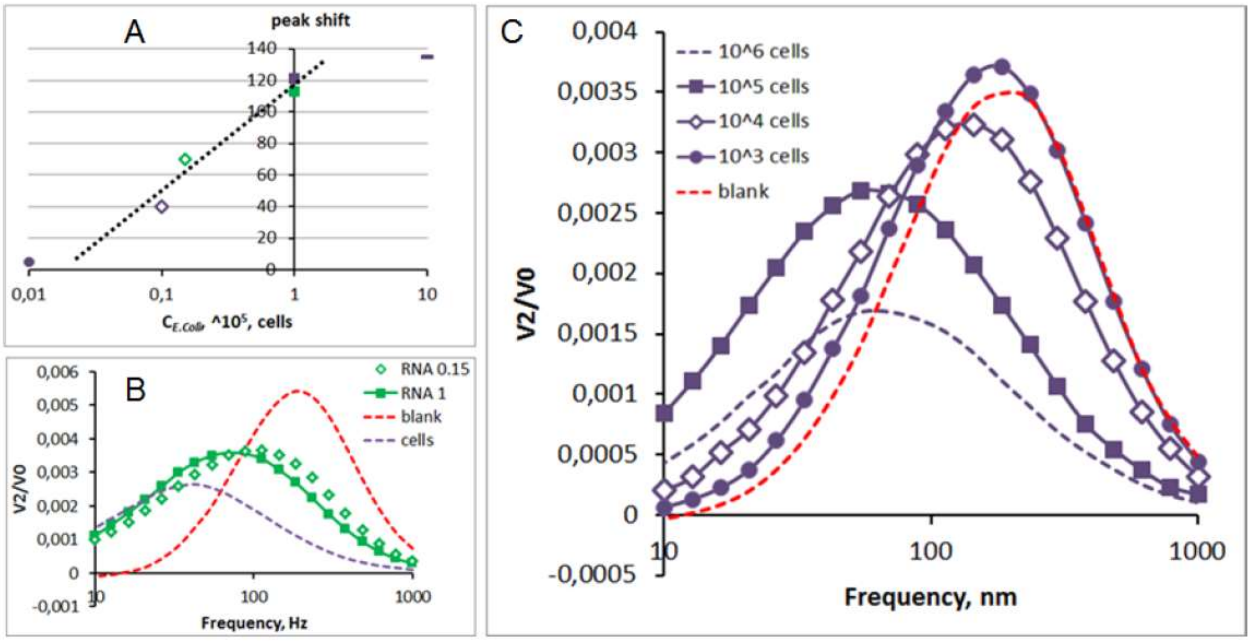

Figure 6: ***Direct amplification from bacteria samples*** *Determination of the LOD (threshold time 25 min). The LOD 3000 cells (50.000 cells/ml) was measured for series of 10 µl cell samples (final volumes 60 µl). The concentration range (X axis) is represented in a logarithmic form. The green points correspond to readout from the extracted RNA+DNA sample (B). The violet points correspond to readout from the living cells (C). B. Comparison of optomagnetic signal for the highest possible cell concentration (10^6 cells/mL), the highest (1 µg) and the middle (150 ng) RNA+DNA concentration; 1 µg of the whole nucleic acid cell content is equal to 2·105 cells under the same conditions (LAMP with beads from the beginning, 15 min). C. Dose response curves for different concentration of E.coli cells (target) after LAMP amplification.*

4 Independent Auditor's report

Financial report and statement from KPMG are included separately.