Project full title:

RAPID BIOTECHNIQUES BASED ON IMMUNOSENSORS FOR IN-SITU DETECTION OF LEGIONELLA IN INDUSTRIAL AND ENVIRONMENTAL WATER SAMPLES

Project acronym:

IMMUNOLEGIO

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[Periodic Activity Report]

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Publishable Executive Summary

Legionella are a common environmental group of bacterial organisms that are well adapted to surviving in a variety of water systems and are widely present at low levels in lakes, streams, rivers, freshwater ponds, and mud. The levels of Legionella found in the natural environment are so low that it is unlikely that an individual will contract the disease from these sources. However when Legionella enters manmade water systems it may well find suitable conditions for its amplification. Stagnant water, mild temperatures, and the presence of biofilms facilitate the growth of Legionella. Human infection occurs through the inhalation of aerosols contaminated by Legionella spp. An estimated 8,000 to 18,000 people are stricken with Legionnaires' disease in EU-15 alone each year. Up to 30% of people who have Legionnaires' disease die.

Legionnaires' disease occurs in the presence of a contaminated water source. Appropriate maintenance of water distribution systems is thus critical in controlling Legionella growth. Many countries have developed guidelines to prevent and control Legionnaire's disease and much legislation considers monitoring water for Legionella, as a crucial part of any prevention program. These have a strict regulation fixing the security threshold level for Legionella content at **100 CFU L^{-1}**. However a fundamental problem in the battle against Legionella exists- that of detecting the presence of the bacteria.

Current regulations impose annual screening for Legionella in all buildings that have a sanitary hot water recirculation system, SHW, and a quarterly analysis in cooling towers and such systems. This analysis must be quantitative and carried out in a selective culture according to the ISO-11731 standard "Water Quality: Detection and Enumeration of Legionella". Moreover, the laboratories in which these analyses are carried out must be accredited. However, in light of the frequent errors and large fluctuations inherent with cell counting, these analyses prove to be of little use. Besides, in the event of the outbreak of disease, quantitative counting on a selective culture does not offer real-time data and thus cannot enable immediate reaction to reduce the magnitude of the outbreak.

Typical assessment of facilities may take over a week. During this period, the bacteria can multiply to sufficiently high levels as to bring about a legionellosis outbreak. This is the main reason why current methods, such as BCYE (buffered charcoal yeast extract), Polymerase Chain Reaction (PCR), ELISA (Enzyme Linked ImmunoSorbent Assay are inefficient in preventing and minimising the spread of disease. In the case of cooling towers, when the concentration of Legionella lies between 1000 and 10,000 CFU L-1, a shock disinfection protocol has to be implemented. If Legionella exceed 10,000 CFU L-1, facilities are shut down until their exhaustive disinfection measures have been carried out.

The main objective of the present proposal is to develop an alternative method for the detection of Legionella species with high sensitivity and short detection time in industrial and environmental waters. This alternative will be formed into an analytical instrument suitable for portable applications for the rapid in-situ detection of Legionella in water systems, such as re-cooling open towers and hot water distribution networks in buildings.

The possibility of obtaining a rapid and in-situ quantification of the bacteria levels constitutes a relevant objective in this proposal with significant public health implications. The fact that a device like the one proposed is not able to distinguish between live and dead cells, has minor impacts in the success of the present project taking account that Immunolegio device opens up the possibility of obtaining a rapid quantification of the bacteria levels thus enabling a rapid identification of potential contamination focus. Immunolegio will allow a rapid detection analysis (about 30 minutes) with a minimal enrichment stage and a low detection limit, improving significantly the problems of conventional culture methods in terms of sensitivity and time. Moreover, an assay like the one proposed, allows doing repetitive measurements at different timing points, which enables to constantly monitor the bacterial growth and to assess adequate

disinfection procedures. It also represents major improvements comparing to other conventional methods such as PCR in terms of automatization, portability and no need for specialised personal or instrumentation.

The consortium of partners participating in the project consists of a number of Small and Medium Enterprises (SMEs), willing to exploit the results of the project, and a number of Research Organisations (RTDs), which are responsible for the main research and developments tasks. Among the RTDs, the Department of Biological and Environmental Science, University of Jyväskylä (UJ) contributes to the project with their expertise in nanotechnology. Centro Nacional de Microelectronica (CNM) will take a major role in the biosensor design and technology validation during the project. Finally, Feltalálói és Kutató Központ és Szolgáltató Kft (FKK) is responsible for the hardware and software development of the prototype instrument. In addition, FKK also coordinates the project. The group of SMEs in the consortium contains a well-balanced number of technological companies which can lead the commercialisation of the instrument (Ademtech S.A., JCB Electromecanica S.L., Applied Research using OMIC Sciences and BVT Technologies, A.S.), as well as “end-user” companies, interested in the instrument itself due to its potential applications in their particular business (AquaTreatment, Aqualab, S.L., Decsa Srl, Microniser S.A.).

After the first year of the project, the characterization of the superparamagnetic particles, identification of antibodies against Legionella pneumophila and antibody coating have been successfully developed and characterized. Laboratory measurements have investigated and demonstrated the size and shape distribution of commercial superparamagnetic particles, the concentration of antibody coating. Besides, different GMR and microinductive sensors have been characterized and developed to reach the sensitivity aimed in the project. Hardware elements for the magnetic sensor, process control, signal conditioning as well as sample preparation have been designed and manufactured. The user interface has been created in this period too.

During the second year of the project, the performance of the prototype will be systematically characterized and optimized and the complete system will be soundly tested and validated. On the other hand, it is foreseen that the SMEs will proceed with the exploitation of the project results by patenting the developed technology, carrying out a detailed market analysis, and producing an adequate exploitation plan.

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1 Project Objectives and Major Achievements

1.1 Project Objectives

General objectives

The conventional method for detection of Legionella is a complicated procedure involving isolation in a selective medium. Several problems are encountered with this method, including the presence of viable but non-culturable pathogens, loss of viability after collection and the long time required for culture and confirmation, which takes several days. In addition, detection proves even more difficult, since Legionella can be inhibited and masked by the rapid or abundant growth of other microorganisms. The time required to carry out a detection test may well be longer than one week, by which time the bacteria may have grown to levels which could already have caused an outbreak of disease. Current detection methods are thus unacceptable as response times do not enable colony blossom prevention.

The main objective of the present proposal is to develop an alternative method for the detection of Legionella species with high sensitivity and short detection time in industrial and environmental waters. In short this project delivers the following strategic objectives:

- Increasing the competitiveness and knowledge base of a large community of European SMEs involved in water monitoring and control by providing them with a fast, easy to use sensor for the in-situ detection of legionella in industrial and environmental water.
- Enabling rapid detection and isolation of legionella in sanitary water systems, cooling towers, evaporative condensers, ornamental fountains, hot-springs and other, preventing public health risks, which will contribute to safeguarding the health and safety of European citizens.
- Placing Europe in a leading position in terms of innovation in novel biosensors, a key growth area and one which will contribute to the creation of value-added jobs.

The **wider social objective** of *immulolegio* is the integration of SME's from across Europe whose will work towards developing a technology, which will bring benefits to a large cross-section of European industries and also in the development of best practices and standards for European *Legionella* management systems for improved public safety. The results of this project will assist in protecting workers and the general public from exposure to harmful and potentially lethal *Legionella*.

Scientific and technological objectives

The scientific objective of this project is to develop an analytical instrument suitable for portable applications for the rapid in-situ detection of Legionella in water systems, such as re-cooling open towers and hot water distribution networks in buildings.

The possibility of obtaining a rapid and in-situ quantification of the bacteria levels constitutes a relevant objective in this proposal with significant public health implications. The fact that a device like the one proposed is not able to distinguish between live and dead cells, has minor impacts in the success of the present project taking account that Immunolegio device opens up the possibility of obtaining a rapid quantification of the bacteria levels thus enabling a rapid identification of potential contamination focus. Immunolegio will allow a rapid detection analysis (about 30 minutes) with a minimal enrichment stage and a low detection limit, improving significantly the problems of conventional culture methods in terms of sensitivity and time. Moreover, an assay like the one proposed, allows doing repetitive measurements at different timing points, which enables to constantly monitor the bacterial growth and to assess adequate

disinfection procedures. It also represents major improvements comparing to other conventional methods such as PCR in terms of automatization, portability and no need for specialized personal or instrumentation.

The Immunolegio instrument will be based on a magnetoresistive biosensor and on the use of magnetic particles as biological markers. It will prove low cost, easy to use and handle and will offer high sensitivity and specificity.

The project aims to achieve the following **technical objectives**:

- Identification of antibodies specific to the *Legionella* species for both their immobilization on the sensor surface and their affixing to the magnetic particles (WP1).
- Development of the superparamagnetic particles coated with these specific antibodies for the detection of the *Legionella pneumophilla* and optimised for their magnetic detection (WP2). These magnetic beads will act as highly specific bio/electro interface.
- Development of the functionalized sensor surface (WP3). Different immobilising methods for sensitive antibodies will be developed, and different approaches to enhance sensitivity, reproducibility, life-time, detection range, detection limit, and/or other specifications will be assessed.
- Development of the microinductive device for measuring the magnetisation of the magnetic beads. The development of a custom design of the signal conditioning electronics as well as the associated software will be necessary to achieve optimal portability, low cost and easy operation (WP4 & WP5).
- Development of a “plug and play” cartridge system to house the biochip. This disposable cartridge will securely house the analysis and provide a sealed chamber in whereby the antibody-binding assay can occur in a contamination-free environment. (WP5)
- Design of a fully automatic measuring system enabling a complete analysis in a total test time of 30 minutes with a minimal enrichment stage.
- Legionella microbiological analyser with a targeted manufacturing cost of under € 900. A targeted cost of between 0.5 – 3 € is expected for the disposable cartridges.
- Detection of a minimum amount of 50 CFU L^{-1} in order to ensure the security level of 100 bacteria.

Objectives for the first year

The main objective for the first year was to demonstrate the feasibility of the technology proposed, according to the results obtained in the different workpackages in which the project was structured. In general terms, the main objectives for the first year are summarized in the table below:

Workpackage	Month	Objective
WP1	M3	To gain a detailed understanding of Europe-wide market needs in terms of Legionella detection in open cooling towers and distribution networks in buildings, legislation, costs, technical and design requirements
	M3	Definition of the *Immunolegio* specifications including the required speed of the system, required sensitivity and the limit detection required for the different applications as well as price that the market can tolerate
WP2	M6	Coating of supermagnetic particles and testing for immunoreactivity
	M13	Evaluation of of the size distribution and coating density as well as the integrity of the superparamagnetic particles
WP3	M8	The antibody immobilisation over the chip surface. Evaluation of modified substrate to evaluate the successful immobilisation process. Layer thickness (ellipsometry), uniformity (AFM) used for loading (surface coverage) and topology evaluation. Surface characterisation
	M13	To validate the appropriate sensor structure that meets the requirements of sensitivity, repeatability
WP4	M16	To develop a dedicated biosensor software package capable of combining numerical integration and global data analysis in an easy-to-use format. The control software for the reader system is designed and planned, and the most appropriate communication strategy between the electronics and the control software is evaluated. Ease of use and the flexibility of programming are key goals and elements such as the construction of a graphical user interface in hand-held equipment
WP5	M18	Prototype system suitable for the evaluation at laboratory scale and the validation in field

Objectives for the second year

The main objective for the second year was two fold, the development of a prototype hardware implementing the results and methodologies worked-out in the first year, and the validation of the technological concept. In general terms, the main objectives for the second year are summarized in the table below:

Workpackage	Month	Objective
WP2	M13	Evaluation of of the size distribution and coating density as well as the integrity of the superparamagnetic particles (finishing task).
WP3	M13	To validate the appropriate sensor structure that meets the requirements of sensitivity, repeatability (finishing task)
WP4	M16	To develop a dedicated biosensor software package capable of combining numerical integration and global data analysis in an easy-to-use format. The control software for the reader system is designed and planned, and the most appropriate communication strategy between the electronics and the control software is evaluated. Ease of use and the flexibility of programming are key goals and elements such as the construction of a graphical user interface in hand-held equipment
WP5	M18	Development of the prototype hardware for the automated sample preparation, including the control electronics and micro controller programming. Development of the Sensor electronics, and the Test Stick and it fitting system to the senor.
WP6	M27	Validate the Immunolegio concept at laboratory scale using inoculum production of pathogens. All parts will be validated individually before validating the complete concept as a whole. The data gathered during the laboratory tests will be evaluated in order to evaluate the efficacy, the specificity, and the sensitivity of the immunosensor as well as to establish the detection limits.
WP8	M30	Investigate the results from the point of view of intellectual property protection. Disseminate activities within the partnership as well as beyond the consortium to a wider audience. Promote the exploitation of the result of the project. Absorption of results by proposers.

1.2 Work done and major achievements

During this project the members of the consortium are developing a system for the rapid detection of *Legionaella* in industrial fields. The technology is based on magnetic labeling of the targeted pathogens so that the detection of *Legionella* is achieved by means of magnetic media detection.

Superparamagnetic particles design and characterization

One of the main tasks in the first year of the project was the research on super-paramagnetic particles as immunological labels, including their characterization, functionalization with antibodies and the laboratory validation of selectivity and specificity. This work was performed within the frame of WP2 and was finished in the beginning of the second project year. This work was perfomed by UJ in close cooperation with AROMICS, ADEM, ALAB and ATREAT.

According to the proposed schedule, in the first year of the project UJ carried out the analysis of commercial superparamagnetic particles and different types of antibodies (mouse and rabbit functionalized). Four types of magnetic particles have been investigated that differ from each other in size: 300, 500, 1000 and 2800 nm particles. The size distribution was soundly studied, because this parameter is crucial during the antibody coating, the sample preparation as well as during the magnetic detection. Coating of the superparamagnetic particles with the selected antibodies had been successfully carried out and their distribution was determined for each kind of magnetic particles. Besides, the success of antibody loading and their distribution was investigated by means of Fluorescent Spectroscopy. The fore-mentioned size and shape distribution of the commercial superparamagnetic particles was conducted by Transmission Electron Microscopy (TEM). During the investigation of the coated particles occurred a new task in the project: the amount of Legionella bacteria that stick to the coated magnetic particles can vary. The grade of the variation is not cleared yet, however it is crucial in the measurement evaluation process.

In summary, the mouse (MAB) monoclonal antibody clone 2F10 and rabbit (RAB) polyclonal antibody to Legionella, along with their isotype (ISO) controls in order to control for unspecific immunoglobin binding, and negative (N) control antibodies have been selected and used in coating the magnetic particle.

As most of the research of the WP was carried out within the first reporting period it should be recalled here shortly for better understanding of the follow up results. In the first year the background research part was performed, as described shortly in following. The size distribution was soundly studied, because this parameter is crucial during the antibody coating, the sample preparation as well as during the magnetic detection. Coating of the super-paramagnetic particles with the selected antibodies had been successfully carried out and their distribution was determined for each kind of magnetic particles. Besides, the success of antibody loading and their distribution was investigated by means of Fluorescent Spectroscopy. The fore-mentioned size and shape distribution of the commercial super-paramagnetic particles was conducted by Transmission Electron Microscopy (TEM). During the investigation of the coated particles occurred a new task in the project: the investigation of the variation in the amount of Legionella bacteria sticking to the coated magnetic particles.

The focus of the work falling in the period of the second year was to develop appropriate procedures for functionalization and for the immunoreactions to be applied in the IMMUNOLEGIO system.

Biosensor design and surface antibody immobilization

The other main tasks in the first year of the project was the research on surface antibody immobilization, and the Biosensor design including the selection of a suitable magnetic sensor.

This work was performed within the frame of WP2 and was finished in the beginning of the second project year. This work was performed by CNM in close cooperation with AROMICS, ALAB and ATREAT, as well as BVT and JCB in the magnetic sensor part.

In parallel with the magnetic particle design the biosensor development and the antibody immobilization onto the surface of the biosensor chip have been carried out. In order to gain a reliable structure the antibodies were immobilized not directly onto the surface of the sensor but onto an active spot (defined by a thin deposited gold layer) of a specific Test Stick designed to exactly match the sensors active area. CNM laboratories are not qualified to research studies with Legionella, so they used E. Coli, that is similar to Legionella in size. At this stage the immobilization protocol itself has a key role in the biosensor design. The immobilization of Legionella antibodies will be executed at UJ's facilities. CNM conducted measurements on the reproducibility of the immobilization process and presented promising results. Besides, the biological stability of the immobilized antibodies was investigated in case of dry and liquid storage. The surface of the gold layer was deeply studied by atomic force microscopy impedance spectroscopy and cyclic voltammetry.

As it was mentioned before, the *Legionella* sensor is based on superparamagnetic media detection. In the Description of Work (DoW) giant magnetoresistive (GMR) sensors alone were intended to use, and research work was started applying this type of sensor. CNM purchased two types of commercial GMR sensors and started to characterize them. One is from the NVE Corp. (US) and one from INSEC (Portugal). Both sensors were tested. At this point of the project the desired sensitivity could not be reached by GMR sensors. After the investigation of available magnetic sensors a new task was defined in the project: The development of a new magnetic sensor, as the commercial available sensors turned out to be not suitable for the IMMUNOLEGIO application.

The focus of the work in the second year was accordingly the development of the new magnetic sensor and the appropriate the biosensor design. As it was mentioned before, the *Legionella* sensor is based on the detection of super-paramagnetic beads, which are used as immuno-labels for Legionella detection. In the original Work plan (GA Annex I - DoW) a giant magnetoresistive (GMR) type sensors was planned to be used, however the first results showed that GMR type sensors are not suitable for the IMMUNOLEGO application. Thus it was decided by the consortium to redirect resources for the development of a new micro-inductive (MI) type of magneto sensor, which was the focus of the work in the second year within WP3. For this reason WP3 was completed in month 28 instead of the originally planned month 13. CNM fabricated the new micro-inductive sensor prototypes and worked out a proper encapsulation in close cooperation with BVT. BVT was the partner actually fabricating the encapsulation. Encapsulation was a critical issue, as the protection of the sensor and the electric contacts has to be realized, while the sensitive area of the sensor needs to be accessible with the test stick to be measured. The development and fabrication of the new micro-inductive sensor chip was finished in month 21. The subsequent development of a suitable encapsulation and sensor housing was finished in month 28 of the project. (These unplanned but critical developments were the major reason for the required extension of the project runtime.)

Development of hardware and software elements

To achieve the complete Immunolegio system there are numerous hardware and software elements that envelope the fore-mentioned magnetic sensor and provide easy-to-use and flexible user interface. In the following the developed hardware and software will be briefly presented.

FKK designed and manufactured a microcontroller board that provides flexible process control, collects and processes measurements data and robust communication towards the user interface. The system is preconditioned for receiving the signal of either the GMR or the microinductive sensor. Signal conditioning and sensor electronics containing an electromagnet were designed for the GMR sensor. However, the development of sensor electronics and other supplementary

devices is suspended, because the consortium decided to wait for more laboratory test results on both types of magnetic sensors. Other sensor- independent parts of the system was designed and partly manufactured (despite the fact that it was scheduled for M16). These are the robust mechanical frame and the vibration system for the sample preparation process.

In parallel with the hardware design, the development of the user interface and analysis software was started. The first version of the user interface is done and provides guidance for the operator throughout the measurement procedure, data management and storage. The interface was designed for embedded computer class device. The developer of the signal analysis part of the software is waiting for information about the selected sensor.

Software development (of the graphical user interface) was performed mainly in the first year, however from the hardware only the concept and conceptual design was developed in the first year. The second year's main task was the development and fabrication of the hardware prototype of IMMUNOLEGIO device together with all the associated control software. Also in case of this task there was a significant change in technology in contrast to the originally planned concept. This task was performed by FKK and JCB within WP5 with the instructional support of SMEs ATREAT, ALAB, DECSA, and MICRON. In the following the developed hardware and software will be briefly presented.

The hardware consists of following parts: controller electronics, power electronics, sensor electronics, and the mechanical hardware for automated sample preparation, including all control software required for operation.

FKK designed and manufactured a microcontroller board that provides flexible process control, collects and processes measurements data and robust communication towards the user interface. FKK also developed sensor electronics for the newly developed micro-inductive sensor, including the required frequency generator circuit.

FKK also designed and prototyped the hardware for the automated sample preparation procedure according to the final concept agreed on at M15 (final concept design was presented at M18). Beside the development of a robust mechanical frame and an XY-mechanics for the automated pipetting arm, FKK also developed the power electronics required to drive the positioning step motors and the peristaltic pumps used to operate the pipetting mechanism.

Suporting the developed hardware components FKK developed appropriate control software implemented in the microcontroller on the controller board controlling both the automated sample preparation process as well as the sensor electronics for the magnetic evaluation of the Test Stick. To support the configuration of the sample preparation procedure a PC user interface was also developed.

Validation of the IMMUNOLEGIO concept at laboratory scale

This task was performed by STL with the support of AROMICS ATREAT, ALAB, DECSA, MICRON and ICHNT within WP6. The laboratory validation included the validation of the functionalization developed for the Test Sticks, tests for the specificity of the functionalized beads, combined tests of the IMMUNOLEGIO concept with Legionella + Marker Beads and Test Stick in laboratory scale.

Validation of the IMMUNOLEGIO concept using real industrial samples

This task was performed in a reduced manner integrated into WP6 by providing real industrial samples for the laboratory tests. These tests were also performed within the laboratories of STL. The consortium partners decided to continue with this validation tasks even after the official end of the project.

Knowledge Management

This task was jointly performed by all partners under WP8. This includes patent search, dissemination, technology watch, as well as the preparations for the exploitation.

1.3 Major problems and corrective actions

1.3.1 Reconsideration of the flow-through concept in favor of batch concept

Since the beginning of the project several draw-back of the flow-though concept were detected, which are not conformable with the above identified requirements.

- Handling of dirty samples: High risk of blockage of the micro flow-channel in case of dirty samples by (dust, rust, ect.). Scientific literature reveals blocking as major problems in micro channel systems carrying nano particles. Some improvement can be achieved by using guided flow, however this seem not to be a suitable option in case of magnetic detection.
- Cost: Unnecessary measurement costs, because of the need of integration of the flow channels with the sensor in a cartridge, which could be use only for one measurement.
- Cost: In a flow through method high amount of paramagnetic beads are required to enable a continuous adding and mixing with the sample. Because the functionalized paramagnetic beads were identified as main cost factor this procedure would yield unacceptable high measurement costs.
- Long testing (flow through) time in case of large sample volumes

The alternative method proposed in the final system specifications avoids upper mentioned problems of a flow through system, by utilizing an incubation type method known from laboratory analysis. That means the paramagnetic marker beads are mixed to the bulk sample. During the incubation time the immunoreaction takes place marking the bacteria, and the dirt has time for sedimentation. After the bacteria are marked with the magnetic particles immobilization on an activated surface for sensing can be enhanced by a magnetic field for further separation from dirt, and it can also be cleaned from non-immobilized paramagnetic beads using a contrary magnetic field. The advantages are:

- Amount of utilized paramagnetic bead markers can be optimized, finding a compromise between bead amount and required incubation time. However incubation time can also be shortened by using increased temperatures and by micro-mixing. This allows the minimization of the required amount of the costly beads.
- Dirty samples can more robustly be handled in the bulk. Sedimentation process supports the separation of dirt and the activated surface, which is introduced only at the end of incubation to the sample. Immobilization speed will be increase in this case by magnetic fields instead of mixing.
- Separation of sample handling and sensor possible, which allows the construction of a reusable sensors, saving testing costs.

1.3.2 Need for sensor development

In the DOW it was foreseen to use a commercially available GMR sensor as detector for the super-paramagnetic immuno-markes used in the IMMUNOLEGIO concept. However, the results obtained with commercially available GMR sensors showed that these sensors are not suitable for the IMUNOLEGIO application. The main problem with GMR sensors was their extremely short

detection range that means if the super-paramagnetic bead was not directly on top of the sensitive GMR line it could not be detected by the sensor. These reasons made it necessary to develop a new sensor specific for IMMUNOLEGIO with following two options:

- Development of a new GMR sensor design. Achieving a high density of GMR lines to avoid undetected particles between two lines. However this option was considered to be too risky to be realized within the available time and resources. Increasing the width of the GMR lines was not an option as this would significantly decrease the sensitivity of sensor.
- Redesigning an already established micro-coil type sensor design for the requirements of IMMUNOLEGIO. This option was considered by the consortium to be less risky, as the RTD partner CNM has already experience with the development of such kind of sensors.

The second option was selected by the consortium, which defined a completely new task for the RTD partners. Following new tasks were defined:

- Evaluating existing prototypes of Micro-coil type (micro-inductive) sensors for the detection of super-paramagnetic beads. (RTD: CNM)
- Fabricating a set of micro-inductive type sensors with the available design and evaluating their detection limit for super-paramagnetic beads. (RTD: CNM)
- Develop and fabricate a micro-coil sensor chip design suitable for the IMMUNOLEGIO application. (RTD: CNM)
- Develop a special encapsulation for the newly developed micro-inductive sensor protecting the sensor and the electric contacts while leaving the sensitive area of the sensor needs is accessible for the measurements and allowing a precise fit of the Test Stick to be measured. (RTD: CNM, MFKK; SME with significant role: BVT).
- To design and fabricate new Test Stick appropriate for new sensor design (RTD: CNM, MFKK; SME with significant role: BVT).

This was a significant but required change in the project work plan, which required a considerable redistribution of the recourses and resulted in huge delays in the project execution, as all these new tasks are very time consuming. For these reasons the consortium decided to take away resources from the originally planned industrial validation tasks, for the benefit of this unexpected extra research work, and to ask for an extension of the project runtime.

The SME partners of the consortium decided to perform the industrial validation at their own cost in the post project (exploitation) phase as the SME partners have all the required competences to perform these tests by their own. However for the development of the sensor being a critical part of the IMMUNOLEGIO system they definitely need the competence and resources of the RTD partners within the project. The consortium decided to prepare industrial validation as far as possible within the laboratory validations by including industrial samples in the laboratory validation plan.

1.3.3 Problems during sensor development

Even though the sensor and Test Stick development could be finished within the extended project runtime, the associated system integration works (encapsulated sensor + housing + electronics) could only be finished with M29 of the project. However the encapsulation of the sensor and the housing introduced parasitic capacitances in an unexpected amount hiding the direct measurable inductive effect. This made the developed sensor electronics unable to measure the inductivity of the sensor carrying the useful information. In the last project month a new electric model was developed for the encapsulated sensor accounting for the parasitic capacitances. (In the standard encapsulation of the sensor used in the initial tests these parasitic capacitances did not have a significant influence.)

New measurement parameters could be identified for the encapsulated sensor based on electric simulations performed with the developed and verified electric model of the encapsulated sensor. A frequency range could be identified where the measured signal carries the sensor information. Minimal modifications to the sensor electronics will allow to measure in these frequency ranges. However, due to this additional delay the original validation plans for laboratory validation including industrial samples could be performed with the final integrated sensor within the project run time. Despite of this the sensor prototype itself was promisingly validated and it was shown by simulations that similar results can be achieved even the encapsulated sensor.

All system components of IMMUNOLEGIO could be validated as separate components making the integrated function feasible even without a combined system validation. The SME partners received all technologies required for a successful pro-market development, and agreed to complete full-system validation in the post project phase. The RTD partners assured their support regarding the full assimilation of the results, in case the provided written documentation would leave some details unclear.

2 Workpackage progress of the period

2.1 Workpackage 1. "Market Research & System Specifications"

2.1.1 Results of the Market research

A market research was conducted as described in the previous section. The results of the market research can be concluded as follows.

The market requirements outline a low-cost, simple-to-use, portable equipment. Portability and easy of use was one of the major requirements found in the market. The test procedure should not require special qualifications of the operator. The interest on the possibility of on-line monitoring was low.

There was a high need and interest on the market for fast and low cost testing of *Legionella*. Thus the targeted system should allow to obtain the test results on site, in time and at low costs. Most companies (currently not using any test) would only be interested in using such a sensor system if they could perform the tests at low costs. Providing a testing possibility at low costs would obviously lead to an increased testing frequency.

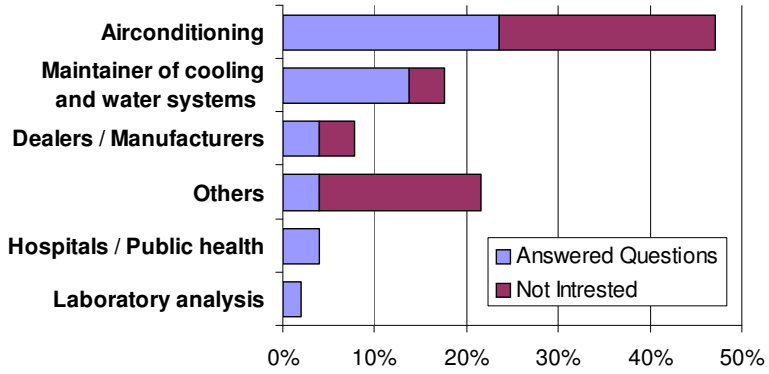
The requirement on the sensitivity of the device was simply to fulfill the actual regulations for testing. A reasonable target application could be cooling tower monitoring, water system monitoring by easy and fast on site test.

Recently a new case of multiple legionella illnesses due to the contamination of the water supply system of a hotel in Phuket was reported showing the need for more frequent monitoring. The hotel was closed until the water and air-conditioning systems are disinfected. It takes weeks to clean the system of the whole hotel.

Unfortunately participation was very poor, only 51 companies answered the queries out of 850 contacted all over Europe. The answers came from 7 different countries. IMMUNOLEGIO project partners reported that their partners or customers contacted by them stated during an ongoing customer contact interest for the project, however excused filling the query due to lack of time.

Company profiles

Approximately half of the interviewed companies showed interest on the proposed new sensor. Figure 1 shows the distribution of the interest in the several branches. All public health institutes and laboratories were highly interested. Although most of the companies in the cooling tower and water system sector showed interest, however some stated using continuous disinfection without the need for testing *legionella*.



- **Figure 1: distribution of the companies showed interest on the proposed new sensor**

2.1.2 General Target Requirements

Based on the collected and obtained information following general design criteria could be defined for the aimed IMMUNOLEGIO system.

Decided general target requirements of the IMMUNOLEGIO system:

- Portable equipment for on sight use.
- Ease of use, targeting test kit like handling.
- No extra efforts for automatization for the pre-competitive prototype.
- Easy sample preparation, without the need of a qualified person.
- Low cost testing should be targeted.
- Detection limit (sensitivity) of at least 100 CFU/ℓ.
- System should be able to process 250 to 1000 mℓ sample volumes.
- Reducing the required sample volume while fulfilling required sensitivity would be advantageous.
- An extra pre-concentration step should be avoided if possible. However a risk of un-sufficient sensitivity was identified in the realization of this requirement.
- The required marker and antibody consumption of the test procedure should be aimed to achieve the requirements for low cost testing.
- The costs of the disposable parts should be minimized (low cost requirement).

2.1.3 Basic Technical Specifications

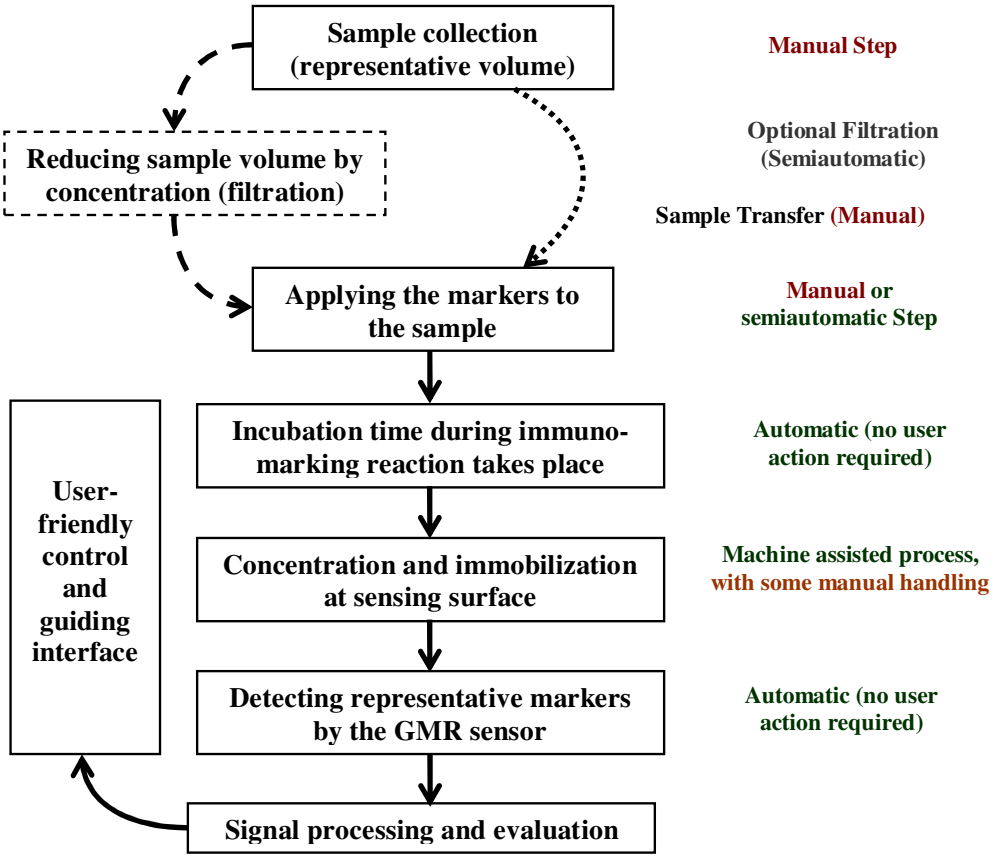
Basic technical conditions of the IMMUNOLEGIO system:

- Using a GMR sensor for detecting the markers.
- Using paramagnetic nano-particles as immuno-marker.
- Using monoclonal as well as polyclonal antibodies.
- Targeting a “control strip” like component to immobilize legionella for sensing.

- Targeting a flexible system, capable to measure detect the whole range of legionella serotypes as well as also specific single serotypes controlled by the applied marker set.
- Using DC magnetic field for the sensor measurement, to allow the use of metallic layer for the antibody immobilization methods.

2.1.4 Specified Test Procedure of the IMMUNOLEGIO System

The test procedure in general was defined as follows:



- **Figure 2: The test procedure**

More details on market research and system specification can be found in Deliverable 1.

2.2 Workpackage 2. "Superparamagnetic Particles Design"

WP2: Superparamagnetic Particle Design with following goals:

- *Technical assessments*
- *Coating of superparamagnetic particles*
- *Size distribution & coating density and*
- *Imaging & magnetic properties*

This WP was performed by UJ in the first year of the project. Only a few tasks were left for the beginning of the second year. Here a is only sort summery of the results is provided, as this was discussed in detail in the first year report.

2.2.1 Summery of work performed in the first year

WP2: Superparamagnetic Particle Design for UJ commenced physically in Dec 06. Our goals were to provide:

- *Technical assessments*
- *Coating of superparamagnetic particles*
- *Size distribution & coating density and*
- *Imaging & magnetic properties*

Due to the delay in the start date of this project, physical work commenced directly after the kickoff meeting in November 06. The following is the timeline of the work conducted since December 06 (Our physical month 1).

Timeline of Our Work	Our Physical Months
Identification of antibodies for Coating and Surface Immobilization	Month 1-3
Identification of standard magnetic beads to validate characterization methods conducted on ADEMTECH beads	Month 1-6
Conclusion that 500, 1000 and 2800 nm beads would be the focus beads in this project.	Month 6
Design of suitable reagents and functionalization procedures	Month 1-6
Characterization of the standard magnetic beads and ADEMTECH Beads with respect to shape, size, uniformity of size within a population, and antibody distribution	Month 1-present
Culturing procedures for Legionella	Month 9-present
Definition of operative measuring protocols	Month 1-present

The following is a synopsis of the results at particular milestones within the project:

MONTH 3 Feb 07: Functionalization of the magnetic particles became a routine procedure and showed that unspecific binding is very low. From initial Transmission Electronic Microscopy (TEM) studies and frequency of these size ranges, Ademtech beads and many of the standard magnetic beads smaller than 1000 nm are heterogeneous in size and shape and there is a wide distribution of sizes in the magnetic beads. Due to the heterogeneity of the beads, difficulties in quantitation and consistent antibody loading appeared. This heterogeneity is a significant issue in obtaining

consistent and validated results in any experiments conducted. Based on this, 300, 500 and 1000 nm magnetic beads became the focus of the project.

MONTH 6 May 07: Characterization of shape, size, uniformity of size within a population, and antibody distribution was conducted with TEM and Fluorescent Spectroscopy (FS) was used to investigate the antibody loading and density of the

functionalized beads. As seen, these beads were coated well with the secondary antibody with very little unspecific background. Photon Correlation Spectroscopy (PCS) demonstrated the distribution of sizes within the bead population, which supported the TEM results. Again frequency of size ranges for each bead showed that there is a wide distribution of sizes in the magnetic beads smaller than 1000 nm. UV/VIS Spectroscopy allowed concentration of functionalized beads as well as determination of the amount of bead loss. Unfortunately, due to the wide size distribution and variable streptavidin coating of the 300 and 500 beads, Flow Cytomety, Fluorescent Correlation Spectroscopy, and Confocal Microscopy were proven to be useless in analyzing antibody coating and density. For these reasons, 500, 1000 and 2800 became the focus of our efforts and TEM, FS, PCS and UV/VIS were the choice of techniques to analyze the magnetic beads.

MONTH 9 Aug 07: The ADEMTECH 500 nm (500), Dynabeads 1 micron (1000), and Dynabeads 2.8 micron (2800) are now our focus. TEM, FS, PCS and UV/VIS studies concluded that there is better homogeneity in size shape, size, uniformity of size within a population, and antibody distribution with 1000 and 2800 nm magnetic beads. UV/VIS indicated that approximately 50% of 500, 30% of 1000 and 10% of 2800 nm beads are lost after the functionalization procedure.

MONTH 11 Oct 07: Growth of *Legionella* has become standard procedure within our facilities. With grown *Legionella*, western blot analysis was conducted to see if the functionalized superparamagnetic beads bind specifically to *Legionella pneumonia* serotype 1. These experiments indicated that MAB and RAB 500, 1000 and 2800 functionalized beads do specifically bind to *Legionella.* An additional property of the magnetic beads that has been and is being conducted is the magnetic integrity of the functionalized beads compared to its unfunctionalized partner. This task is being completed with our partner CNM and when completed with in month 12, we will be able to comment on if the functionalization procedure, with the antibodies, has an influence on the magnetic properties of the beads.

MONTH 12 Nov 07: Additional, work with CNM in UJ will be conducted on determining if one bacterium with one magnetic particle can be obtained. It is important to bring together the functionalized magnetic particles with the bacteria and with the sensor chip. In addition the specific binding and integrity of this binding to *Legionella* with the sensor chip is concurrently being conducted. After this physical month 12, we will be able to conclude if one bacterium, one magnetic particle is feasible.

In conclusion, we have conducted analysis of 8 different commercially available superparamagnetic beads in regards to shape, size, uniformity of size within a population, and antibody distribution. We have successfully loaded antibodies on the surface of the magnetic beads. These antibodies included the mouse (MAB) monoclonal antibody clone 2F10 and rabbit (RAB) polyclonal antibody to Legionella, along with their isotype (ISO) controls in order to control for unspecific immunoglobin binding, and negative (N) control antibodies. We have demonstrated that the MAB and RAB 500, 1000 and 280 nm functionalized beads do bind to *Legionella*. We could achieve functionalization of 3 different beads with consistent properties and they are ready to be utilized by our partners. Within this month and the next to aid our partners, we will be able to comment on the specific binding of the functionalized beads and on the integrity of this binding to *Legionella* with the sensor chip within our physical month 12 (Nov 07). Upcoming tasks that will be concluded within the last two months will be the magnetic properties with the aid of CNM and the last experiments to be conduct to help CNM will be to conclude if one bacterium, one magnetic particle is feasible. In addition, due to available resources, additional experiments involving testing

of water sources and the magnetic particles, under the direction of STL will be conducted to aid in fulfilling WP6.

The complete characterization of the coated magnetic particles in terms of shape, size, uniformity in size within a population, antibody loading, antibody distribution, and antibody density were major tasks of work package 2 conducted in the first 6 months. In addition to this, suitable reagents and functionalisation procedures as well as the definition of the operative measuring protocols, together with guidance from ADEM has commenced. Characterization of shape, size, uniformity of size within a population, and antibody distribution was conducted by Transmission Electronic Microscopy (TEM). IMAGEJ software was then used on scanned TEM images to extract the defined information. After coating of the magnetic beads with the primary antibody, successful loading of antibodies on the surface of the magnetic beads as well as the two - dimensional antibody coating density was visualized utilizing immunogold TEM techniques. Analysis for unspecific binding of gold particles demonstrated minimal loose gold materi al. Numerous magnetic standard beads validated this method.

Fluorescent Spectroscopy was used to investigate success of antibody loading, antibody distribution, and antibody density. Generally, spectroscopy is the study of matter and its properties by investigating light, sound, or particles that are emitted, absorbed or scattered by the matter under investigation. For our purposes, trace amount of fluorescently labeled antibody (Alexa 488; secondary antibody) reactive to the Legionella antibody used in co ating was mixed with the coated beads. The measured recovery of fluorescence with a known amount of standard fluorescently labeled antibody was conducted.

Flow cytometry (FC) has also been used to collaborate size and density of the particles. Simultaneo us multiparametric analysis of the physical characteristics of single magnetic particles could be contacted with this technique. However, due to the significant variability in size within the bead population present in most commercial beads tested, this te chnique proved futile when extracting individual magnetic bead characteristics.

Antibody density of the particles was confirmed with Fluorescent Correlation Spectroscopy (FCS) and Confocal Microscopy. FCS is a type of spectroscopy based on the measurement of fluorescence intensity and the analysis of it fluctuations, which can be due to the diffusion of the observed fluorescent entity in the excitation volume or the changes in the fluorescence quantum yield arising from chemical reactions. Again secondary antibodies were used to visualize the particles and provide size distribution curves, diffusion coefficients, the number of fluorescent molecules being analyzed, the amount of the magnetic particles and antibody specificities. In addition, integrity and concentration of the particles with respect to antibody coating were investigated with exposure to hard detergents (urea).

2.2.2 Technical assessments of Superparamagnetic particles

Identification of antibodies against Legionella pneumophila (Legionella) suitable for coating of the magnetic particles and one antibody for surface immobilization

A search on commercial Legionella antibodies was done, focusing first on monoclonal antibodies due to their higher homoge neity and selectivity. Two monoclonal antibodies have been selected; clone 2F10 for coating and clone 5F4 for detection. Both antibodies are raised against LPS (bacterial membrane) from

Legionella pneumophila (Legionella), and are recommended by the manufacturers for sandwich assays. Through discussions with Aromics, we sought to increase the serotype diversity detected by the immunosensor, thereby including a polyclonal antibody that reacts with Legionella serogroups 1-12 in our antibody repertoire. In summary, the mouse (MAB) monoclonal antibody clone 2F10 and rabbit (RAB) polyclonal antibody to Legionella, along with their isotype (ISO) controls in order to control for unspecific immunoglobin binding, and negative (N) control

antibodies have been selected and used in coating the magnetic particle. The following table describes the amounts of antibodies used in coating procedures:

Antibody for coating: 10 ug per mg of beads	Concentration of antibody (mg/ml)	ADEMTECH 300 nm want 2.5 ug antibody (ul)	ADEMTECH 500 nm want 7.5 ug antibody (ul)	DyanBeads 1 micron (ul)
MAB	2.2	1.5	3.5	3.5
RAB	4	0.75	1.75	1.75
ISO MAB	0.5	25	65	65
ISO RAB	2	6.25	11	11
N MAB	0.1	25	75	75
N RAB	0.1	25	75	75

Unless specified in the table, commercial beads were coated with parameters indicated for ADEMTECH 300 beads. Amounts of antibody coating were based on the specific binding capacity of 10 ug /mL and15 ug/mL for ADEMTECH 300 and 500 beads, respectively.

Identification of standard magnetic beads to validate characterization methods conducted on ADEMTECH beads

A search for streptavidin -coated commercial magnetic beads to act as standard beads when analyzing ADEMTECH 300 and 500 nm beads has been conducted. The following table describes the commercial beads used and their specifications (if known) initially characterized to serve as standard beads:

	150	250	280	300 ± 15 nm	500 ± 20 nm	860	1000 ± 3%
Binding Capacity	can pull down 10^6 cells			10 ug /mg	15 ug/mg	4-5 mg/g beads	20 ug/mg beads
Concentration		10 mg/mL		5 mg/mL	10 mg/mL		10 mg/mL
Number of particles			$4.97*10^{11}$ beads/mL	$2.42*10^{11}$ beads/mL	$6.32*10^{10}$ beads/mL	$1.6*10^{10}$ beads/mL	$7\text{-}12*10^{9}$ beads/mL

Specific parameters of the beads ar e listed, if known. The following acronyms are now used for the following: MagCellet 150 nm (150 nm), Micromod 250 nm (250 nm), Estapor 280 nm (280 nm), ADEMTECH 300 nm (300nm), ADEMTECH 500 nm (500), Estapro 860 nm (860), and Dyanbeads 1 micron (1000).

Cost Analysis of Magnetic Beads

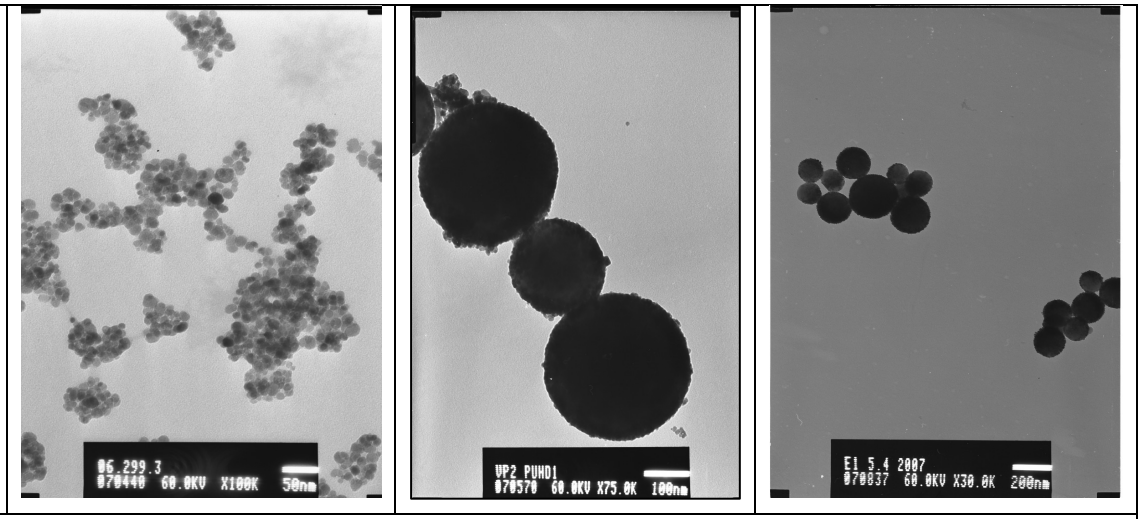
Cost Analysis of ADEMTECH beads and antibodies used to coat the beads are as stated below.

Bead / antibody	Shipping	Price €
MagCel lect Streptavidin Ferrofluid 150 nm (1 ml)	20	380
Micromod nanomag-D 250 streptavidin 10 mg/mL (1 mL)	20	125
Estapor Streptavidin Coated Magnetic Microspheres 1x 2mL of Size (µm) 0.31 Ferrite (%) 40 Product number BE-M08/03	20	181
ADEMTECH 300 5mL	20	946
ADEMTECH 500 5mL	20	581
Estapor Streptavidin Coated Magnetic Microspheres 1 x 2 mL Size (µm) 0.86 Ferrite (%) 55 Product number BE-M08/08	20	179
Mouse anti- *Legionella pneumophila* biotin conjugated Biodesign 2F10 2.2 mg/ml 1 mg/vial	20	681
Rabbit anti- *Legionella pneumophila* biotin conjugated Biodesign 5 mg/mL 1 ml vial	20	349

2.2.3 Characterization of Superparamagnetic particles

The following figure represents TEM micrographs of the magnetic beads analyzed. Population uniformity, shape, size, and antibody loading as a count of nanogold particle is indicated.

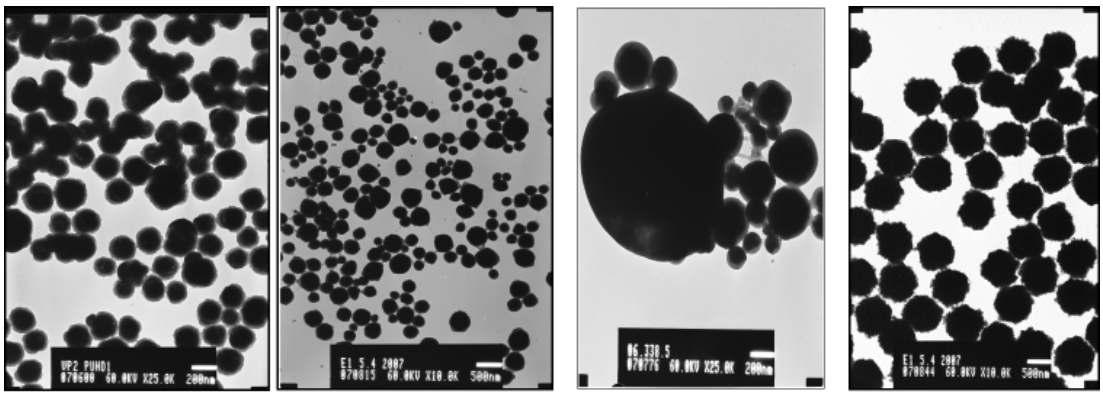
	150	250	280
population uniformity	uniform in size	not uniform in size	not uniform in size
Shape	spherical	spherical	spherical
Size	15 nm N=129	133 ±57 N=200	167 ±52 N=73



- **Figure 3: TEM micrographs of 150, 250 and 280 nm beads**

Population, shape size are seen in the representative TEM micrographs. Nanogold per bead is indicative of the secondary 15nm gold conjugate antibody used to label MAB, ISO MAB, N MAB, RAB, ISO RAB, AND N RAB.

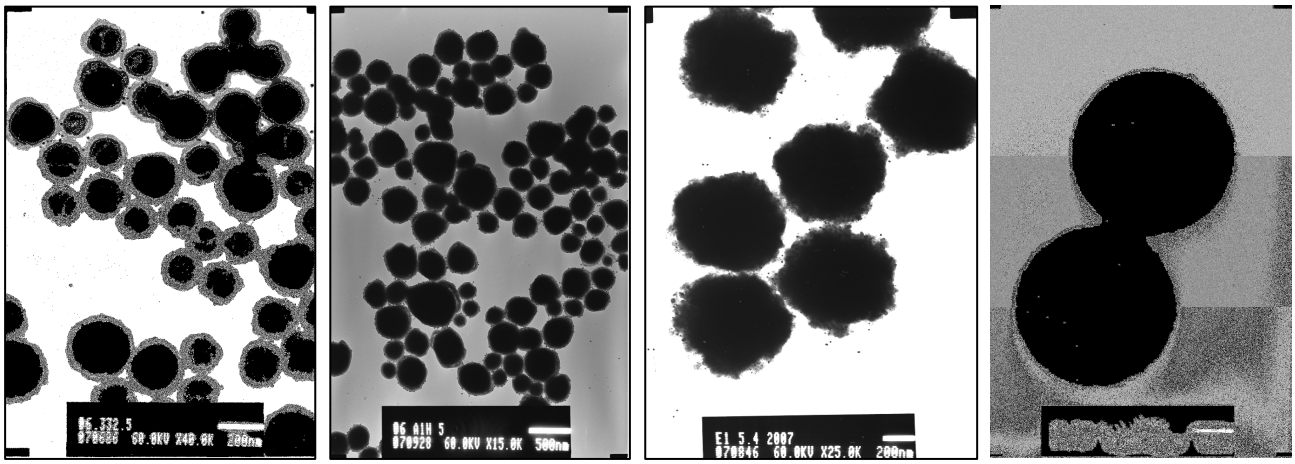
	300	500	860	1000
Population uniformity	not uniform in size	not uniform in size	not uniform in size	uniform in size
Shape	spherical	spherical	spherical	spherical with rough edges
Size (nm)	217 ±122 N=128	288 ±145 N=677	277 ±223 N=146	809±74 N=180
Nanogold per bead	MAB 10,16 ISO MAB 0.55 N MAB 0.09 N=30 RAB 6.95 ISO RAB 11.47	MAB 13.69 ISO MAB 1.7 N MAB 0.2 N=80 RAB 42 ISO RAB 3	MAB 14 ISO MAB 19.92 N MAB 2.03 N=13 RAB 12.31 ISO RAB 11.38	MAB 8.20 ISO MAB 0.6 N MAB 1.1 N=10 RAB 54.5 ISO RAB 18.2



- **Figure 4: TEM micrographs of 150, 250 and 280 nm beads**

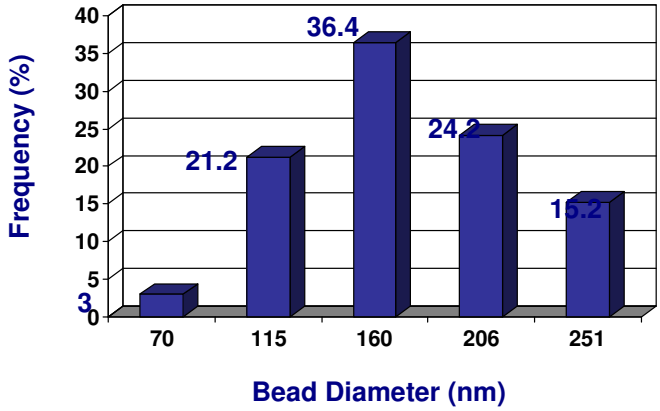
Population, shape and size are seen in the representative TEM micrographs. Nanogold per bead is indicative of the secondary 1 5nm gold conjugate gold antibody used to label MAB, ISO MAB, N MAB, RAB, ISO RAB, AND N RAB.

The antibody loading, distribution and density from immunogold labeled 300, 500, 1000 and 2800 beads are portrayed in Figure 5. As can be seen, these beads were coated well with the secondary antibody with very little unspecific background.

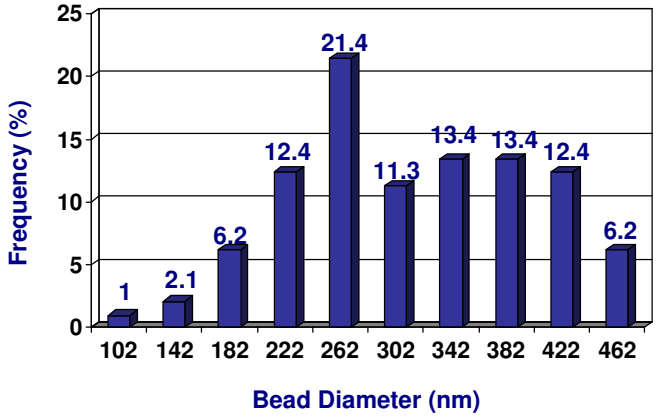


- **Figure 5: 300 nm, 500nm, 1000nm, 2800 nm beads**

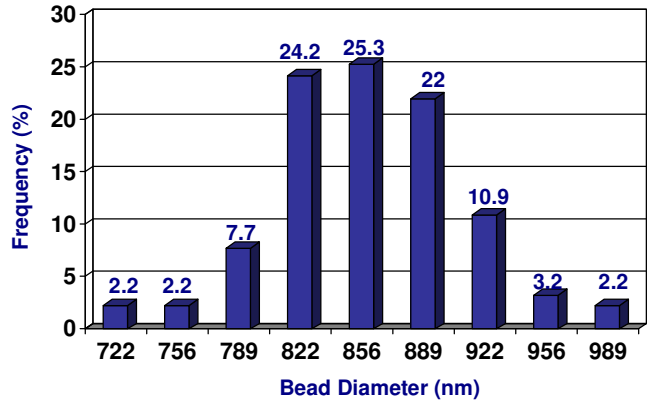
Frequency of size ranges for each bead is shown with the corresponding inserts:



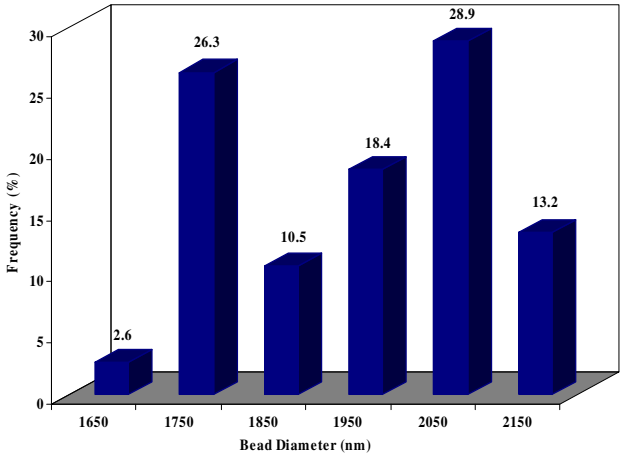
- **Figure 6: Frequency of size ranges of 300 nm beads**



- **Figure 7: Frequency of size ranges of 500 nm beads**

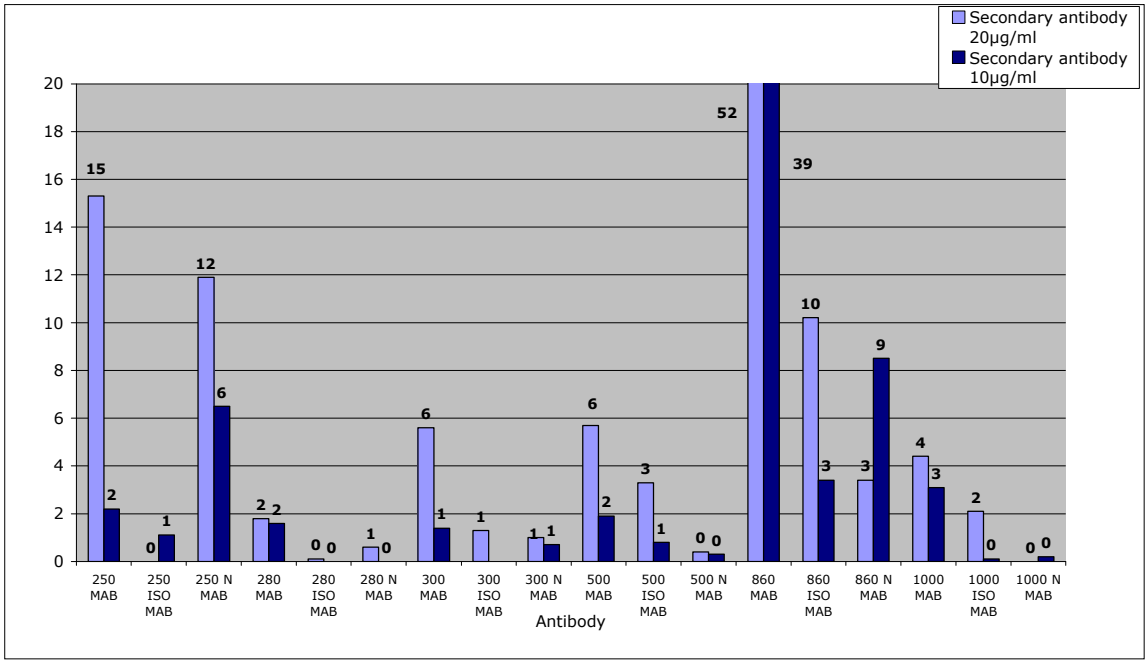


- **Figure 8: Frequency of size ranges of 1000 nm beads**

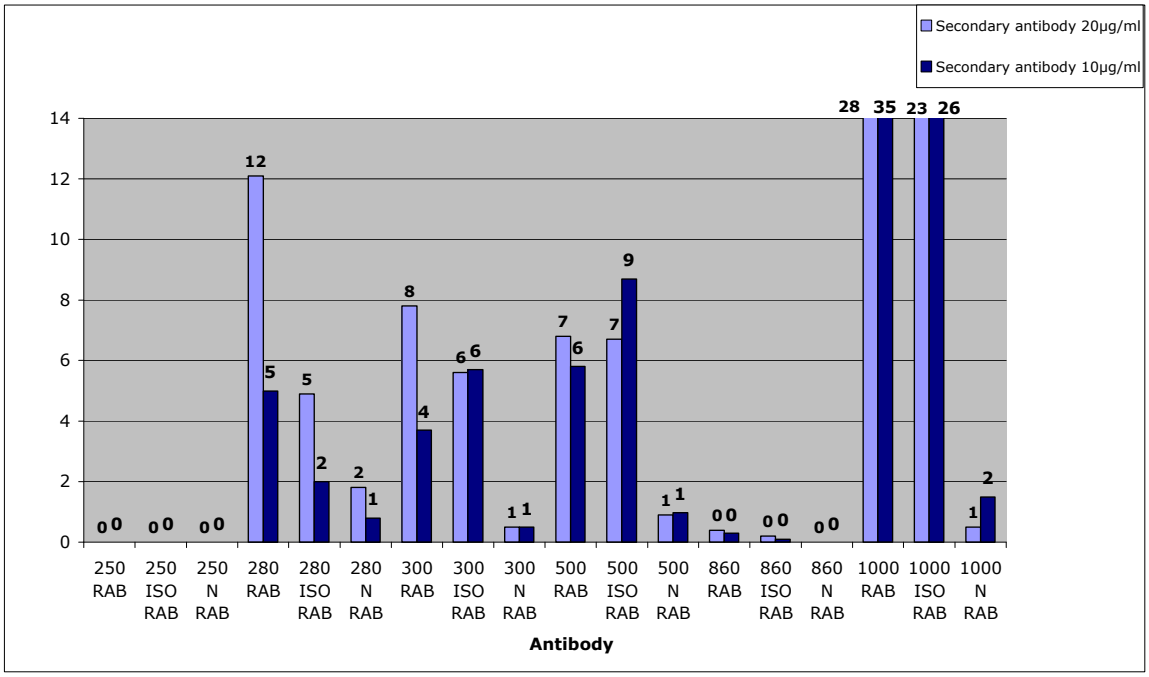


- **Figure 9: Frequency of size ranges of 2800 nm beads**

Fluorescent spectroscopy was used to investigate the antibody density on the magnetic beads. A standard curve for secondary antibody Alexa 488 concentration was used as a guideline for establishing antibody concentration on coated beads. Figure 10 provides antibody concentration of the magnetic beads coated with MABs and Figure 11 provides concentration amounts for the magnetic beads coated with RABs.



- **Figure 10: Fluorescent Spectroscopy of Mouse Functionalized Magnetic Beads**



- **Figure 11: Fluorescent Spectroscopy of Rabbit Functionalized Magnetic Beads**

2.2.4 Design of suitable reagents and functionalisation procedures

The following is the coating procedure used for all beads. Ideally the solutions need to be filtered before use.

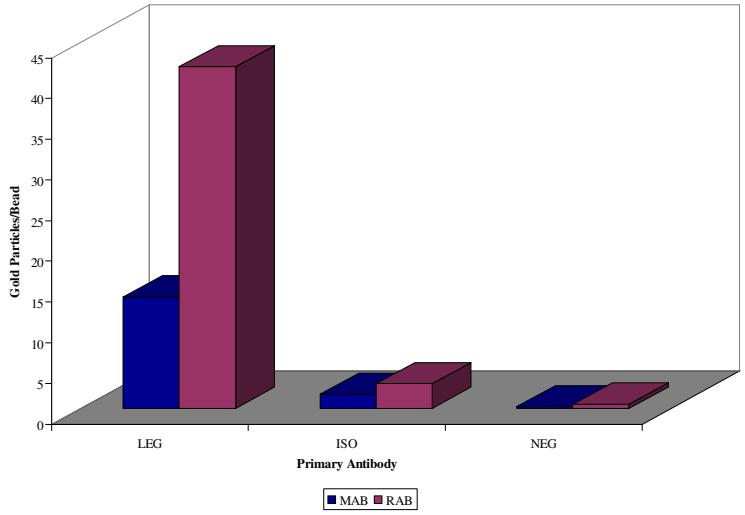
1. Resuspend beads thoroughly
2. 50 ul beads (500 ug) wash 3 x PBS (100 ul) -let beads settle to the bottom with magnet between each step or use the pic-pen
3. Resuspend particles in 50 ul 1 % BSA/PBS for 10 min -let beads settle

to the bottom with magnet between each step or use the pic -pen

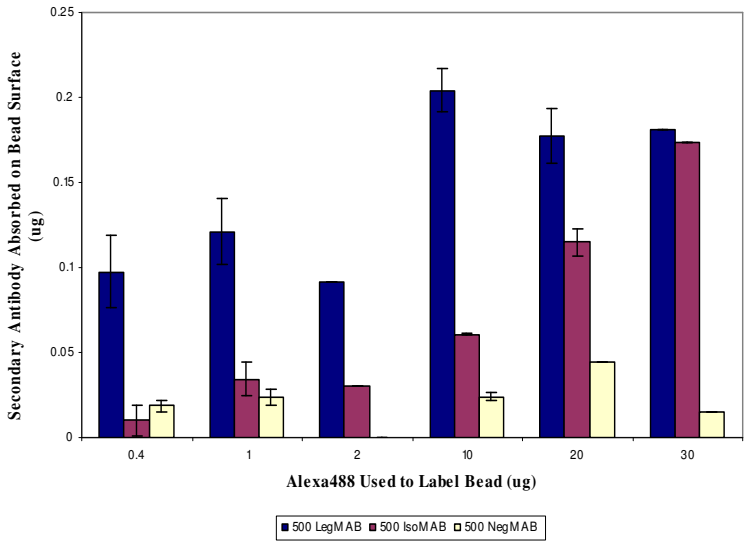
4. Add primary antibody (50 ul total volume) in 1% BSA/PBS
5. Incubate 30 RT rocking.
6. Wash 3 x PBS (100 ul) -let beads settle to the bottom with magnet between each step or use the pic-pen
7. Resuspend in 40 ul PBS

Analysis of antibody loading:

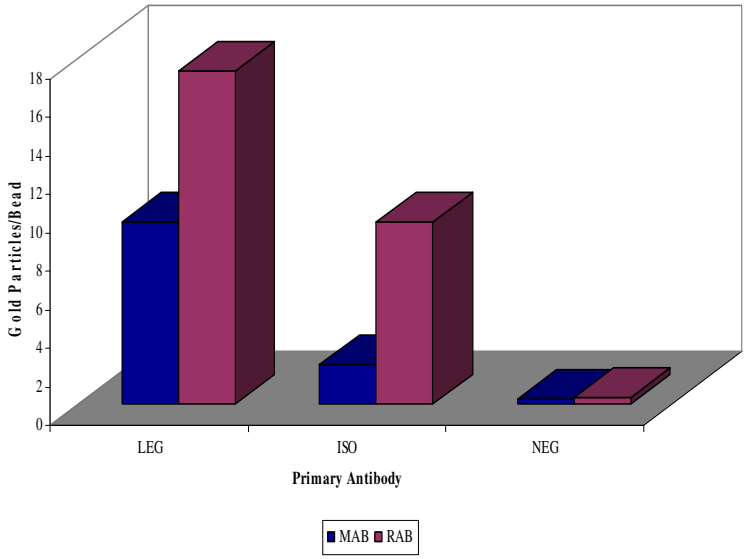
- TEM characterization of antibody loading on 500 beads using secondary antibody conjugated to 15nm gold particles.
- Fluorescent spectroscopic evaluation of antibody loading on 500 beads using Alexa488 conjugated secondary antibody



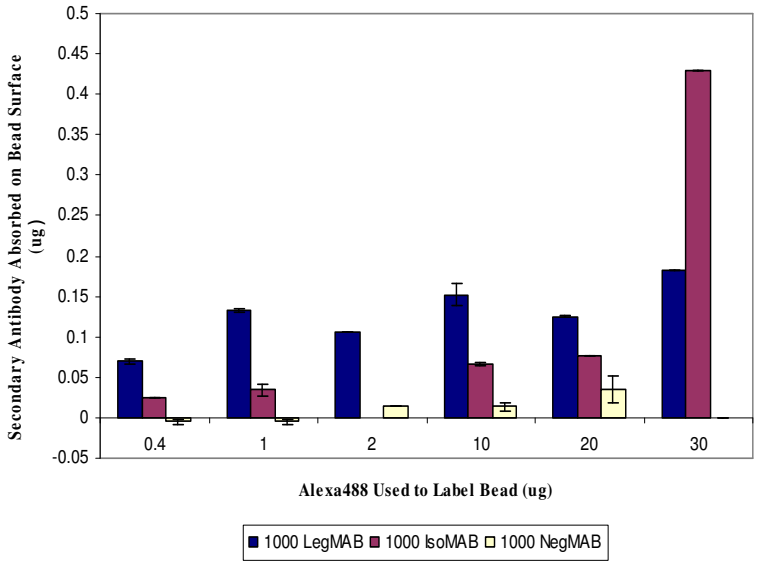
- **Figure 12: Analysis of primary antibody loading on 500 nm beads**



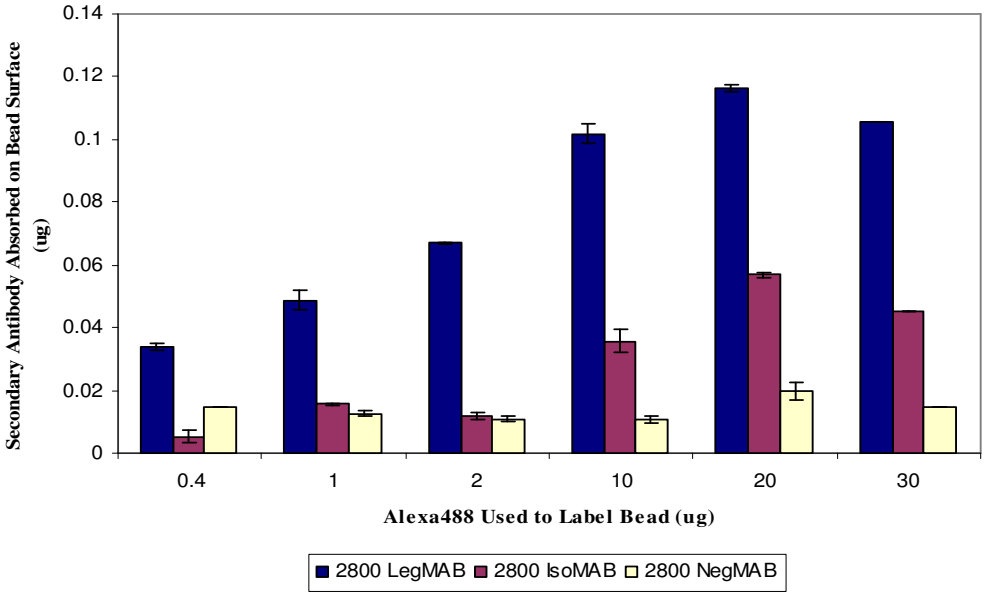
- **Figure 13: Analysis of secondary antibody loading on 500 nm beads**

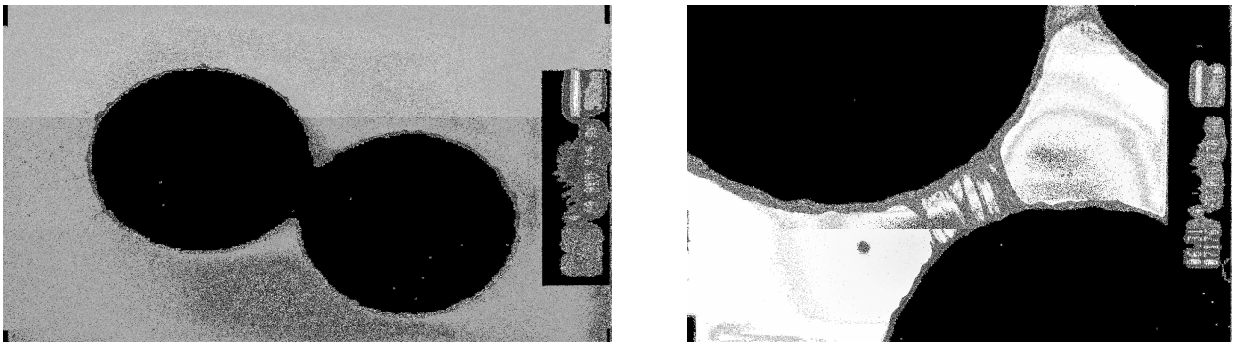


- **Figure 14: Analysis of primary antibody loading on 1000 nm beads**



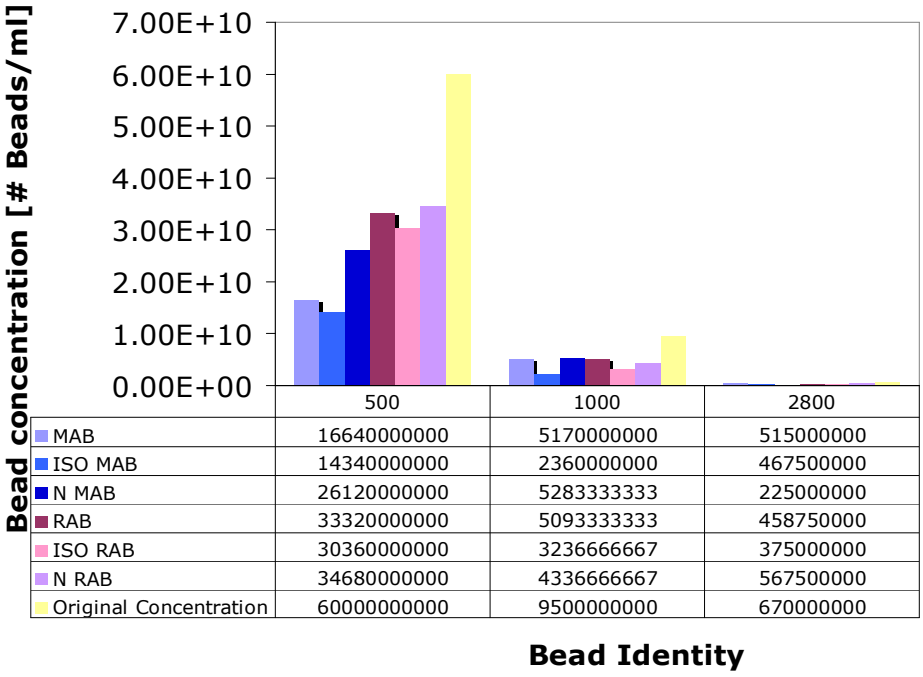
- **Figure 15: Analysis of secondary antibody loading on 1000 nm beads**





- **Figure 16: Analysis of secondary antibody loading on 2800 nm beads**

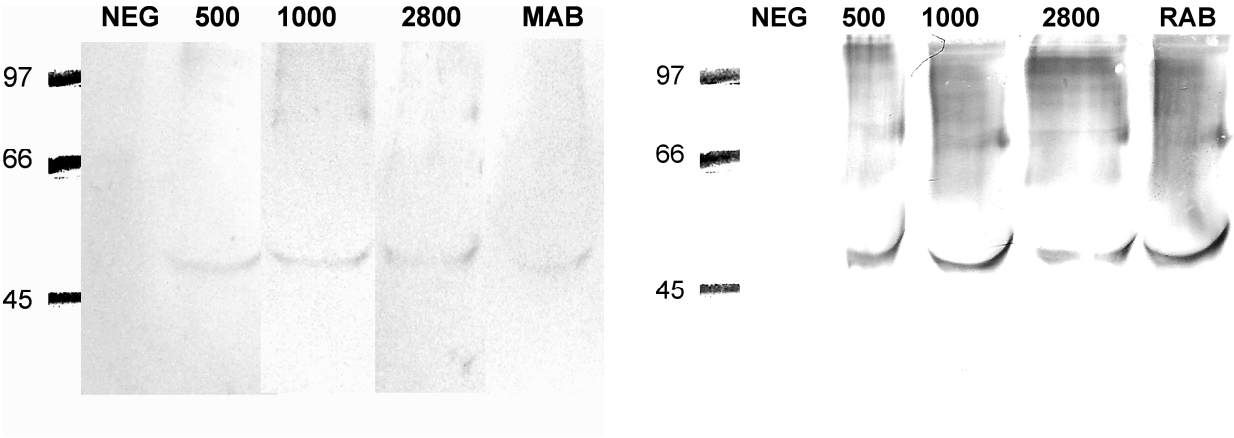
Concentration analysis UV/ Vis Spectroscopy



	500	1000	2800
MAB	16640000000	5170000000	515000000
ISO MAB	14340000000	2360000000	467500000
N MAB	26120000000	5283333333	225000000
RAB	33320000000	5093333333	458750000
ISO RAB	30360000000	3236666667	375000000
N RAB	34680000000	4336666667	567500000
Original Concentration	60000000000	9500000000	670000000

- **Figure 17: UV/Vis Spectroscopy for identification of concentration of functionalized beads**

Fuctionalized Magnetic Particles Recognize Legionella LPS



- **Figure 18: Recognition of *Legionella* by functionalized 500, 1000 and 2800 beads. *Left:* Beads functionalized with Mouse Monoclonal Antibody (MAB) against *Legionella* LPS. *Right*:. Beads functionalized with Rabbit Polyclonal Antibody (RAB) against *Legionella* LPS. NEG-Negative Control using *Borrelia***

2.2.5 Work performed and achieved in the second year within WP2

To give support to the TEM findings, Photon Correlation Spectroscopy (PCS; see Annex 1 for procedure) was conducted to provide particle size when the samples were in a liquid environment. This technique also provided size distribution for the particle of interest. Figure 5A-C provides size and size distribution on the 500, 1000, and 2800 beads, respectively. Validating beads for PCS are seen in Figure 5D-E in Annex 2.

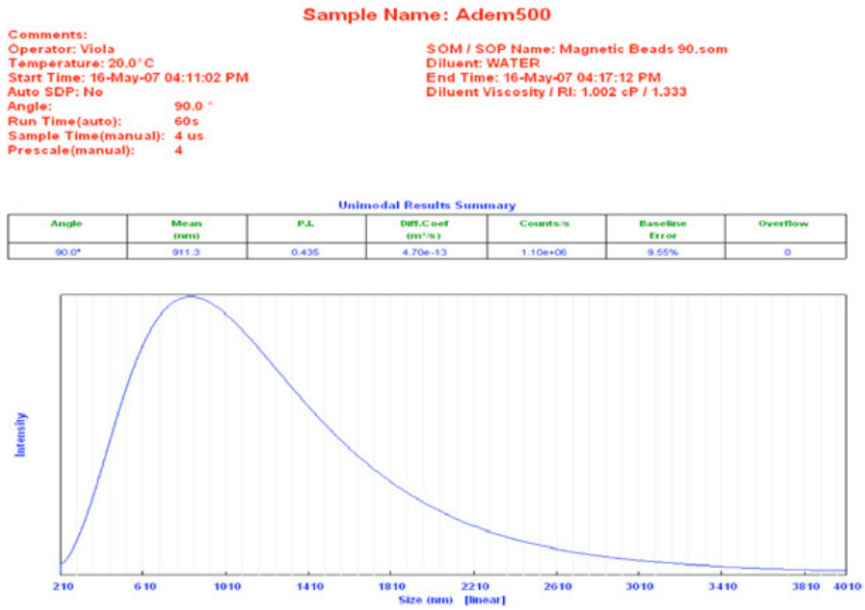


Figure 5A. PCS analysis. Size frequency and population distribution of 500 using angle setting of 90°.

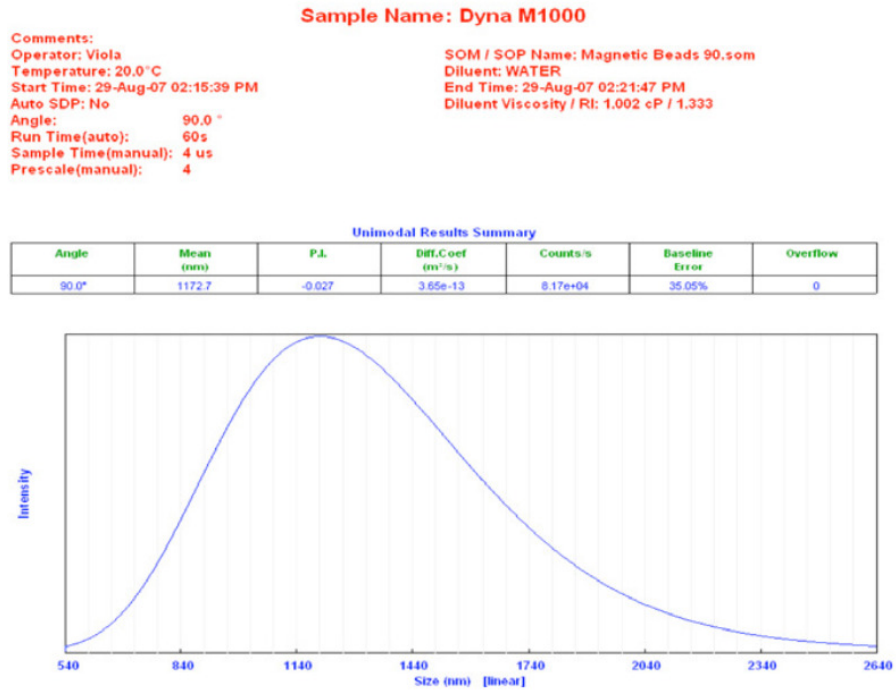


Figure 5B. PCS analysis. Size frequency and population distribution of 1000 using angle setting of 90°.

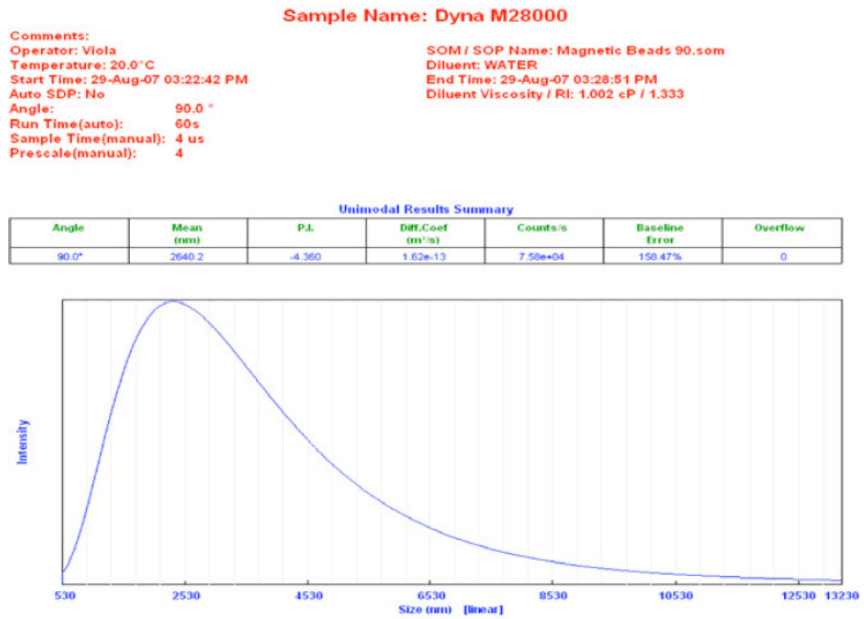
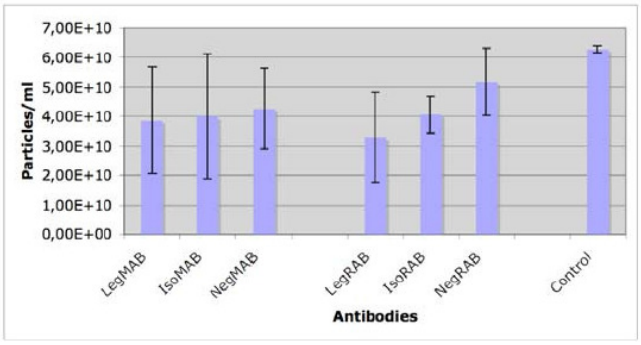


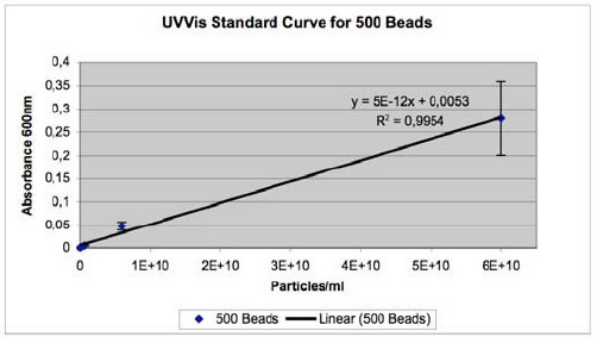
Figure 5C. PCS analysis. Size frequency and population distribution of 2800 using angle setting of 90°.

PCS studies (Figure 5A-C) indicated that 500 gave inconsistent sizes with a mean of 911 nm and the distribution of the sizes is very board ranging from 610–1270 nm. 1000 gave consistent size value of a mean of 1172 nm and a narrow size distribution range of 1000–1250 nm. The 2800 demonstrated consistent sizes with a mean of 2640 nm and the distribution of the sizes was much narrower with a range of 2500-2900 nm. Latex 300 nm and 1000 nm beads were used as validating beads for PCS.

UV/Vis Spectroscopy (UV/Vis: see Annex 1 for procedure) was used to analyze bead concentration after functionalization. Up to this point, it was not feasible to obtain information of the amount of particles per mL due to the fact of the inconsistent size and uniformity of shape of the 500 beads. Instead UV/Vis was employed to aid in obtaining the concentration of the beads after functionalization. In addition, the amount of beads lost after the functionalization step can be deduced with this analysis. With this technique, we used unfunctionalized beads to establish a standard curve that was then used to compare the functionalized beads with. By performing the standard curve at a minimum of 5 times, statistically significant standards were created and used to compare the functionalized beads. Figure 8A-C provides (a) information of the concentration of the functionalized beads with the unfunctionalized beads as a control. In addition, (b) the standard curve for the bead of interest is provided.

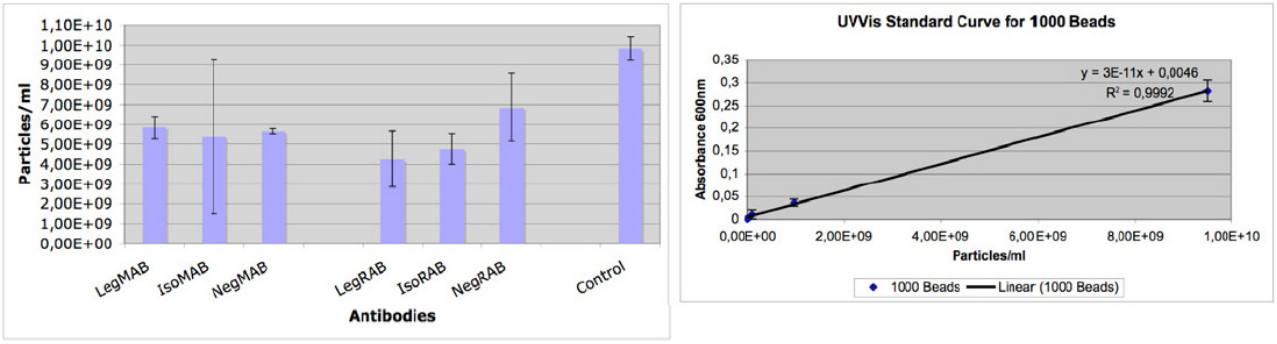


a.



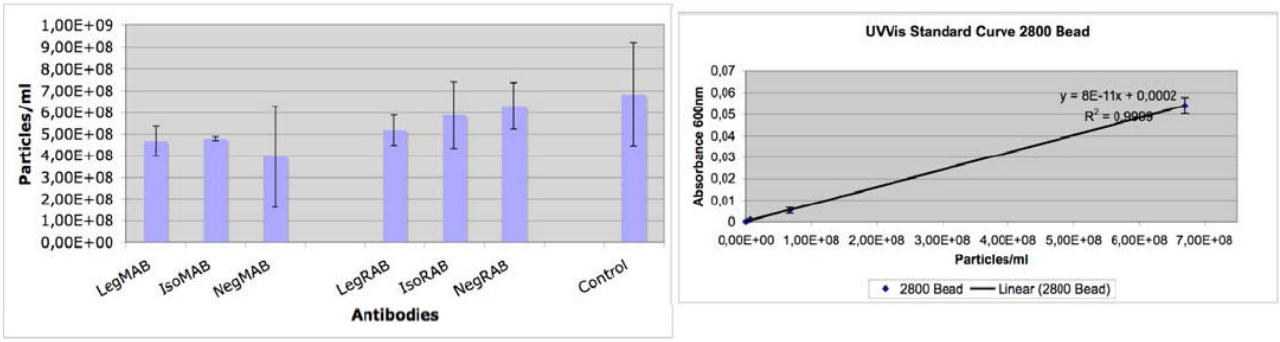
b.

Figure 8A. UV/Vis Spectroscopy analysis for 500. a. Concentration of functionalized beads. Control is unfunctionalized beads. b. Standard curve for 500 beads. See Annex 1 for specific details.



a. b.

Figure 8B. UV/Vis Spectroscopy analysis for 1000. a. Concentration of functionalized beads. Control is unfunctionalized beads. b. Standard curve for 1000 beads. See Annex 1 for specific details.



a. b.

Figure 8C. UV/Vis Spectroscopy analysis for 2800. a. Concentration of functionalized beads. Control is unfunctionalized beads. b. Standard curve for 2800 beads. See Annex 1 for specific details.

Superconducting Quantum Interference Device (SQUID; see Annex 1 for procedure) was a collaborative effort with the R&D group of CNM. Together we were able to obtain preliminary data on the changes of magnetic field or flux density properties of the functionalized beads. Figure 10 provides evidence that the functionalized beads do not change magnetic properties of the bead.

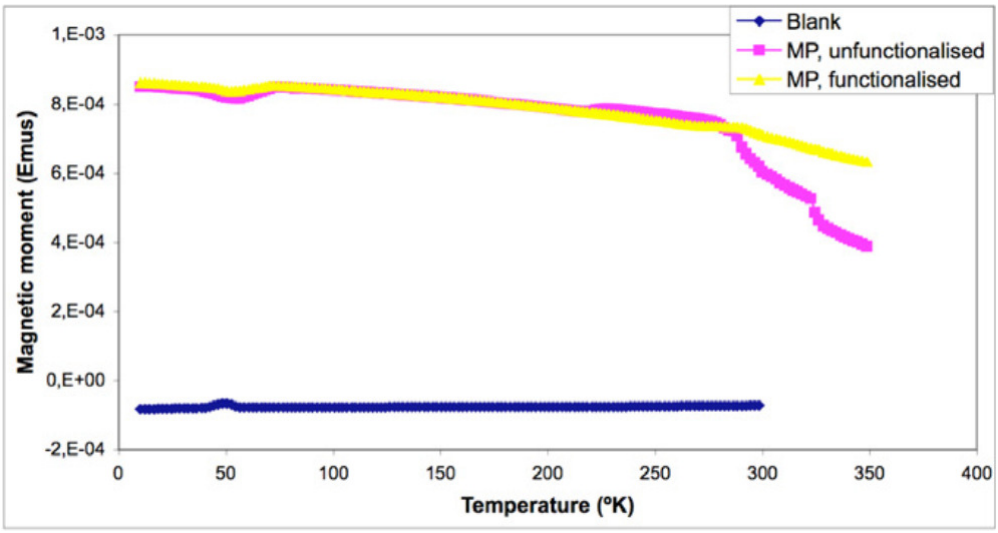


Figure 10. SQUID Analysis of 2800. Functionalized magnetic particles (MP) and unfunctionalized MP of 2800 were analyzed by squid with water as a control. The detection of magnetic moment change is displayed.

SQUID provides preliminary evidence that the magnetic moment does not change in functionalized beads.

Proof of principle tests

Proof of principle tests were performed in cooperation with CNM. Collaborative efforts allowed obtainment of proof of principal that the detection beads and the capture surface could be employed within this project. What is worth mentioning, is that the following Figure 11 provides a glimpse of the functionalized beads detecting Legionella. Here the coated particles were added to a methonal fixed Legionella sample. After a series of washes the beads-Legionella complex was mounted with DAPI hardset Vectashield mounting media. DAPI stain allow visualization of the DNA of the Legionella and Differential Interface Contrast (DIC) imaging allowed visualization of the bead and bacteria together. These confocal images were taken to focus on the bead itself, and then on the bacteria for DNA visualization. In short the detection of the Legionella functionalized beads is doable, but the optimization of the detection procedure should be optimized to give consistent results. It is the intent of UJ to continue this work with CNM even after M13.

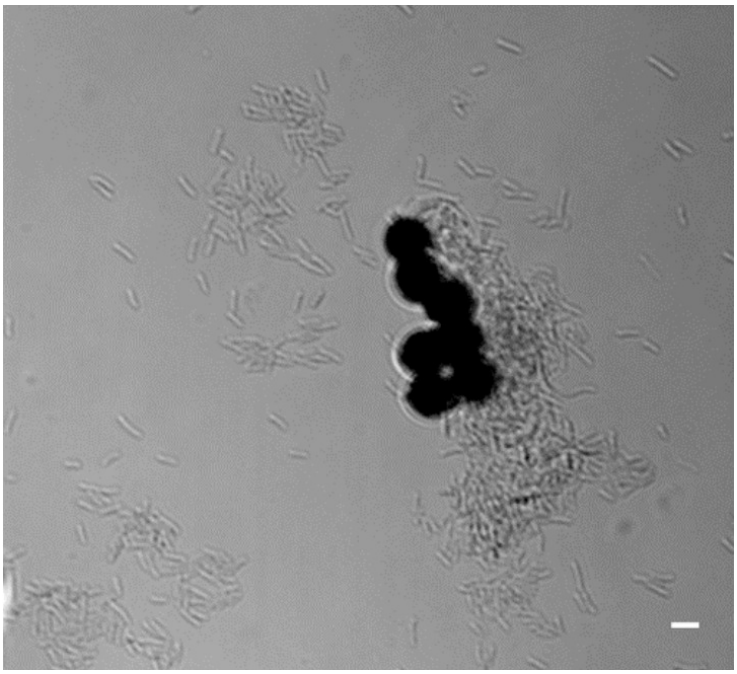


Figure 11. Confocal Analysis of Bead on Legionella. RAB 2800 functionalized magnetic bead bound to Legionella. DIC image. Bar 2 microns.

As the main focus of this work package was to evaluate 300 and 500 beads to be used as the choice bead for our detection beads, numerous techniques were not employed. These techniques included TEM for number of particles per mL of sample, Confocal/Spectral Imaging to investigate antibody loading, as well as Flow Cytometry and Atomic Force Microscopy to support population uniformity and size of the beads. In addition, MicroBCA™Protein Assay Kit was also initially used to provide data on concentration amounts of the beads. This was not followed through for all the beads and data on this technique is not provided within this report. We have however kept this technique in the Operative Measuring Protocols; Annex 1 because we feel that this technique could be a valuable tool once a defined bead with uniform size distribution and shape is chosen.

The exclusion of these various techniques is due to the fact that the inconsistent sizes seen within 500 beads provided inconsistent and irreproducible results in most evaluation points studied within this report. Even if the experiments were conducted minimum of three times, inconsistent and irreproducible results were obtained with high error bars. The idea detection bead would consist of consistent bead size, uniform in shape and a narrow size distribution. This would then provide consistent and reproducible results in size, shape, and antibody loading and binding of the bacteria. This was obtained with 1000 and 2800 beads. Despite the redirecting of techniques to analyze the beads, D6 provides evidence that supports the successful preparation of superparamagnetic particles coated with specific Legionella antibodies. This means that the progress of the overall project should continue.

2.3 Workpackage 3. "Biosensor Design"

During the first year of our activities in WP3 we have place special efforts both antibodies identification and surface antibody immobilization & evaluation. In parallel we have been working in the selection of optimum magnetic structures as sensing devices, being GMRs (giant magnetoresistive) and MI (micro-inductive) devices the structures chosen.

Antibodies identification (M1-M3)

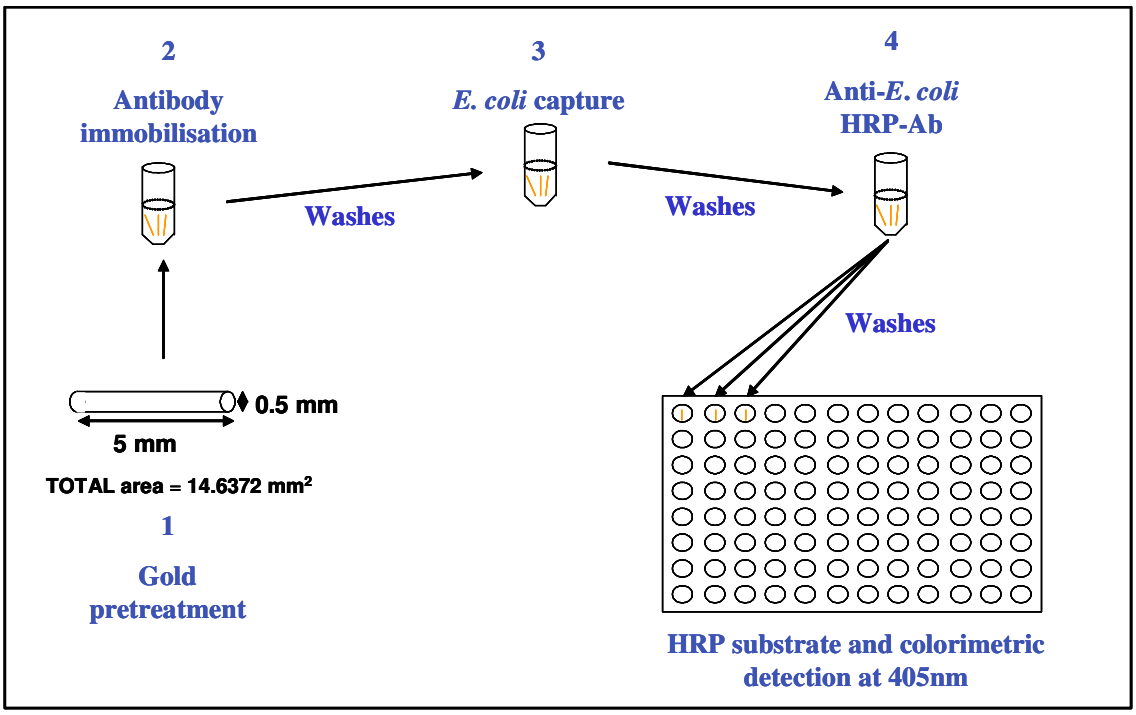
Identifying, selecting and obtaining antibodies (M1-M2)

This work was realised collaborating with UJ group. The provide the criteria for antibodies selection.

Surface antibody immobilization & evaluation (M2-M8)

This document reports the optimisation, characterisation and validation of different recognising surfaces for bacteria detection, produced by immobilising antibodies (Ab) on gold surfaces. Gold is one of the favourite materials used for biosensor fabrication due to its inertness, stability and lack of toxicity. With this aim, two main alternative strategies have been considered. In the first case, Ab were chemically conjugated to self-assembled monolayers (SAM) of different type and composition. In this context, molecule combinations and conjugation procedures previously optimised for protein detection by our group, and thus having shown good performance, were exploited. The different procedures were re-formatted for bacteria detection by determining each step optimal reagent concentration, incubation time, incubation temperature, etc. In the second case, we took advantage of random physisorption of either Ab or Ab-capture proteins (e.g.: biotin-binding proteins, protein A, and anti-mouse Ab). The goal of this part of the work was to establish which the most efficient protocols were, determining the performance of each Ab-modified gold surface in terms of low level of unspecific binding generated while high Ab functionality recovered, using *E. coli* as the model microorganism. The best performing surfaces, in terms of highest efficacy, specificity and sensitivity in the gold model format, were to be transferred to the IMMUNOLEGIO gold-coated sensing surfaces for *Legionella* detection.

The optimisation of the different functionalisation protocols required the manipulation of high numbers of sensing surfaces. The difficulty to have such devices continually and regularly provided, and in the wait for the magnetic sensor to be selected/optimised, it was decided to initially work on cheaper and reusable surfaces and a more convenient detection system. For the exposed reasons, and in the need for fast results, the enzymatic colorimetric detection of a sandwich assay was developed on pure gold as a model (figure 1). The following experiments have been carried out on gold wires cut into rods of similar lengths and weights, and thus similar surface size. The bare gold rods under study are easier and faster to obtain and can be effectively regenerated, and thus reused, through piranha treatment. In addition, a higher number can be manipulated in each experiment, as espectrophotometric detection takes place in 96-well microtitter plates, compared to the one-by-one electrode magnetic detection.



- **Figure 19: Scheme of immunoassay realised on gold rods.**

Reagents and components

A biotinylated anti-*E. coli* Rabbit Polyclonal antibody (α-Eco Bt-Ab) and an anti-*E. coli* HRP-labelled Rabbit Polyclonal antibody (Ab-HRP) were provided by *AbCam* (Cambridge, UK; Refs. ab20425 and *ab20640)*. An un-modified anti-*E. coli* Rabbit Polyclonal antibody provided by USBiological (α-Eco Ab; Ref. E3500-26) was alternatively used in the immobilisation experiments. Three monoclonal Ab against *Salmonella* were obtained from AbCam and immobilised in parallel (clone 10D9H, Ref. ab8272; clone 513, Ref. ab20949 and clone 5D12A, Ref. ab8273). Their performance was compared to a rabbit polyclonal Ab from the same company (Ref. ab21118). Detection was carried out in all cases in a sandwich format using an HRP-labelled polyclonal Ab (Ref. ab2077).The performance of the Ab had been previously assayed by traditional ELISA in microtitter plates. *Escherichia coli* K12 (ATCC 10536), *Salmonella typhimurium* ATCC 14028, *Pseudomonas putida* KT 2442 and *Staphylococcus aureus* were obtained from the American Type Cells Collection. Unless otherwise stated, all the biocomponents were diluted and incubated in 10mM phosphate buffer saline (PBS). Washes were carried out with either PBS or PBS containing 0.05% of Tween 20x (PBS-tween).

The following reagents were provided by Sigma. CM-dextran is carboxymethylated dextran. BSA is Bovine Serum Albumin. As the HRP enzymatic substrate we used (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), liquid substrate, Ref. 3219, mixed in a 10:1 proportion with (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) Enhancer, Ref. A1227 and preincubated at RT for some minutes in the dark previous to use, as recommended by the provider.

Gold surface pre-treatment.

The gold rods are sistematically pretreated by immersion in a freshly made Piranha solution (1:3 hydrogen peroxide 30% : sulfuric acid) for 3 minutes, in order to both wash away any organic depositions and render the surface hydrophilic. Our results show that extending such a treatment (3 minutes to over-night tested) does not improve the surface and/or assay quality. The gold rods are then roughly washed with water, sonicated for 3 minutes in isopropanol and washed with

ethanol and water before being immediately used. For the rest of the process, the rods are distributed into eppendorf tubes, three to six replicates per tube, in an attempt to avoid them lying on a surface and thus interfering with homogeneous coverage. Values showed along the text are the mean of at least 3 replicates.

2.3.1 Testing SAM method for immobilization (M2-M5)

The group has extensively worked in the past in the optimisation of SAM-based immuno-functionalisation strategies for protein detection. In this context, a number of thiolated molecules of different length and chemical characteristics were assayed, and several approaches for the chemical conjugation of Ab were optimised. The best performing strategies have been re-formatted for bacteria detection in the present work by incorporating to the selected SAM-based surfaces an anti *E. coli* Ab (α-Eco Ab), followed by *E. coli* capture and incubation with an anti *E. coli* HRP-labelled Ab (Ab-HRP). As a negative control for specificity, both surfaces lacking Ab immobilised and surfaces bearing BSA instead were assayed in the presence of bacteria. We will show as an example part of the results obtained.

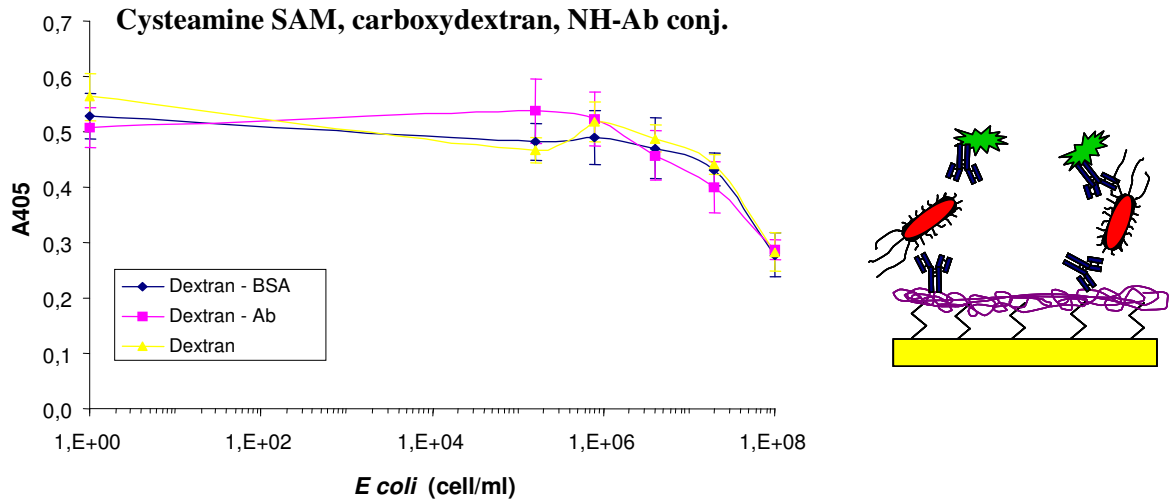
Cysteamine SAM plus EDC/NHS-activated dextran and NH-Ab

According to our previous results, a gold surface modified with a cysteamine SAM and conjugated to EDC/NHS-activated CM-dextran can induce a decrease in biomolecule unspecific adsorption of up to 60% compared to bare gold. The EDC/NHS active groups incorporated to the CM-dextran can be additionally exploited for Ab chemical incorporation via their NH- groups in a very simple reaction. The obtained surface showed a good performance for protein detection in the past. This surface was thus selected to optimise Ab conjugation for bacteria detection in the first place.

The initial protocol and parameters under study/optimisation (*in brackets*) were as follows:

1.) Piranha treatment of the gold rods as previously described.
2.) Incubate the gold rods for 30 minutes at RT in a cysteamine solution (1mM in ethanol).
3.) Wash twice with ethanol and twice with distilled water.
4.) Add the CM-dextran, dissolved to a final concentration 8mg/ml in PBS pH 7.2-7.4 containing 100mM EDC/NHS, and incubate for 15 minutes at RT.
5.) Wash three times with PBS.
6.) Add the appropriate antibodies (or no antibodies / BSA to the negative controls) diluted in PBS to a concentration (*1.25 to 10µg/ml*), and allow to conjugate for 1h at (*37ºC or RT*).
7.) Wash twice with PBS-tween and twice with PBS.
8.) Block the unreacted active groups for 15 minutes with 1M ethanolamine.
9.) Wash three times with PBS.
10.) (Optional) Incubate for 2h at 37ºC in the presence of 2% (w/v) of BSA
11.) Wash three times with PBS-tween.
12.) Add an *E. coli* dilution series and incubate for 1h at 37ºC.
13.) Wash three times with PBS.
14.) Incubate for 1h at 37ºC with the Ab-HRP diluted 1:750 in PBS-tween.
15.) Wash 4 times with PBS-tween.
16.) Transfer the gold rods to the wells of a microtitter plate and add 100µl/well of substrate + enhancer,. Incubate for 20 minutes at RT and read A405.

Results: Extensive optimisation was performed on the different parameters of the functionalisation protocol. However, and in spite of the promising results previously obtained or protein detection, bacteria detection was completely unsuccessful. As shown in the following figure 2, similar trends were obtained in the presence / absence of α-Eco Ab in the surface. The increase in the concentration of Ab-HRP did only generate higher levels of background signal, accompanied by negative signals presumably induced by bacterial unspecific adsorption at the highest titters, and thus a certain level of physical blocking against the subsequent Ab-HRP adsorption.



- **Figure 20: Curves of gold functionalized by Cysteamine SAM with carboxydextran conjugated.**

Cysteamine SAM plus EDC/NHS-activated dextran, ethylenediamine blocking and conjugation to EDC/NHS preactivated COOH-Ab

In the fear that the NH-Ab modification may partly interfere with its functionality, the optimisation of an alternate protocol, consisting on Ab conjugation through its COOH-groups was attempted. The protocol is as follows:

1.) Piranha treatment of the gold as previously described.
2.) Wash twice with ethanol and twice with distilled water.
3.) Incubate the gold rods for 30minutes at RT in a cysteamine solution (1mM in ethanol).
4.) Wash twice with ethanol and twice with PBS or distilled water.
5.) Add the CM-dextran, dissolved to a final concentration 8mg/ml in PBS pH 7.2-7.4 containing 100mM EDC/NHS, and incubate for 15 minutes at RT.
6.) Wash three times with PBS.
7.) Incubate the modified rods for 1h at 37ºC in a 1M solution of an ethylenediamine / ethanolamine (*10:1, 1:1 or 1:10*) molar ratio mixture.
8.) Wash four times with PBS-tween
9.) Preactivate the Ab for 15minutes at RT in MES pH 5.3 containing 100mM EDC/NHS.
10.) Eliminate part of the remaining unreacted products by centrifuging for 2-3 minutes at 12000rpm through a microcon device at least twice, adding each time fresh PBS.
11.) Incubate the EDC/NHS-activated Abs with the gold rods for 1h at 37ºC
12.) Wash four times with PBS-tween.
13.) Block the unreacted active groups for 15 minutes with 1M ethanolamine.
14.) Wash three times with PBS.
15.) Proceed as previously described

Results: According to our preliminary data, Ab immobilisation via their COOH- groups performed slightly better than Ab linked through their NH-. Nevertheless, this approach did not improve bacteria detectability.

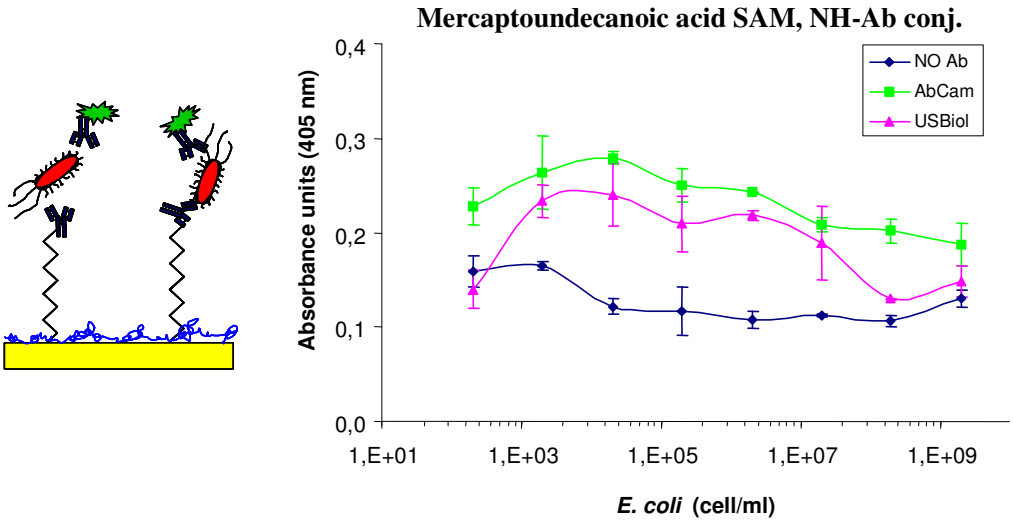
Mercaptoundecanoic acid SAM, EDC/NHS activation and NH-Ab CONJUGATION

Our previous results indicated that the longest the thiolated chain used for SAM self-assembly, the lowest the level of biomolecule unspecific adsorption recorded. The longest molecule we have tested is mercaptoundecanoic acid (MUA), which generated in our assays a reduction in biomolecule random adsorption of up to 50% compared to bare gold.

The initial protocol and parameters under study/optimisation (*in brackets*) were as follows:

1.) Piranha treatment of the gold rods as previously described.
2.) Incubate the gold rods for 1h at RT in a MUA solution (10mM in ethanol).
3.) Wash twice with ethanol and twice with distilled water.
4.) Incubate for 15 minutes at RT in PBS pH 7.2-7.4 containing 100mM EDC/NHS.
5.) Wash three times with PBS.
6.) Add the appropriate antibodies (or no antibodies / BSA to the negative controls) diluted in PBS to a concentration (*1.25 to 10µg/ml*), and allow to conjugate for 1h at 37ºC.
7.) Wash twice with PBS-tween and twice with PBS.
8.) Block the unreacted active groups for 15 minutes with 1M ethanolamine.
9.) Wash three times with PBS-tween.
10.) (Optional) Incubate for 2h at 37ºC in the presence of 2% (w/v) of BSA
11.) Wash three times with PBS-tween.
12.) Add an *E. coli* dilution series and incubate for 1h at 37ºC.
13.) Wash three times with PBS.
14.) Incubate for 1h at 37ºC with the Ab-HRP diluted 1:750 in PBS-tween.
15.) Wash 4 times with PBS-tween.
16.) Transfer the gold rods to the wells of a microtitter plate and add 100µl/well of substrate + enhancer. Incubate for 20 minutes at RT and read A405.

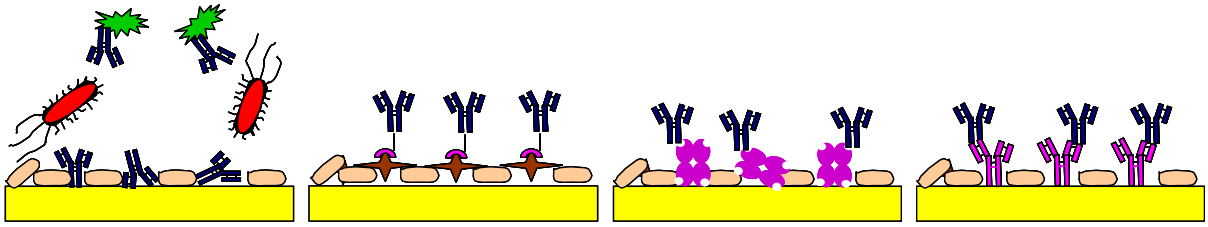
Results: None of the attempts to optimise this protocol allowed the successful detection of *E. coli* (the figure 3 shows the results for two different Ab immobilised in parallel). The physical blocking of the surface with BSA following Ab incorporation, on the other hand, contributed in this case to significantly reduce the level of Ab-HRP unspecific adsorption.



- **Figure 21: Curves of gold functionalized by mercaptoundecanoic acid SAM conjugated.**

2.3.2 RANDOM PHYSISORPTION functionalisation protocol (M6-M8)

As an alternative to SAM-based functionalisation, non-specific adsorption, or physisorption, is the easiest way to immobilise Abs/proteins on a physical substrate. Physisorption consists on just depositing the Ab/protein on the surface and leaving them to interact in a completely random way. One of the main drawbacks associated to this strategy is the fact that, as a consequence of adsorption, proteins may partly denature and thus loose structure and function. Nonetheless, several authors have reported acceptable performances and high stability of the surfaces prepared in this way. In any case, and in order to maximise Ab functionality, we also attempted their indirect capture. This implies that a capture protein is physisorbed on surface, followed by BSA blocking and Ab capture. The later approach should favour Ab orientation and prevent its integrity and function. The different approaches are summarised in the following figure 4:



- **Figure 22: Different functionalisation approaches for gold surfaces based on protein physisorption.**

Ab random physisorption

This was the simplest protocol assayed, and consists of the following steps. The parameters that have been studied/optimised are shown in brackets. Unless otherwise stated, washing consist of two washes with PBS and other two with PBS-tween.

1.) Piranha treatment of the gold rods as previously described.
2.) Incubate the gold rods with the Abs (*0 to 20µg/ml* in PBS) for 1h at 37ºC.
3.) Following washing, incubate for 2h at 37ºC in the presence of (*0-4%* w/v) of BSA.
4.) Wash, add an *E. coli* dilution series and incubate for 1h at 37ºC.
5.) Wash three times with PBS and proceed with the sandwich assay as previously described.

Results: This protocol worked optimally when 15µg/ml of Ab were immobilised, followed by physical blocking with 2% BSA. The functionalised surface allowed detection of *E. coli* in a concentration range between $2x10^6$ to $2x10^8$, with an approximate Limit of Detection (LOD = background + 3 x SD) of $2x10^5$ cell/ml and IC_{50} around 10^7 cell/ml. The curve slope was in all cases the steepest one obtained, what is an indicative of high sensitivity. Even if the unmodified α-Eco Ab showed slightly higher maximal values than the α-Eco Bt-Ab, both Ab generated very similar results in terms of LOD and IC_{50}.

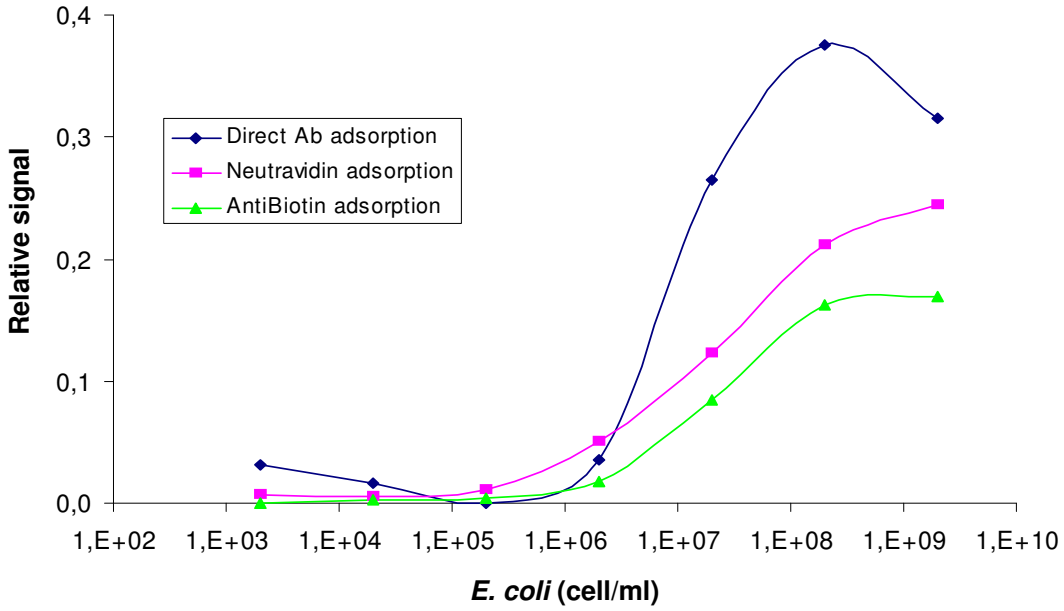
Physisorption of biotin-binding proteins and capture of biotinylated Ab

In this case, we exploited three well known biotin-binding proteins: neutravidin, streptavidin and anti-biotin Abs (α-Bt Ab), followed by the affinity capture of the biotinylated anti *E. coli* Ab (α-Eco Bt-Ab). We did not assayed avidin, as it has been repeatedly reported to generate extremely high levels of bacterial unspecific adsorption. The protocol was as follows:

1.) Piranha treatment of the gold rods as previously described.
2.) Incubate the gold rods with the biotin-binding protein (*0 to 20µg/ml* in PBS) for 1h at 37ºC.
3.) Following washing, incubate for 2h at 37ºC in the presence of 2% (w/v) of BSA.

4.) Wash and incubate for 1h at 37ºC with the α-Eco Bt-Ab (*0 to 20µg/ml* in PBS).
5.) Wash, add an *E. coli* dilution series and incubate for 1h at 37ºC.
6.) Wash three times with PBS and proceed with the sandwich assay as previously described.

Results: Unexpectedly, the Ab indirect capture correlated with significantly lower maximal signals recorded in all cases, and did not seem to improve performance in an important way compared to Ab random physisorption at similar concentrations. Nevertheless, this strategy generated more extended detection ranges, with *E. coli* being detected in some cases between $<10^5$ to $>10^9$ cell/ml, and LODs around or under 10^5. Among the tested molecules, neutravidin was the best performing one, followed by streptavidin and the anti-biotin Ab. The results have been summarised in the following figure 5:



- **Figure 23: Comparative performance of three functionalisation strategies of physisorption.**

Physisorption of protein A and affinity capture of Ab

Protein A and protein G have been described as extremely useful reagents for Ab capture, because they show strong affinity for the Ab COOH-ter. This promotes the orientation of the trapped Ab, which will expose the active target-binding NH-ter. Nonetheless, protein A/G are mainly applied to Ab affinity purification, and very few authors have used them in sandwich immunoassay formats. We worked on the following protocol and parameters:

1.) Piranha treatment of the gold rods as previously described.
2.) Incubate the gold rods with the protein A (*0 to 20µg/ml* in PBS) for 1h at 37ºC.
3.) Following washing, incubate for 2h at 37ºC in the presence of 2% (w/v) of BSA.
4.) Wash and incubate for 1h at 37ºC with the α-Eco Ab (*0 to 20µg/ml* in PBS).
5.) Wash, add an *E. coli* dilution series and incubate for 1h at 37ºC.
6.) Wash three times with PBS and proceed with the sandwich assay as previously described.

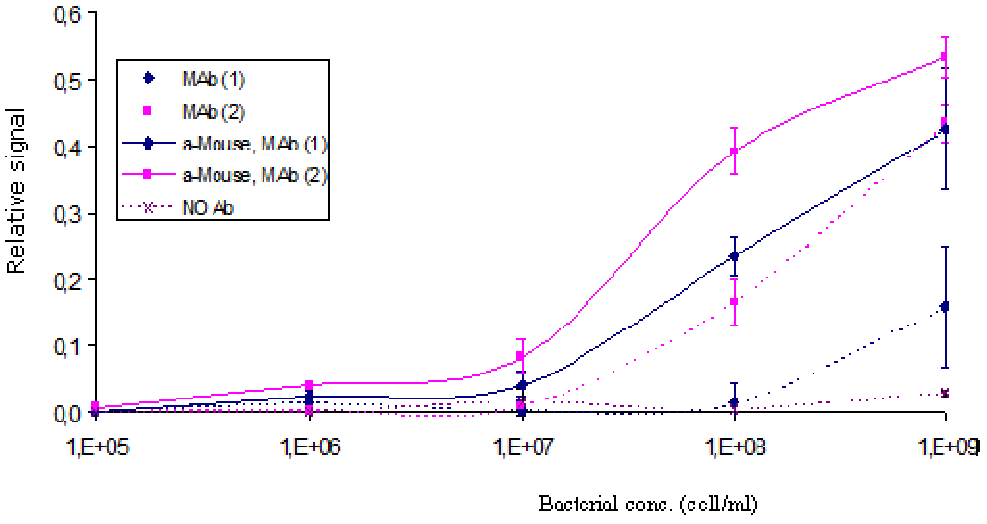
Results: The use of protein A was unsuccessful and high levels of Ab-HRP cross-binding were observed for all the combinations of protein A and α-Eco Ab tested. In this respect, the use of α-Eco Ab at concentrations exceeding 4-fold those of protein A previously physisorbed either did not generate surface saturation, or suffered from later displacement by the Ab-HRP. This strategy was thus discarded.

Physisorption of anti-mouse Ab and immunoaffinity capture of monoclonal Ab

Some reports describe dissimilar performance of certain monoclonal Ab following either physisorption or biotinylation. For this reason, we decided to optimise this alternative protocol in case the previous ones showed limited success for the IMMUNOLEGIO monoclonals of choice. In the lack for monoclonals against the *E. coli* strains we use at our laboratory, we used *Salmonella typhimurium* and anti-*Salmonella* monoclonal Abs. Among the three different monoclonals that were assayed and compared to an anti-*Salmonella* polyclonal, one did not recognise our *Salmonella* strain at all. The other two will be named MAb (1) and MAb (2). The functionalisation protocol was very similar to the ones previously described, except that the concentrations of capture protein were increased. According to the provider, 1mg of anti-mouse Ab binds 0.5mg of MAb.

1.) Piranha treatment of the gold rods as previously described.
2.) Incubate the gold rods with the anti-mouse Ab (*0 to 40µg/ml* in PBS) for 1h at 37ºC.
3.) Following washing, incubate for 2h at 37ºC in the presence of 2% (w/v) of BSA.
4.) Wash and incubate for 1h at 37ºC with the α-Sal MAb (*0 to 20µg/ml* in PBS).
5.) Wash, add an *Salmonella* dilution series and incubate for 1h at 37ºC.
6.) Wash three times with PBS and proceed with the sandwich assay as previously described.

Results: Figure 6 shows the two MAb used showed lower sensitivity for *Salmonella* than the polyclonal Ab we assayed in parallel. Even if MAb (2) showed always higher maximal signals and slightly lower LOD, both MAb performed quite similarly on microtitter plates. However, Mb (1) lost activity when physisorbed on gold, the drop in signal and increase in LOD being evident in the graph shown underneath. Following capture by an anti-mouse Ab (30µg/ml), both MAb (15µg/ml) showed again comparable behaviours.



- **Figure 24: Comparative about monoclonal works directly adsorbed o captured by antibodies.**

2.3.3 SURFACE CHARACTERISATION.

According to the results previously summarised, the best performing functionalisation strategies were considered to be:

(i) Ab random physisorption and

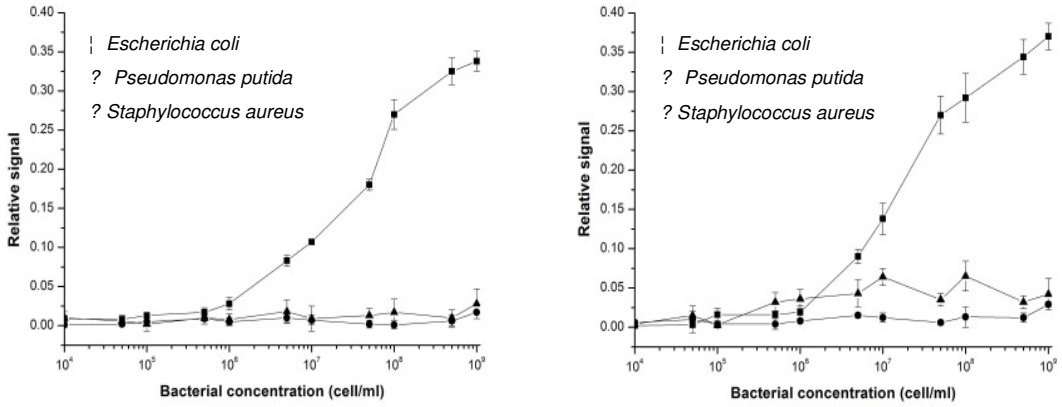
(ii) neutravidin physisorption coupled to capture of a biotinylated Ab

It was considered that a cover of anti-mouse Ab with affinity capture MAb would behave similarly.

These two functionalised surfaces were additionally characterised, as to determine their specificity, stability and reproducibility.

Surface specificity: cross-binding with unrelated bacteria

Gold rods, functionalised as previously described with anti *E. coli* polyclonal Abs, were incubated in parallel with E. coli, *Pseudomonas putida* and *Staphylococcus aureus*. *Pseudomonas* is considered a very "sticky" microorganism. As shown in the following figure, very low levels of cross-binding were observed for *Pseudomonas* and *Staphylococcus,* both onto Ab physisorbed and captured by neutravidin. On the contrary, we observed detectable levels of Ab cross-binding for *Salmonella* at concentrations over 10^7 cell/ml. The level of bacterial unspecific adsorption on similar surfaces lacking Ab (functionalised with BSA and/or neutravidin) were only detectable at titters over 10^8 cell/ml (Fig. 7). We can conclude that surfaces immuno-functionalised by physisorption are highly specific for the microorganism of interest, provided that the Ab used are truly specific for them.



Neutravidin + Biotin-Ab

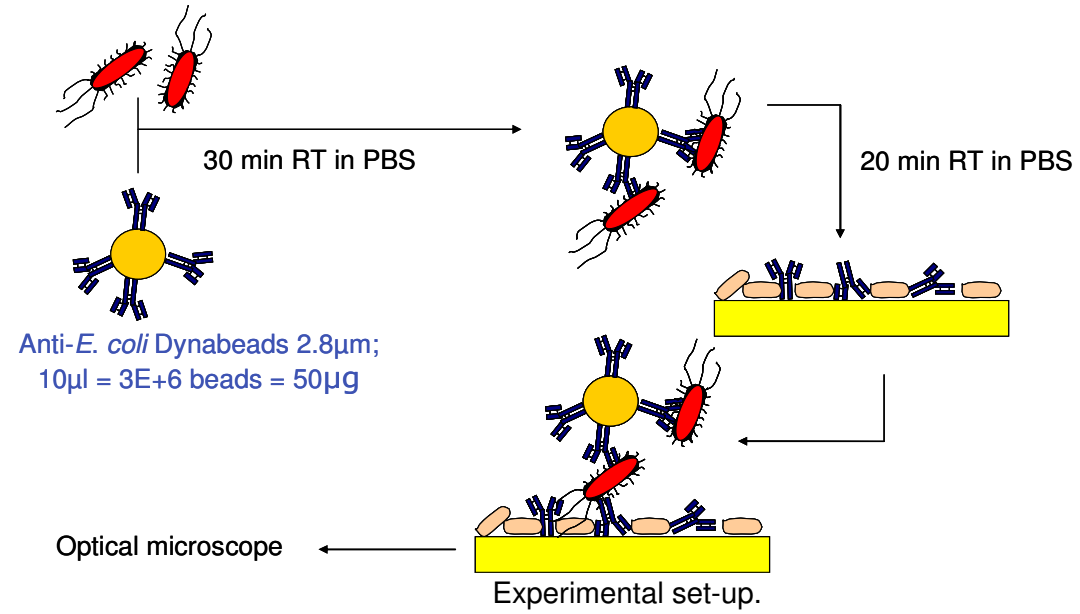
Ab unspecific adsorption

- **Figure 25: Cross-binding study of functionalisation of surfaces vs. other bacteria**

Surface specificity: cross-binding with functionalised magnetic particles

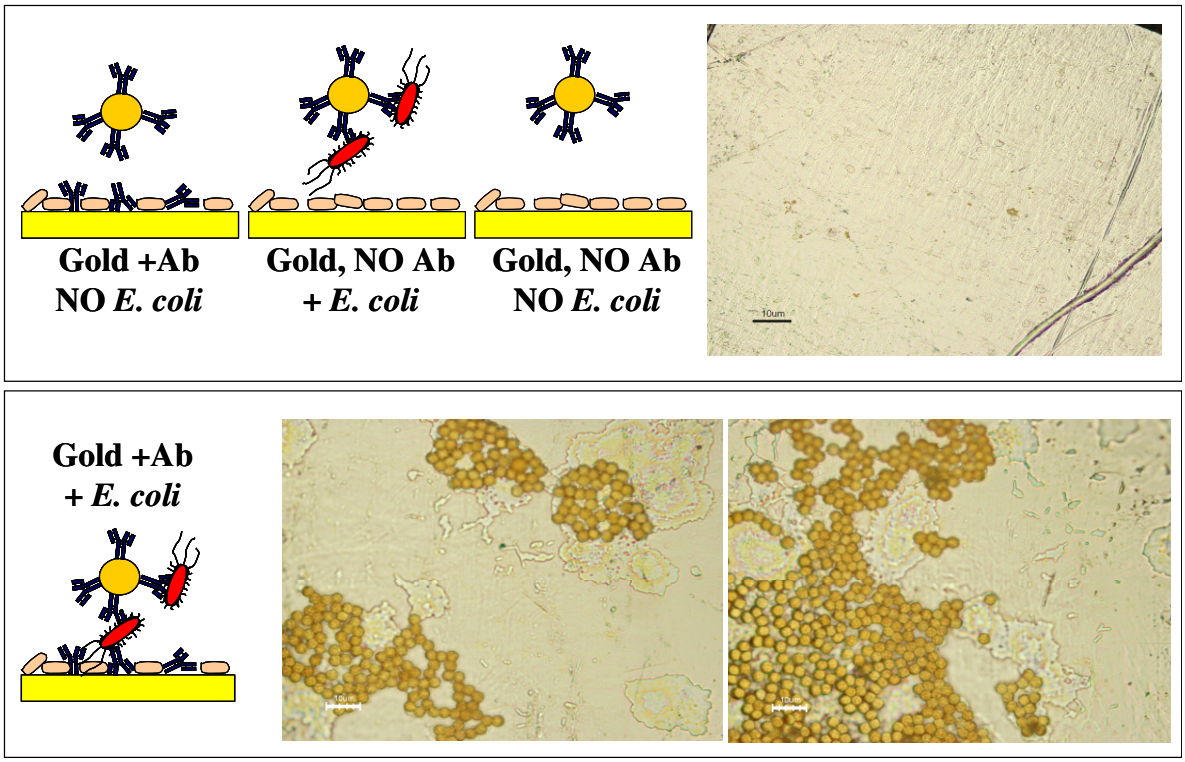
The specificity of the final IMMUNOLEGIO device depended on the specificity of the immuno-functionalised gold surface for bacteria, previously bound by immuno-functionalised magnetic particles. It was thus absolutely necessary for the surface not to capture magnetic particles not bearing bacteria. We investigated this possibility.

We assayed in parallel the anti-*E. coli* magnetic particles commercially provided by Dynal-Invitrogen, which are 2.8 µm in diameter, and streptavidin-coated 500nm magnetic particles from Ademtech, that we fucntionalised with the α-Eco Bt-Ab. The experiment was carried as displayed in the following figure 8:



Experimental set-up.

Functionalised particles (50µg per sample) were incubated in the presence / absence of 10^7-10^9 *E. coli* cell/ml for 30 minutes at room temperature, washed 3 times with PBS-tween, incubated with the gold surface, functionalised with either Ab or BSA, and washed again before being studied under an optical microscope. As shown in the following pictures, only the surfaces functionalised with Ab captured big numbers of bacteria-bearing magnetic particles. The immunofunctionalised surfaces did not cross-bind with the immunofunctionalised magnetic particles. In the same way, surfaces functionalised with only BSA but no Ab did not capture magnetic particles, bearing or not *E. coli*. The 500nm particles, however, showed a certain tendency to adsorb onto surface irregularities (scratches, holes, edges, etc.), as can be seen in Figure 26



- **Figure 26:Unspecific adsorption of the MP with and without captured bacteria, and on surface**

functionalized and not functionalized.

Surface coverage reproducibility

The gold surfaces, immuno-fucntionalised as previously described, were studied by Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). Disappointingly, the images obtained were not informative enough, due to gold rugosity. Cyclic Voltammetry (CV) and Impedance Spectroscopy (IES) were alternatively used to study surface coverage by proteins.

CV allows the study of electron transfer between a solution and the surface of an electrode immersed in it. If the solution contains a know electroactive chemical specie, this translates into the apparition of reduction and oxidation peaks at known voltages. CV was carried on gold electrodes before and after Ab physisorption. We observed in all cases that the modification induces insulation of the electrode, with the redox peaks not being detectable any more on functionalised electrodes. This fact indicated that proteins (Ab, neutravidin and/or BSA) cover an important part of the surface.

IES, on the other hand, allows the study of the resistance offered by an electrode surface against electron transfer through the calculation of a parameter named capacitance. This methodology requires very complex data analysis, but is extremely sensitive. IES was carried out on 13 independent electrodes before and after Ab physisorption. In all cases, a change in capacitance of around 30% was obtained following the modification, with a coefficient of variation between electrodes/experiments of about 8% (% CV, calculated as (SD / Mean) x 100 for the 13 data sets obtained). These results indicate that the level of surface coverage induced by Ab physisorption is highly reproducible.

Surface immuno-functionality reproducibility

We also studied if reproducibility in surface coverage translated into reproducibility of bacteria capture/detection by the immuno-functionalised gold surfaces. With this aim, gold rods were functionalised and *E. coli* dilution series captured and detected as previously described.

To study the intra-assay variability, an experiment was carried out in which 9 replicates per bacteria concentration were manipulated in parallel. The coefficient of variation (CV) was then calculated for each of the assayed *E. coli* concentrations, within the same assay run, as follows: [% CV) = (SD/mean) x 100], in which “mean” represents the average of the 9 values independently obtained for each concentration, and “SD” is the standard deviation of these 9 values. To study the inter-assay variability, 8 independent assays were carried out at different days, using each time freshly prepared functionalised surfaces, solutions and biocomponents. The coefficient of variation (CV) was this time calculated for each of the assayed *E. coli* concentrations, obtained in 8 independent assay runs, as follows: [% CV = (SD/mean) x 100], in which “mean” represents the average of 8 values independently obtained for each concentration, and “SD” is the standard deviation of these 8 values. As shown in the following table 1, the CV was in most of the cases under 10% and in all except one case it was under 15%. This indicates that, in spite of the variability induced by manual handling, the surfaces produces are very reproducible.

Intra-assay variability

E. coli (cell/ml)	Mean	SD	% CV
1,00E+08	0,370	0,028	**7,58**
2,50E+07	0,306	0,034	**11,24**
6,25E+06	0,193	0,029	**14,98**
1,56E+06	0,107	0,012	**11,40**
3,91E+05	0,080	0,007	**8,43**
NO *E. coli*	0,065	0,004	**5,94**

Inter-assay variability

E. coli (cell/ml)	Mean	SD	% CV
1,00E+09	0,461	0,031	**6,75**
1,00E+08	0,363	0,049	**13,63**
1,00E+07	0,271	0,043	**15,96**
1,00E+06	0,153	0,015	**9,52**
1,00E+05	0,084	0,009	**10,16**
NO *E. coli*	0,074	0,008	**10,92**

- **Figure 27: Intra-assay and inter-assay variability**

2.3.4 CONCLUSIONS ABOUT FUNCTIONALISATION

After having assayed a number of gold immuno-functionalisatrion strategies, it was decided that functionalisation by Ab conjugation to SAMs was not adequate for bacteria detection. The best results were obtained on surfaces obtained by (i) Ab random physisorption and (ii) neutravidin physisorption followed by affinity capture on a biotinylated Ab. In both cases, detection ranges of 10^{5-6} to 10^{8-9} cells/ml, LODs of $<10^5$ to $2x10^6$ cell/ml and IC50 around 10^7 cell/ml were obtained using *E...coli* as the model microorganim. In case the selected protocols performed poorly for the IMMUNOLEGIO monoclonal Ab to be immobilised, an additional alternative was optimised, consisting on the physisorption of and anti-mouse Ab, followed by immuno-affinity capture of the monoclonal.

The functionalisation protocols have been optimised step-by-step, and the obtained surfaces additionally characterised. Our results demonstrate that Ab physisorption generates surfaces which are:

- highly stable, remaining unmodified and functional for at lest 10 days in PBS at 37ºC
- reproducible, with a coefficient of variation in coverage (studied by IES) and bacteria detectability (studied by sandwich ELISA) under 10-15%, and
- specific, generating undetectable levels of unspecific adsorption of unrelated bacteria present at titters lower than 10^8 cell/ml, and provided that tha Ab used are specific for the bacteria of interest.

2.3.5 MAGNETIC DEVICES: Validation of the proposed technology

Overview of work and activities performed in WP3 in the M8-M12 period

Our activities during the first year of the project have been covering the definition of a magnetic device and method to measure the magnetic particles concentration and the surface antibody immobilization & evaluation.

Work corresponding to select and test magnetic devices has covered GMR sensors. Two different types of magnetic sensors have been tested: NVE Inc (US) and INESC (P). Both types of GMR devices did not cover expectative regards to required specifications and as a contingency plan last month the group start to explore micro-inductive devices, as a possible alternative.

On the other hand, we optimised several protocols to a good surface antibody immobilisation, which was tested on planar devices simulating different magnetic surfaces.

After six month, alternative task was started and devoted to complement the magnetic sensing methodology to work with micro-inductive devices. In relation to other approaches, inductive devices do not require for the application of magnetic fields during their operation, and show a high compatibility with standard Si technology. These characteristics justify the inclusion of a significant research effort in this technology, in order to achieve the level of maturity of the other sensing strategy. Nevertheless, in these approaches, significant research efforts have been realised to consider “ready for applications to real working devices” especially when they are considered in conjunction with constraints such as the measure of very low (a few) concentrations of MP's and a compact and simple readout electronics.

The work actions in this field were focused on the fabrication and test of suitable sensors models, on the definition of optimal sensor architectures and on the assessment of the technology to use for the sensors realization.

Overview of the secound years activities

The Biological part was finished and completed within the first year. In the second year the newly defined tasks of the sensor development were performed.

The second years work however was extended by following new tasks to deal with the unplanned need for sensor development (see also section 1.3 of this report):

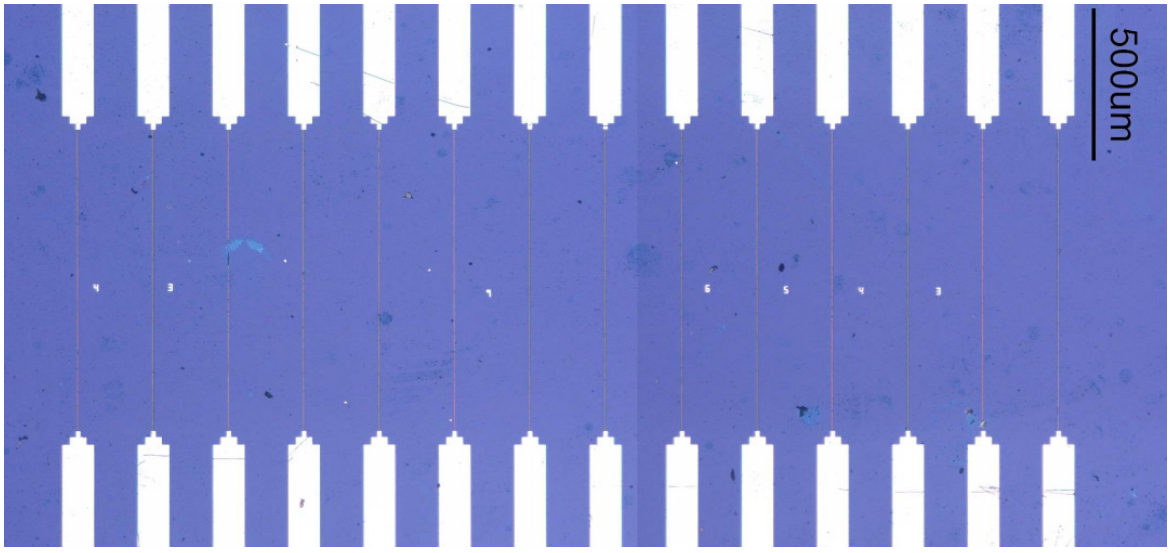
- Evaluating existing prototypes of Micro-coil type (micro-inductive) sensors for the detection of super-paramagnetic beads. (RTD: CNM)
- Evaluating the detection limit micro-inductive type sensors using existing prototypes of micro-inductive sensors. (RTD: CNM)
- Develop and fabricate a micro-coil sensor chip design suitable for the IMMUNOLEGIO application. (RTD: CNM)
- Develop a special encapsulation for the newly developed micro-inductive sensor protecting the sensor and the electric contacts while leaving the sensitive area of the sensor needs is accessible for the measurements and allowing a precise fit of the Test Stick to be measured. (RTD: CNM, MFKK; SME with significant role: BVT).
- To design and fabricate new Test Stick appropriate for new sensor design (RTD: CNM, MFKK; SME with significant role: BVT).

Sensor development is a time and resource consuming task, even if there is significant experience available to build on. For these reason an extension of the project runntime was requested, as the follow-up task cannot be started before the completition of the the sensor development. In following the results of the sensor development are discussed.

Discussion of the results: GMR (M8-M11)

GMR from INESC

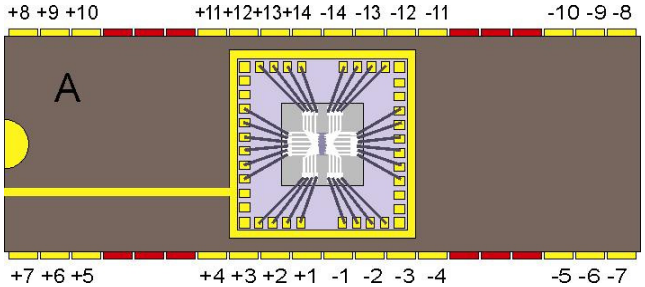
Figure 4 shows two devices were obtained from INESC (Portugal). Each device consists on 14 parallel strips of GMR-material contacted by aluminium paths.



- **Figure 28: General view of the area where the devices fabricated at INESC. (Lisbon. Portugal) are placed. There are 14 devices numbered from right to left.**

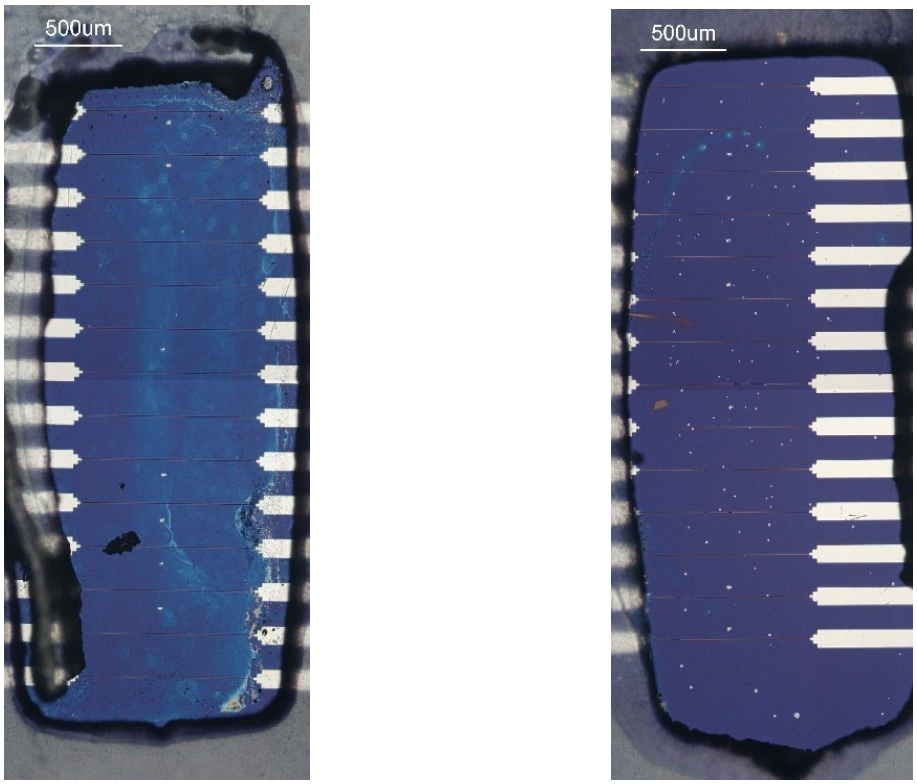
Each strip is 995µm long and 3µm wide. This is equivalent to put 332 squares 3µm side, one next other. Each strip is separated 250µm from each other. So only (995x3/2,5x105) =12x10-3 (1.5x10-4%) of the surface is active.

Both chips have been encapsulated and wire-bonded to a substrate DIL-40 (Fig. 5)



- **Figure 29: Drawing of one chip after encapsulating. The number on each pin corresponds to the number of each GMR.**

To protect the wire-bonding the cavity was filled with a negative photo polymer and was processed to led the active area free of polymer.



- **Figure 30: Image of both devices after encapsulated with polymer. By way of a photolithographic process the active area remains free of polymer.**

Measurements

Initial measurements on device A give the following results:

device	measured with HP 34401A	measured with HP 4145A
GMR #1A	11,0 kΩ	10,923±0,005 kΩ
GMR #2A	11,2 kΩ	5,05 / 11,11 kΩ
GMR #3A	7M / 2,3 MΩ	6,33M / 1,22 MΩ
GMR #4A	11,4 kΩ	11,34 kΩ
GMR #5A	0,5M / 1 MΩ	0,23M / 0,72 MΩ
GMR #6A	24,7 kΩ	10,560±0,005 kΩ
GMR #7A	0,58M / 63 MΩ	0,30M / 14,9 MΩ
GMR #8A	11,5 kΩ	11,435±0,005 kΩ
GMR #9A	11,7 kΩ	11,080±0,005 kΩ
GMR #10A	10,9 kΩ	10,667±0,005 kΩ
GMR #11A	11,5 kΩ	11,173±0,005 kΩ
GMR #12A	26,2 kΩ	26,738±0,005 kΩ
GMR #13A	12 MΩ	11,111±0,005 kΩ
GMR #14A	11,3 kΩ	10,983±0,005 kΩ

There are 8 values very close with a value of 11,3±0,3 kΩ, or 14±6 kΩ if including all the values below 100kΩ

The initial measurements gave as a result that device B was very bad, almost all the GMR show a high resistance value, probably due to a bad contact with aluminium, as it can seen in next table:

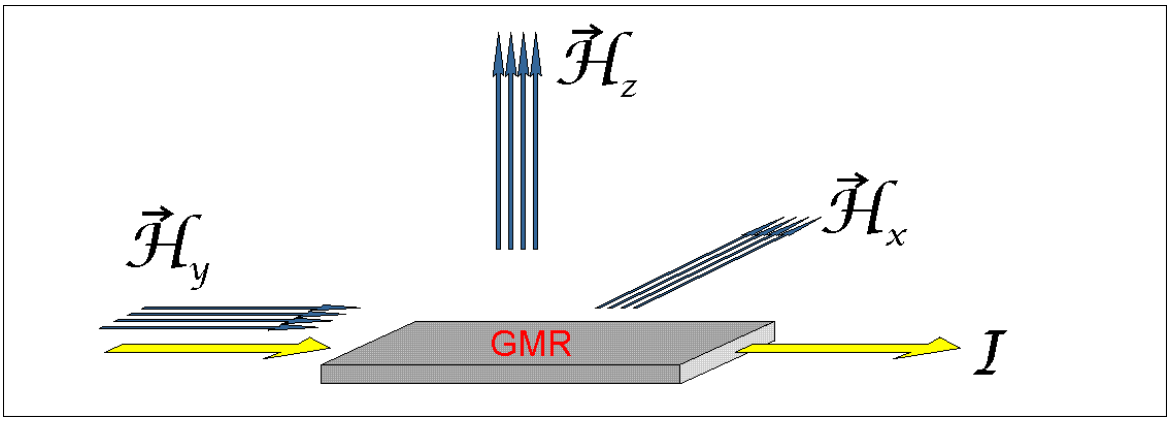
GMR #1B	3 MΩ
GMR #2B	open circuit
GMR #3B	6 MΩ
GMR #4B	open circuit
GMR #5B	6,5 MΩ
GMR #6B	43,8 kΩ
GMR #7B	0,96 MΩ
GMR #8B	2,4 MΩ
GMR #9B	31,32 kΩ
GMR #10B	27 MΩ
GMR #11B	0,5 MΩ
GMR #12B	5,3 MΩ
GMR #13B	25 kΩ
GMR #14B	12,12 kΩ

MEASUREMENTS ON GMR #2A

This device shows an asymmetric behaviour. The resistance was 11,11kΩ for V= -6,5v and 5,05 kΩ for V= +6,5

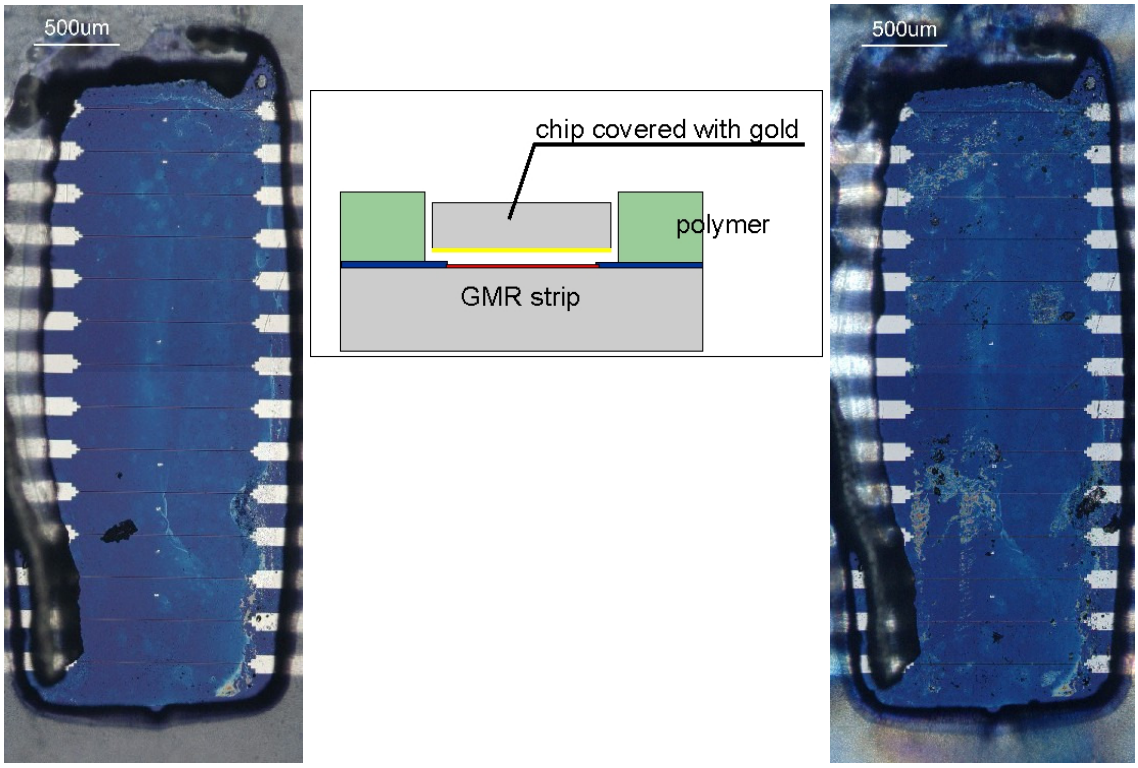
MEASUREMENTS ON GMR #8A

Measuring GMR #8A the resistance is 11,44kΩ at rest and 10,98kΩ in presence of a magnetic field perpendicular to the device but parallel to the device surface (X), 11,07 kΩ with the field oriented in the Y direction and 11,11 kΩ in the Z direction (Fig. 7).

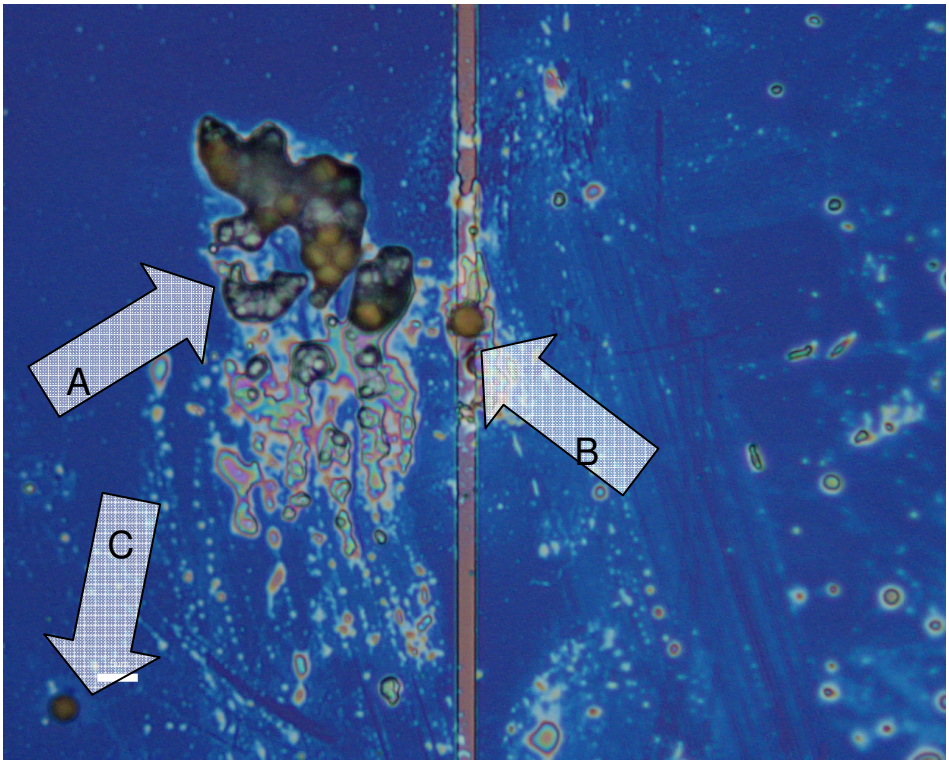


- **Figure 31: The resistance change depends on the magnetic field orientation.**

Figures 8 and 9 show the image of GMRs after place a gold chip with capture MP against the magnetic device. In photograph 9 different situations of several MP can be observed. From 3 different MPs location only B position is able to be quantified.



- **Figure 32: Aspect of chip-A before (left) and after (right) the first set of measurements with gold chips with particles.**



- **Figure 33: Particles attached to the device due to a transfer. A) Cluster of particles. B) Particle well placed to be detected. C) Particle to far from de GMR strip to be detected.**

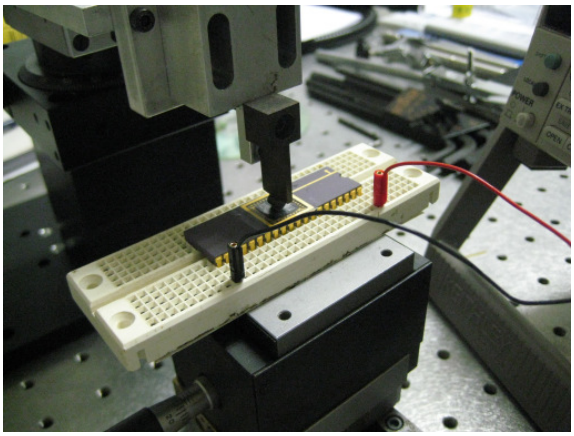
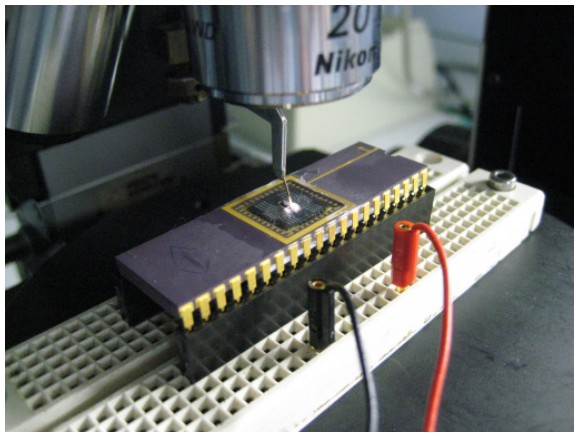
Assuming that one particle placed on the GMR strip can shield 100% of the magnetic field on an area equivalent to the particle diameter (3,0µm×3,0µm), then this particle will increase the resistance of the whole GMR strip in 1,4Ω. That is,

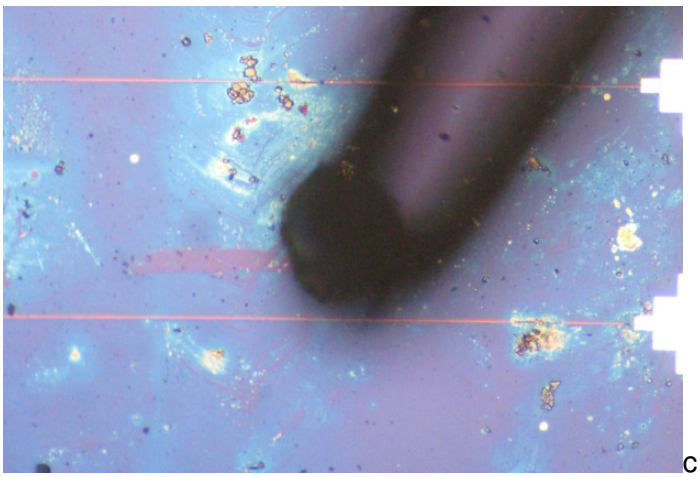
$$\frac{\Delta R_p}{R_{GMR}} = \frac{1}{R_{GMR}}\left(R_{GMR}(B=0) - R_{GMR}(B\neq 0)\right)\times\left(\frac{3}{995}\right) = \frac{11{,}44-10{,}98}{10{,}98}\times\left(\frac{3}{995}\right) = 1{,}3\times 10^{-4}$$

$$\Delta R_p = 1{,}4\Omega$$

SET-UP

Next figure is showing the GMR measurements set-up

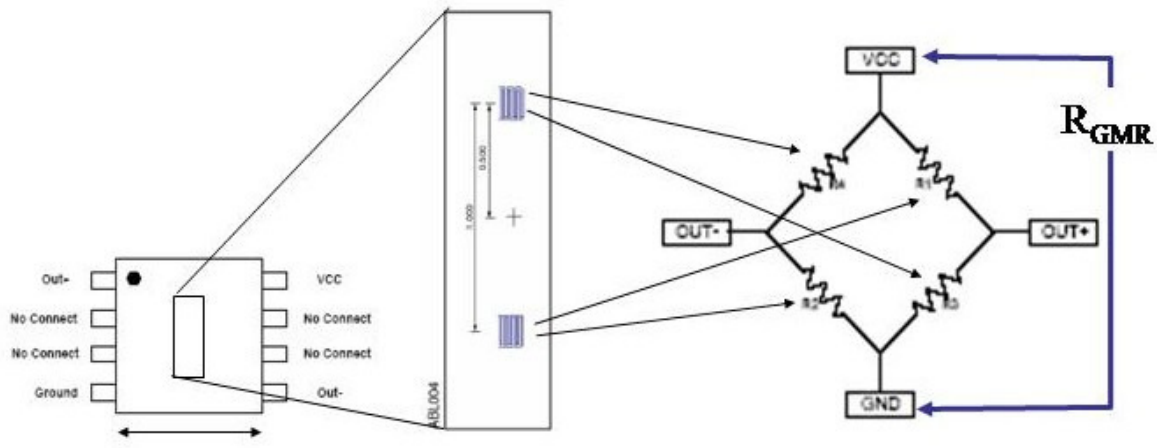

a


b

c

- **Figure 34: Different photograph showing measurements set-up: a,b) DIL-40 position under microscope, and c) magnetic spot moved between GMRs.**

GMR from NVE Corporation

Some devices were bought to NVE Corporation to evaluate the possibility to use them as particle sensor. Each device came encapsulated and configured in a Wheatstone bridge (Fig. 11).



- **Figure 35: Scheme of GMR NVE measurement by Wheatstone bridge.**

Measurements

The initial tests were made measuring the Wheatstone bridge as a unique resistance (between out+ and out-). In Fig 12, a set-up scheme is shown.

GMR alone:

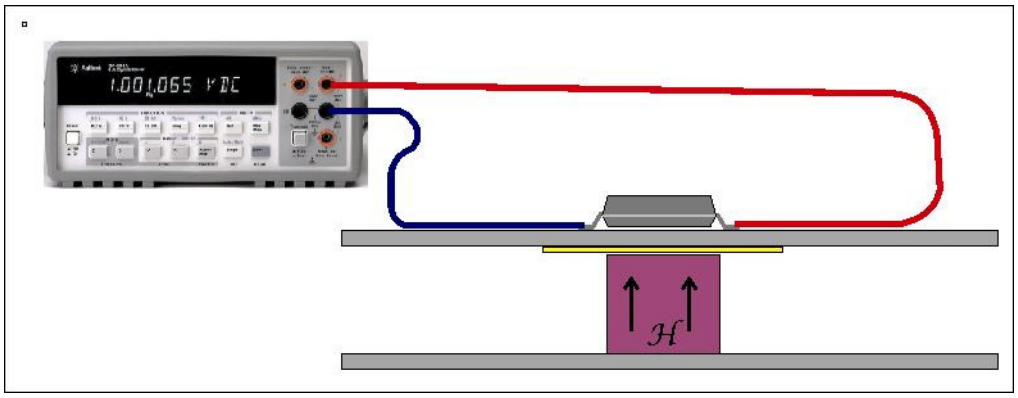
6.294,0 ±20,6 Ω

with magnet and plastic strip without MNP:

5.789,2 ±17,9 Ω

with magnet and plastic strip with MNP

5.823,1 ±20,6 Ω



- **Figure 36: Scheme of the set-up for the measuring.**

The particle concentration was about 104 on 4mm2.

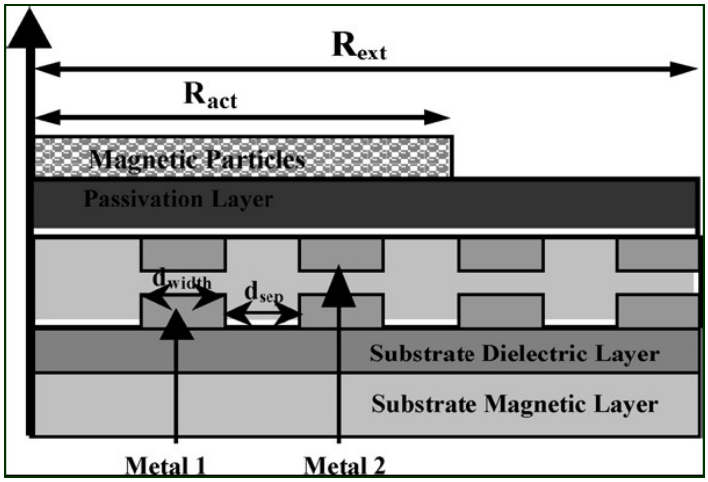
Discussion of the Micro-inductive (MI) devices

Different groups have reported the development of magnetic immuno-biosensors using inductive devices. The detection principle of these systems is based on the inductance changes of a solenoid due to the presence of a certain amount of MP's in the sample under analysis, which is located in the nucleus of the device. Larsson et al. have developed a compact system which uses big silica particles in addition to the MP's. The surface of both the MP's and the silica particles is functionalised with an antigen specific to the analyte to be detected. Then, the interaction between the analyte and the particles determines the fixation of the MP's on the surface of the silica particles, through the formation of antigen–analyte–antigen sandwich complexes. Further improvement of the device sensitivity can be obtained by scaling the device dimensions down to the micrometer and even submicrometer range. This is achieved by the use of processes derived from the standard Si integrated circuits (IC) technology.

In this framework, this work investigates the integration of inductive-based devices in standard Si technology, for the development of low cost and high sensitivity sensors for biodetection applications. This has several potential advantages over the previous developments based on macroscopic coils. First, the decrease of the sensor size would lead to a significant decrease of the volume of the sample under analysis, with a consequent decrease of the reagents cost. This can also allow a decrease of the measuring time, as well as an increase of the device sensitivity, with a potential reduction of the minimum number of detectable MP's.

When compared to the traditional solenoids, the integration of the coils in Si technology has additional potential advantages related to the fabrication cost, yield and reproducibility of the planar devices. Moreover, the use of Si IC processes opens interesting perspectives for the development of more complex devices, such as sensor matrices for the simultaneous analysis of various components. In comparison with other kinds of magnetic biosensors already developed using micrometer – and even submicrometer – resolution processes, the proposed microinductive devices are characterised by a very high simplicity, as well as a full compatibility with standard Si IC technology and materials. These features are especially relevant for the development of low cost devices. On the other hand, the operation of the inductive devices does not need the application of magnetic fields, which simplifies the design of the device and its integration within the whole system.

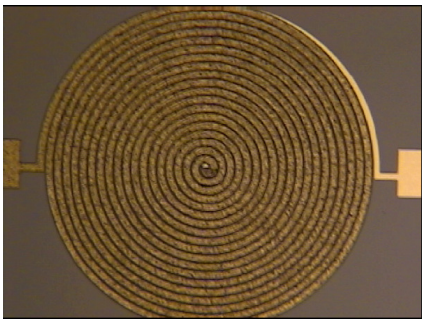
In principle, the maximum magnetic field generated by the coil is located at the centre of the coil, and decreases as the distance to this point increases. According to this, a maximum change of the device inductance is to be expected when a MP is located in the central region of the coil. However, the previous simulations performed for these devices revealed that, for a given density of MP's, the response of the device is higher when the particles are allowed to distribute through the whole coil surface (Ract = Rext in Fig. 13). A further increase of the active surface beyond the coil rings does not lead to a concomitant increase of the sensor response. This is related to the distribution of the magnetic field induced by the coil, which vanishes in the region of the coil plane external to the rings (R > Rext).



- **Figure 37: Half schematic cross-section of the device, referred to its centre (indicated by the vertical symmetry axis), with the different design parameters.**

Fabrication of the integrated coils (1st prototypes):

The devices have been fabricated using processes compatible with standard CMOS technology. The starting wafers were 100mm diameter (1 0 0) Si, and the coils were fabricated with two levels of 1.5 µm thick Al metal tracks, with a lateral separation between metal tracks of 5 µm. For the devices fabricated without magnetic substrate, the first metal level has been defined onto a 800 nm thermal oxide layer (represented by the dielectric substrate layer in Fig. 1). The two metal levels are separated by a 1.5 µm thick plasma-enhanced chemical vapour deposition (PECVD) SiO2 layer. Finally, the upper metal tracks are passivated with a 400 nm SiO2 layer and a final 700 nm Si3N4 coating (both grown by PECVD). The surface of this nitride layer constitutes the active area of the device where the MP's will be fixed. A radius Rext = 500_m has been selected, and three different track widths of 5, 20 and 500 µm have been used. This leads to devices with 50, 19 and 9 turns, with nominal inductance values of 105, 525 and 3500 nH, respectively. According to the previous section, the 50 turns coils are the devices where a higher sensitivity to the presence of the MP's is to be expected. Fig. 14 shows a microscopic image of one of the coils.



- **Figure 38: Microscopic image of the coil fabricated with 2 metal levels, 19 turns.**

Characterisation of the devices ((1st prototypes)

The electrical characterisation of the devices was performed with an Impedance Analyser HP model 4192 A, in the frequency range 0.7–6 MHz. These measurements give values of L which agree with those predicted by the simulation. On the other hand, the presence of the magnetic layer below the coils determines a significant increase of the inductance value which, for the measuring conditions used in this work, is similar for both NiCo and Fe layers. According to this, the devices fabricated with the magnetic substrate layer have a higher value of the quality factor Q. In agreement with the results predicted by the simulation, the increase of L is higher for the coils with more turns, with a relative increase ΔL/L of 39% and 7% for the coils with 50 and 19 turns, respectively.

The deposition of the MP's on the device surface is carried out with a micropipette: a 0.5 µl drop of a solution containing a certain density of MP's is deposited on the coil surface, where

the MP's remain fixed after evaporation of the solvent (usually D.I. water). The number of MP's on the surface is determined by counting them from an optical microscopic image.

The measurements revealed that only the coils with 50 turns, and those with 19 turns and with a magnetic substrate layer, shown experimental response to the presence of the particles. In agreement with the simulation, the coils with the highest number of turns (N= 50) exhibit the highest sensitivity to the presence of the MP's. According to this, the analysis of the devices has been focused on these coils.

Table 1 summarises the experimental results of the inductance variation ΔL (in absolute value) of the coils fabricated with 50 turns, as a function of the estimation of MP's, and for different kinds of substrates. As shown in the table 1, does not seem to correlate with the density of MP's. For the devices without magnetic substrate, the minimum density of particles determining an observable change of inductance is of the order of 250 MP/mm2.

The presence of the magnetic layer in the substrate region leads to a decrease of this parameter down to 143 MP/mm2. This corresponds to less than two particles detected in an equivalent surface of 100 µm×100 µm. According to the active surface area (0.785mm2), this implies the detection of about 110 particles distributed over the whole coil surface. These data also indicate a higher reproducibility of the experimental response of the coils fabricated with a magnetic substrate. These devices also tend to exhibit a smaller uncertainty on the measurement of ΔL (between 3 and 7 nH) as compared to the ones without magnetic film. This agrees with the increase in the quality factor of the coils Q with the implementation of the magnetic substrate.

Table 1. ΔL (in absolute value) vs. density of MP's measured in the coils fabricated with 50 turns, and with different types of substrate.

Density MP's (mm^{-2})	ΔL (nH)		
	No magnetic substrate	Fe substrate layer	NiCo substrate layer
143		20 ± 5	
255	17 ± 4		
313	162 ± 22		
315		23 ± 3	
320		33 ± 7	
460			69 ±5
515			79 ± 5
570			87 ± 6
580			67 ± 7
1030			67 ± 3
1390	16 ± 6		
1600			29 ± 7
2340	22 ± 6		
2900	50 ± 9		
3500	45 ± 18		

These data also indicate a higher reproducibility of the experimental response of the coils fabricated with a magnetic substrate. These devices also tend to exhibit a smaller uncertainty on the measurement of ΔL (between 3 and 7 nH) as compared to the ones without magnetic film.

This agrees with the increase in the quality factor of the coils Q with the implementation of the magnetic substrate.

New and final design of the micro-coil for IMMUNOLEGIO

After the verification of the parameters, as described above, a final sensor layout was developed based on the best performing micro-coil design. The test on the first prototypes showed significant influence of temerature to the coil impedance. The final sensor desing features two micro coils on one chip to allow differential measurement compensating for temerature and other influences. The connection pads were placed further away from the coils which are the sensitive part. This is necessary to allow a good access with the Test Stick to the sensitive part.

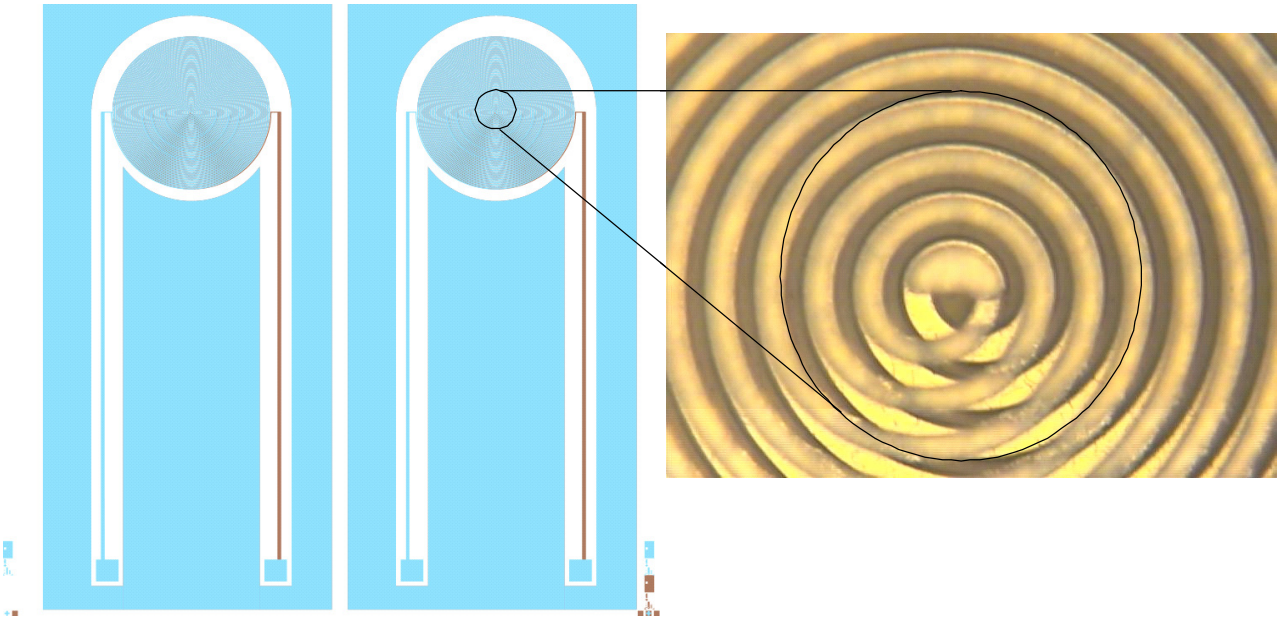


Figure 39: Right: Design of the new differential micro-coil with extended connection wires making place for the alignment of the Test Stick. This sensor chip layout was specifically designed for IMMUNOLEGIO. Left: Microscope image of the final micro-coil after fabrication.

The final sensors were validated in laboratory achieving similar specifications as the selected first prototype design. However the laboratory test were made without encapsulation and with standard encapsulation.

2.3.6 Development of Sensor encapsulation

In order to use a micro-chip based sensor for biosensing applications one of the key issues is a proper encapsulation of the sensor. The initial concept is shown in the figure below, effectivly visualizing the planned system, even though the actual realization developed is quite different.

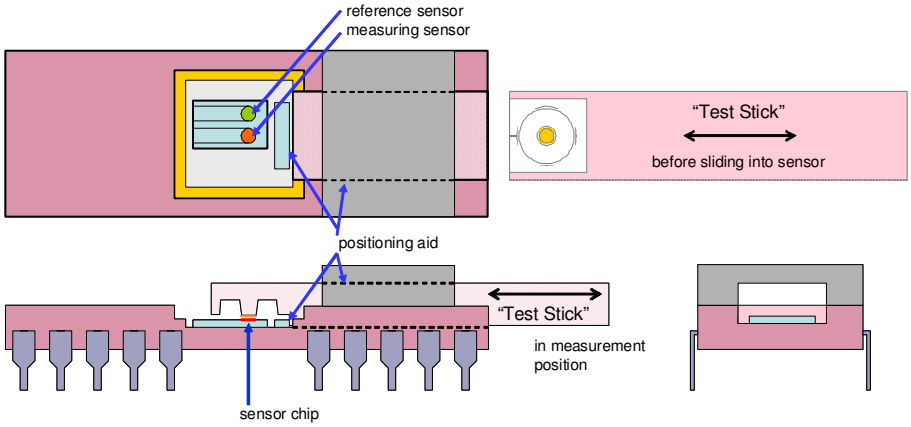


Figure 40: Conceptual drawing of the first sensor encapsulation concept.

The encapsulation has to full fill 4 tasks:

1. protect the micro-chip structures from damage by external influences;
2. Establish electrical connenctions between the micro-chip and the electronics (i.e. electric interfacing)
3. Enable the physical access to the sensitive areas by the media to be measured,
4. To provide a precise guidance for the Test Stick, to allow an exact match of the Test Stick's active area and the sensitive area.

For technological reasons the sensor encapsulation and the sensor housing was developed as separate modules. The sensor encapsulation targets to fullfill mainly the first three requiremnts, and will only partly provide guidance for the Test Stick. The development of the encapsulation was a joint effort of MFKK and BVT.

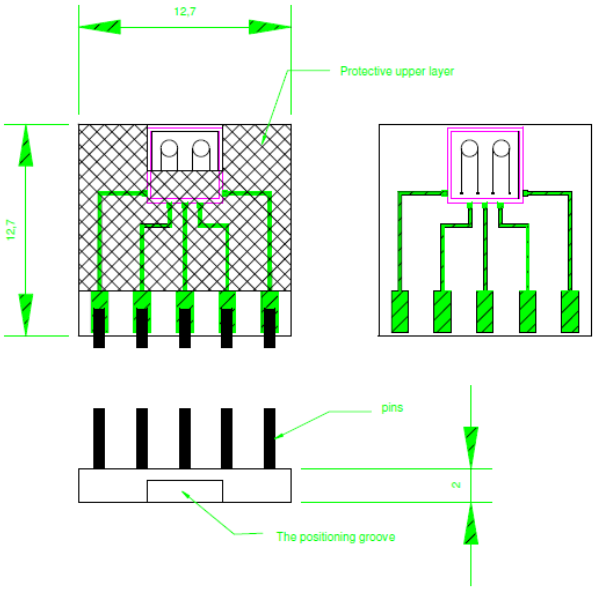


Figure 41: Final design of the Sensor encapsulation fulfilling the three requirements for encapsulation. The electric connections of the sensor chip are wire bonded and lead out to solderable pads.

The encapsulation is fabricated on a ceramic base with thickfilm printed wiring. The Senor chip is precisly aligned and fixed by glue to the base. The electric connections are wirebonded to the thickfilm wiring. The electric wiring and the sensor-chip is protected from the top by a glass structure leaving the sensitiva areas of the sensor open. The glass structure is precision aligned only in the length direction (perpendicular to the axis built by the centerpoint of the two micro-coils).

2.3.7 Development of Test Stick

The Test Stick desing was developed by MFKK in cooperation with CNM and BVT. The test Stick has to fullfill the following features:

1. A defined active (functionalized) area in the size of the micro-coil diameter, which is the sensitive area of the sensor for the immobilization of the bacteria to be detected;
2. A technologicaly equivalent but (immunologicaly) passive area oposit the refernce coil;
3. It has to provide reference surfaces or points to support precise mechanical alignement;
4. A long grip for the fingers alowing safe handling by the operator, without touching the active spot.



Figure 42: Final design of the IMMUNOLEGIO Test Stick featuring two active spots, which exactly match the sensitive areas of the sensor.

The prototypes of the Test Stick were fabricated on a silizium substrate. The active and the reference area were defined by a spot of gold layer, which was deposited by spattering structured by a litograficaly created mask. The final design and the dimensions can be seen in the above figure. These fabrication method was considered to be best availabe for prototyping within the consortium. The Test Stick prototypes were fabricated by CNM.

For the final product following technologies were suggested to realize the same function at lower cost:

1. Using a streptavidin coating instead of gold to define the active area for functionalization.
2. Using cheaper base material, such as glass or hard polymer
3. There is the possibility to have different precision thus materials and technolgy for the fabrication of the active part and the handle.

2.3.8 Development of the sensor housing

The sensor housing is planned to fullfill following functions:

1. Incoperating the encapsulated sensor and the sensor electronics in a housing. The Prototype was designed in adition to be fully magnetically shielded.
2. Providing a precise guadance for the Test Stick alining with the sensor.
3. Ease of use by the operator

The Sensor housing was developed by MFKK and BVT in close cooperation.

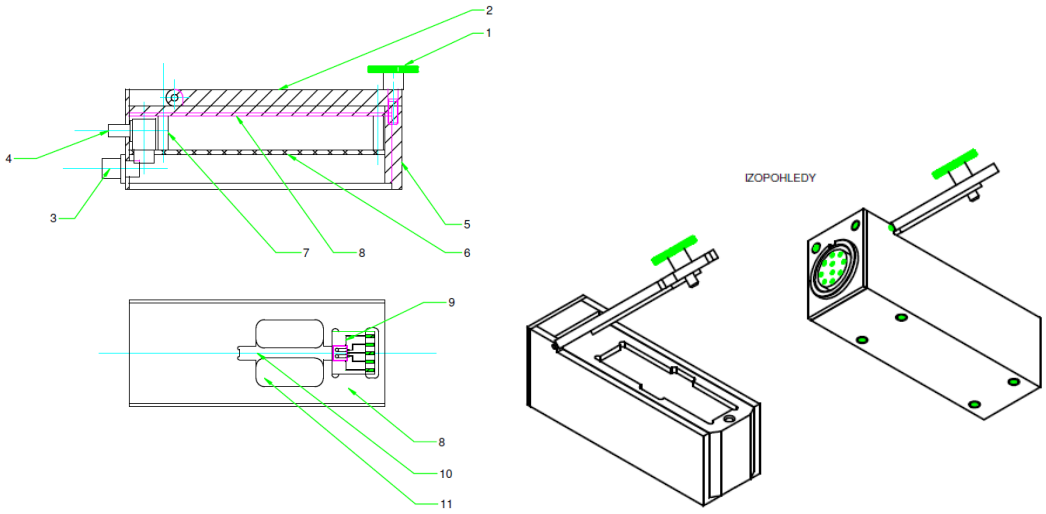


Figure 43: Design of the sensor housing prototype incorporating the encapsulated sensor and the sensor electronics, and providing a precision fit for the Test Stick with a closable top.

The sensor housing was fabricated out of solid iron to provide best magnetic shilding. The precision fit for the Test Stick was foreseen under a swing cover. The encapsulated sensor is precisly aligned and fixed by gluing to the housing. The sensor electronics was integrated within the housing with the electric conectors faced to the backplane of the housing.

The sensor housing desing was considered to be most robust without any unnecessary risk for the validation of the concept. Never-the-less a concept study was made for a more automated and more compact sensor housing concept, which may be a thus commertially more viable option incase the sensing concept and the electronics measurement are fully validated and stable. The figure below ilustrates the the concept study prepared for commertial sensor housing with automated Test Stick handling.

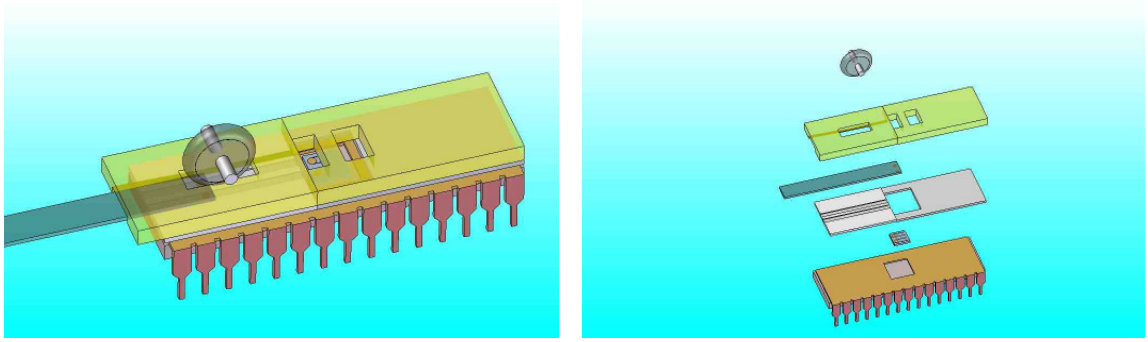


Figure 44: Conceptual design of a more advance sensor housing, this is a possible desing for later commercialization featuring automated Test Stick movement.

2.4 Workpackage 4. "Development of the Software"

2.4.1 Development of a graphical user interface

In this workpackage the development of the user interface and the software analysis are included. According to the proposed schedule the development of this task was started at M8. A significant part of the user interface is dependent on the applied measurement system, so it will be modified in the following months.

As it was mentioned above, the Immunolegio system is intended to include a pocket PC for process control and data acquisition. This is the visual user interface that displays important messages during the whole process and the results of the measurement. The software has been developed in Microsoft Visual Studio and an embedded Pocket PC emulator has been used to visualize the program. In the following the first draft of the user interface with screenshots of some phases will be presented.

In the main menu one can choose of three actions: starting new measurements, data management, and measurement settings. The software has been developed in a manner that it provides an easy-to-use platform. The whole process (including sample preparation, adding marker and incubation, immobilization) can be easily carried out following the program's instructions. All the steps that the user has to execute are clearly illustrated (see Figure 45 and Figure 46), so that the instructions are clear and definite.



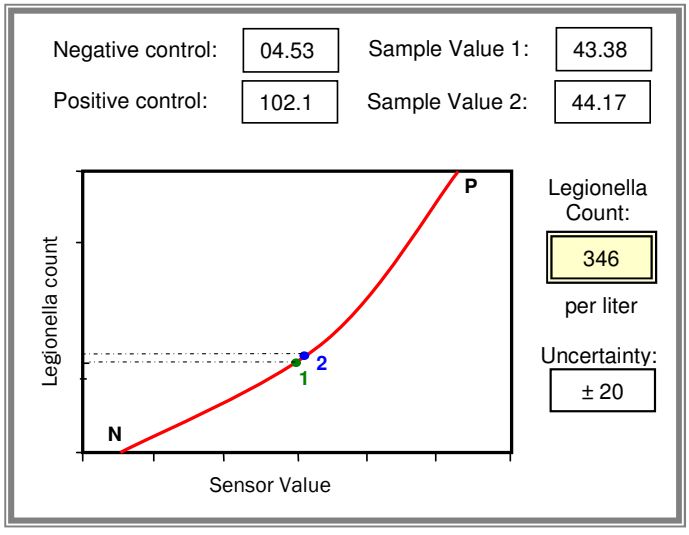
- **Figure 45: Initial screenshots of the measurement software**

After the measurement is done the result (Colony Forming Units, CFU/l) can be seen immediately and details can be displayed with the "View Report" command.



- **Figure 46: Screenshots during the measurement**

The development of the measurement analysis was started, but shortly was pendent due to the lack of information about the magnetic sensor output signal. The development of the signal processing software will be started right after the microinductive sensors are extensively tested. The visualization of the measurement results will have a form like Figure 47



- **Figure 47: Output form**

2.4.2 Development of a configuration and control software

The graphical user interface for the high level control of the IMMUNOLEGIO device and for measurement data management was developed within the first year of the project. In the second year of the project the software development was focused on the low level hardware control algorithms and the realization of the process control in the micro controller. The controller software was designed in a way to allow also a direct on divice control of the device functions in addition to the control possibilities though the graphical user interface.

The software of the micro-controller on the controller board implements following modules:

- Module for driving the on device LCD display
- Module for the handling of the on device direct control keys
- Module for the step motor control of the XY-actuator with absolute positioning feature
- Module for the two-directional PWM control of the 3 peristaltic pumps
- Module for the PC communication (through serial or USB interface)
- Module for downloading (from PC), storing and executing of the process sequence to be executed on the IMMUNOLEGIO hardware.
- Module for controlling the sensors signal generator
- Module for reading and preprocessing the sensor signal

The operation sequence of the automated sample preparation can be individually configured by defining the processing protocol step-by-step. Currently following base steps are implemented to define the processing sequence:

- Display message on LCD display (to inform or to instruct the user)
- Wait for user input (key press to confirm a specific action or instruction)
- To calibrate the step counter of the X axis
- To calibrate the step counter of the Y axis
- To move on the X axis to an absolute position in steps
- To move on the Y axis to an absolute position in steps
- To wait a given amount of time (defined in seconds)
- To operate Pump 1 (pipetting) forward or backward for a specified time (in milliseconds)
- To operate Pump 2 (fresh water pump) for a specified time (in milliseconds)
- To operate Pump 3 (filtration pump) for a specified time (in seconds)

The pumps have predefined pumping rates defined in ml/s. In the current realization the pump speed is hardcoded in the microcontroller program. However, the pump driving module was developed in a way to enable variable pump speeds if required.

To enable the user to edit the automated sample preparation procedure, e.g. to adapt it to an existing protocol MFKK developed PC based procedure editor. The procedure editor features also buttons for the manual control of the device functions, allowing the user to test on positions or required pumping times.

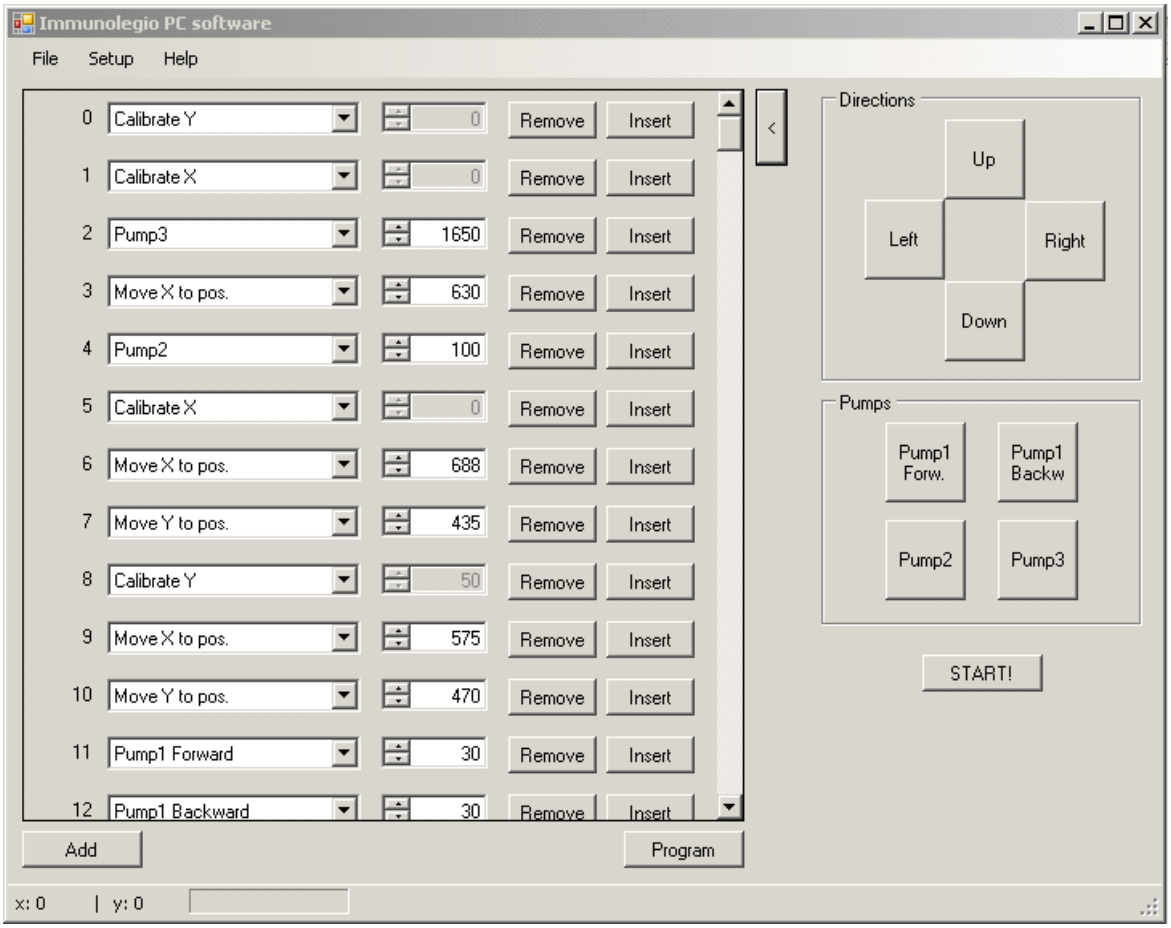


Figure 48: Screen shoot of the IMMUNOLEGIO procedure editor.

Procedure editor includes functions to save and reload the defined procedure to PC, as well as for downloading the procedure to the IMMUNOLEGIO device controller. Also this is not the main user interface used for the actual operation of the device there is the possibility the start the last downloaded procedure for testing purposes.

A complete procedure is exemplary listed in Attachment 1 An overview of the IMMUNOLEGIO procedure is discussed in the next section.

2.5 Workpackage 5. "Hardware Development"

After having the final biological procedure defined at M12 the hardware development concept had to be redesinged to the final procedure, requiring more then the initially planned mixining and incubation device. So it was decided to develop the IMMUNOLEGIO hardware on a completely new basis. The developed concept and the hardware are presented as follows.

2.5.1 Automation Concept for Sample Preparation and hardware description

In follows the basic functional units of the developed hardware design for the automation of the sample preparation will be discussed, an overview is shown in Figure 49.

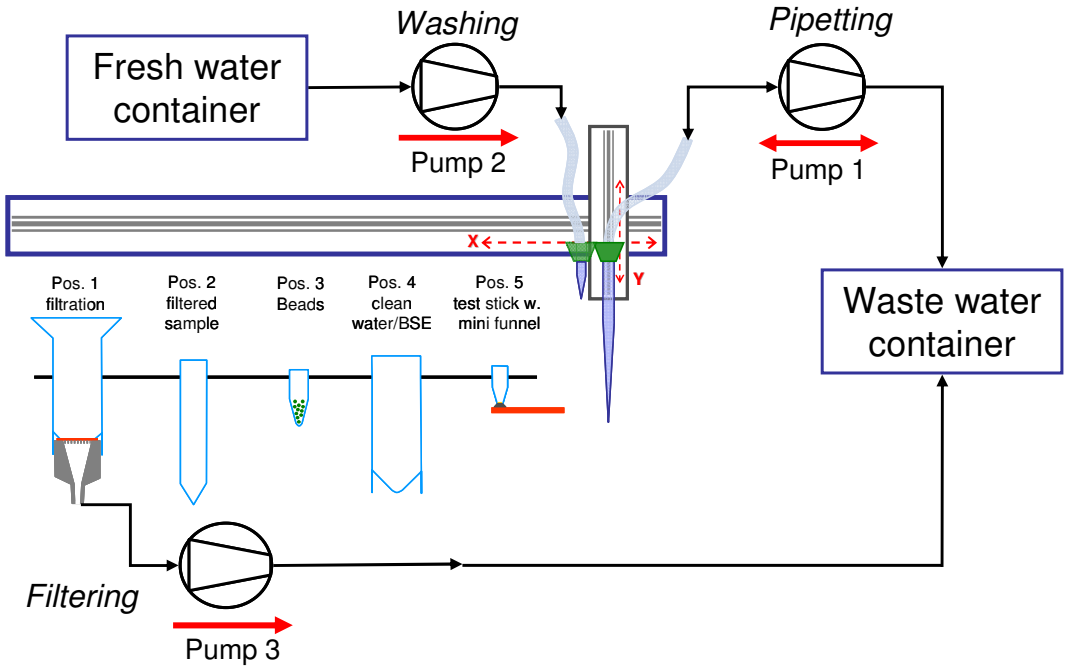


Figure 49 Conceptual drawing of the operative units of the automated sample preparation system

Overview of the procedure:

- **Step 1:** Filtration of the original 1000 ml sample and resuspending in 10 ml.
- **Step 2:** Transfer resuspended sample (10 ml) into sample concentration container.
- **Step 3:** Pipetting of the beads and adding to the sample.
- **Step 4:** Incubation for (bead - bacteria) immuno-reaction, while keeping the beads in suspension. Then washing to selectively clean the sample with the help of a magnetic fixation, including the concentration of the sample down to about 0.1 mL.
- **Step 5:** Cleaning/changing pipette tip, and pre-filling the pumping tube with clean water to achieve a more precise pumping.
- **Step 6:** Transfer of concentrated sample from sample preparation vessel to the Test Stick (mini-funnel) by precision pipetting.
- **Step 7:** Incubation for immobilization on the active surface of the test stick. Support manual steps in washing and drying the Test Stick.
- **Step 8:** Cleaning and disinfection of the device (non disposable parts).

Remaining Manual Steps

The automation procedure was developed in a way that manual actions are only necessary at the beginning and at the end of the process. This consists of steps which cannot be automated, or which were considered to be not worth to be automated because of an unreasonable increase in overall device costs.

Based on the developed sampling procedure the automation hardware was developed and the construction plans were worked out using the Pro/E Engineering CAD tool. The figure below shows a 3D skeleton view of the construction, which was generated from the plans. The detailed plans of the pre-competitive prototype hardware are transferred electronically to the SME partners. As it can be seen in the figure, the device integrates tree peristaltic pumps (red and brown), the electronics box (black), the fresh water and the waste water containers, as well as the linear actors. The the CAD design of the manufactured prototype can be seen in Figure 50.

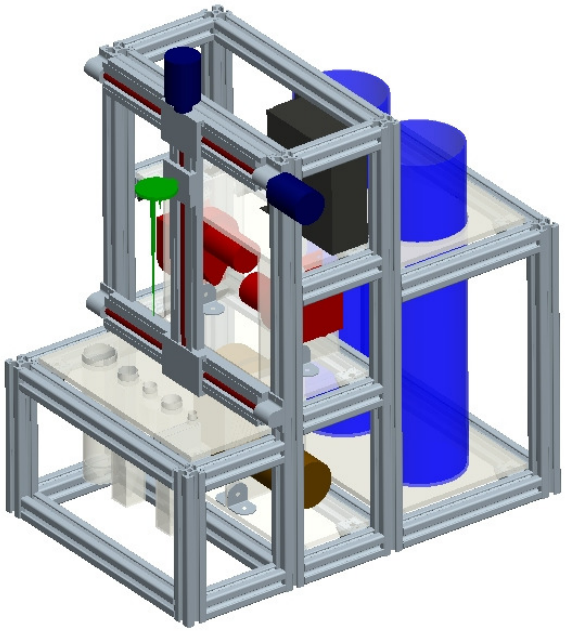


Figure 50 3D skeleton view of the construction

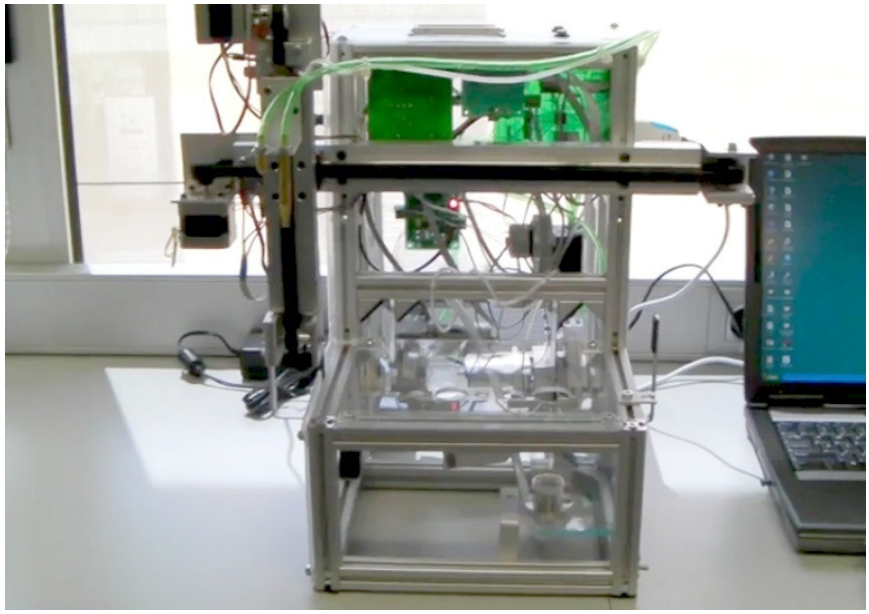


Figure 51 Photo of the IMMUNOLEGIO prototype

The system was planned that manual steps are only required at the beginning and at the end of the automated procedure, allowing the user to deal with other tasks in between.

Manual Steps required in preparation of the automated procedure:

- Assembling filter, placing containers and “Test Stick” into the device
- Filling original sample (1L) into filtration funnel continuously during the filtration process.

Manual Steps after automated procedure is finished:

- Adding Disinfection fluid (this could also be done at the beginning of the process).
- Removing “Test Stick”, washing and drying
- Placing “Test Stick” into sensor for detection

2.5.2 Associated electronics development

The electronics can be divided into two parts. The sensor electronics was developed specifically for the developed micro-coil sensor, as well as the control electronics, which was dimensioned for the current device. For the driver electronics for the pumps and for the XY pipetting actor ready electronics designs were used, to avoid unnecessary development costs.

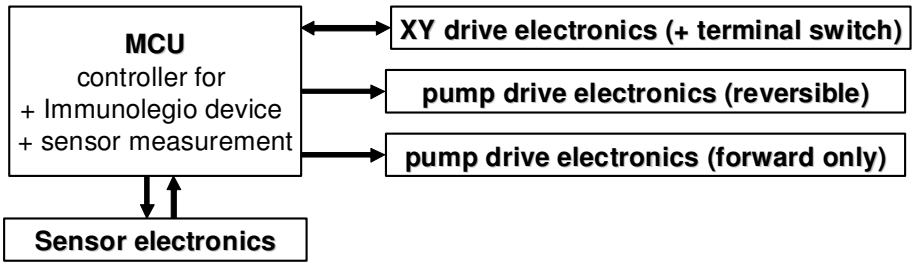


Figure 52 Illustration of the functional modules of the Automated Sample Preparation Equipment.

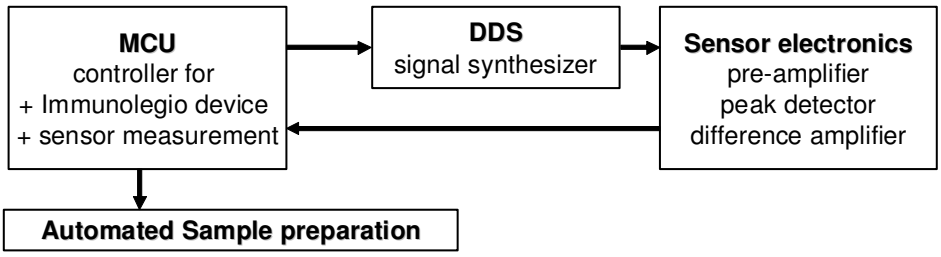


Figure 53: Blockdiagram of the fuctional units of the sensor electronics.

The schematics and the PCB layout plans of the developed electronics can be found in Attachment 2. The schematics for the step-engine driver electronics and for the PWM driver electronics is based on schematics published at http://make.rrrf.org/ under general public license. All other electronics are own development.

2.5.3 Step by step description of the automated procedure

- **Step 1:**
  - User fills up filter funnel
  - Filtration pump running (Pump 3)
  - User fills remaining sample into funnel (continuously)
  - Filtration pump is stopped when funnel is empty
  - Drain tube below filter is closed
  - Filter filled up with 10mL clean water (Pump 2)
  - Procedure for re-suspending bacteria from filter
- **Step 2:**
  - Sample +→ empty vessel
  - Pipetting sample from Pos.1
    for transfer to Pos.2 (Pump 1)
  - Fill of sample into container at Pos.2
    (~ 10mL of concentrated sample)
  - *Maybe: a change of pipette head*
- **Step 3:**
  - Beads +→ Sample
  - Pipetting beads from Pos.3
    for transfer to Pos.2 (Pump 1)
    (~ 1mL diluted bead suspension)
  - Fill beads into Sample at Pos.2 (~ 11mL sample + bead)
- **Step 4:**
  - Incubation of the sample
    for x minutes (~30 min.) with
    slight mixing to avoid sedimentation of beads
    → immuno-reaction

- Activating magnet to secure beads in vessel
- Pumping remaining sample water away
  (Pump 1 to waste water container)
- Deactivating magnet
- Filling clean water (Pump 2 from clean water container)
  to resusped bacteria marked with beads (→ washing)
- Repeating washing (2x), final resuspend in 100µL

- **Step 5:**
  - Clean pipette at Pos.5
    in clean water (or BSE) vessel
    Alternatively: change of pipette head
  - Pre-fill pipette with some clean water (Pump 2)
  - Create small air gap (Pump 2)
    (*To be able to pipette the 100µL sample more precisely*)
- **Step 6:**
  - Pipette concentrated sample
    (~100µL) to transfer to sensor
    (Pos.2 → Pos.5)
- **Step 7:**
  - Filling concentrated sample
    (~100µL) to micro-funnel for
    immobilization on the “Test Stick”
  - Incubation time (~30min)
  -
  - Followed by manual steps
    for washing, drying, and
    sensing.

2.6 Validation of the Immunolegio concept at laboratory scale

2.6.1 Specificity of the beads: Do the beads bind with non-target organisms?

Bacause of the time delay caused by the newly introduced sensor development task, the a suitable magnetic sensor was not available during the laboratory testing task. For this reason this task was performed by using alternative evaluation methods available in the laboratories of the consortium. It has to be considered that these methods (i.e. optical microscopy) are in some regards less effective for the given task then the magnetic sensor would be.

Metodology

Organisms used:
Escherichia coli
Pseudomonas aeroginosa
Klebsiella aerogenes
Entrococcus faecalis
Salmonella poona
Listeria innocula
Staphylococcus aureus

For each organism a lenticule was reconstituted in 1 ml of maximal recovery diluent.

100 µl from each sample was transferred to an Eppendorf that contained 300 µl of water to make the final volume 1 ml.

1 µl of the beads were added to the mixed bacterial solution. The tubes were mixed by rotation at 20 rpm for one hour at room temperature (~28 °C). The beads were collected using magnetic separation, the supernatant was discarded and the beads washed with 1 ml PBS. This was repeated two more times, giving the beads a total of 3 washings. The beads were finally re-suspended in 50µl water and put onto a slide to dry. They were treated with Methanol twice to inactivate any bacteria that may be associated with the beads.

Also tested was a functionalised chip (on a stick) to see if the polyclonal antibody bound non-target organisms. Method was as above except the beads were substituted for by the chip. The chip was removed at the end of the incubation time and rinsed three times with PBS. Then treated with Methanol prior to staining with DAPI. View using a fluorescent microscope.

Procedure for testing Beads, Legionella and Chip together:

- A lenticule of *Legionella pneumophila* serogroup one is reconstituted in 9 ml MRD.
- 1 ml aliquots are transferred into sterile 2 ml screw cap tubes.
- 25 µl of the beads (Immunolegio: 500 MAB and Neg; 1000 MAB and Neg; 2800 MAB and Neg) are added to the 2 ml tube (or 10 µl of the DYNAL beads positive control).
- The functionalised chip (with stick) is added to the tube containing the bacterial-bead mixture.
- The samples are mixed by rotation at 20 rpm, for one hour at room temperature (approx. 26-28 °C)
- After the hour the chip (with stick) is removed from the tube and washed in PBS.
- The magnetic beads are recovered from the mixture and washed with PBS once. The beads are suspended in 50 µl of water and put onto a microscope slide and dried.

- All the samples are fix and the bacteria inactivated by the addition of Methanol to the surface of the chips and microscope slides.
- The samples are then stained with DAPI, prior to viewing with the fluorescent microscope.

For the Negative controls:

- 1 ml of bacterial suspension and 10 µl of DYNAL beads are incubated with:
- unfunctionalised chip and unfunctionalised chip where the surface had been blocked with 2%BSA/PBS.

The samples were sent to UJ to visualize using the equipment available there. These results are also included in this part of the report. Confocal Microscope is an inverted microscope. DIC stands for differential interference contrast (DIC) images.

Methodology applied at UJ:

The beads and chip-on-stick slides were mounted with moviol and left to dry.

Beads and Legionella solution (after the functionalized chip had been incubated for one hour)

Comments by UJ to these measurements:

Beads: They were lot of debris and there were also a lot of the beads in the samples. Large amount debris is normal in case of real samples, and the observed debris should not negatively influence the evaluation by magneto sensor measurement. However, the large number of beads means that the used 25 µl of the beads is too many to get the targeted number of bugs on the beads and that washing must be improved.

The number of bugs on the beads cannot be evaluated if they are captured on the chip with the stick. The chip could be used without the stick, than the beads/bugs can be captured on the chip and than the number of beads/bugs on the chip can be calculated.

Examplary results

MAB beads (Positive):

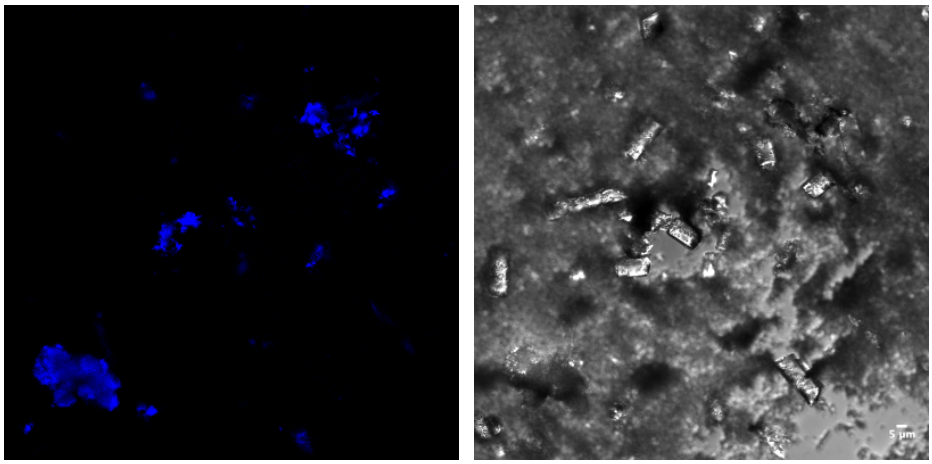


Figure 54: 500 MAB beads with 60x objective, Left: Blue/Dapi. Right: DIC
In spite of the disturbing debris areas with captured *Legionella* (blue) can be localized in the

left picture. Beads itself are hard to identify in the white light picture (right) due their small size (500 nm).

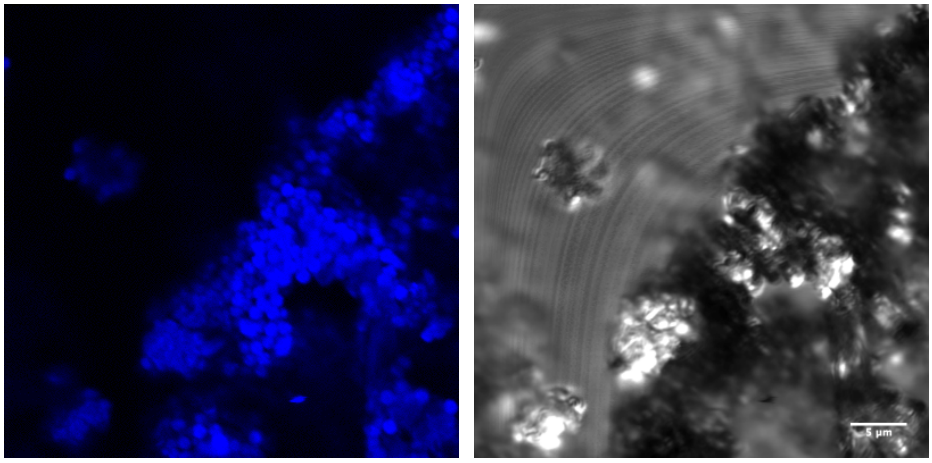


Figure 55: 1000 MAB beads: 40x objective zoomed in. Left: Blue/Dapi. Right: DIC (larger debris pieces provided difficulties in focusing and caused shadows). In spite of the optical difficulties lager areas with captured *Legionella* (blue) can be localized in the left picture.

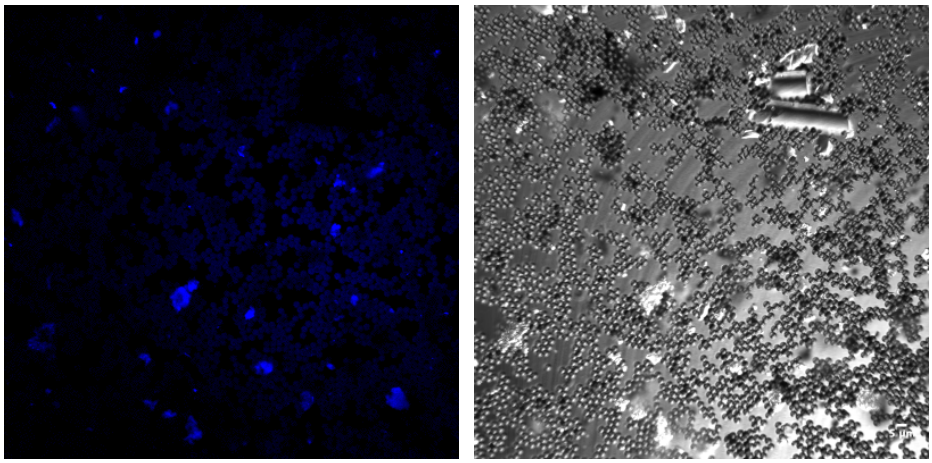


Figure 56: 2800 MAB beads:20x objective. Left: Blue/Dapi. Right: DIC
Due to the larger size of the beads (2800 nm) they are good visible in the white light picture (right). In the fluorescent microscopic picture (left) the DAPI marking of the *Legionella* is faded due to the shadowing by the large beads.

The *Legionella* is more likely between the surface and the beads cached between the surface antibody and bead antibody in a sandwich structure. This is the intended effect in IMMUNOLEGIO. However, there are also some highlighted areas in Figure 57 which could have two explanations: 1) *Legionella* is captured also on top of the beads providing a good DAPI response. This will be not represented in the result of the magnetic measurement. 2) Reflections cased by the debris on the surface or DAPI cached (mechanically) by the debris.

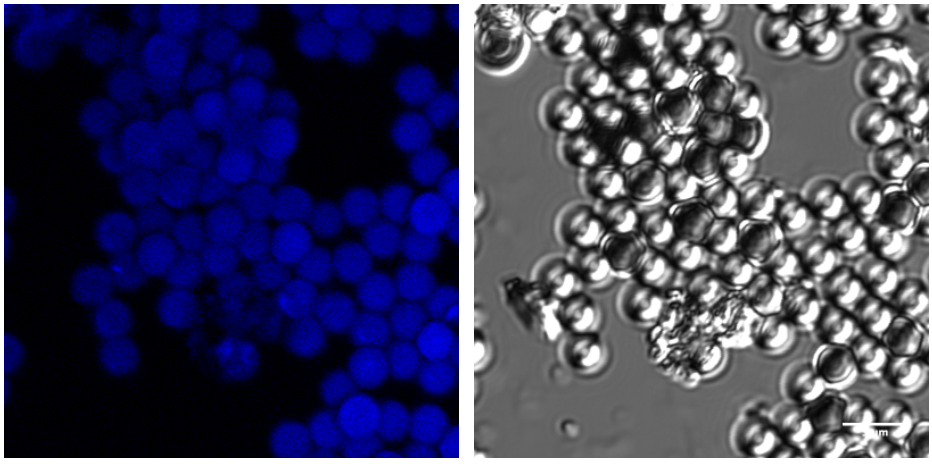


Figure 57: 2800 MAB beads: 60x objective and zoomed. Left: Blue/Dapi. Right: DIC
At higher magnification it can be seen that there is a high density of beads, which increases their shadowing effect making it hard to identify *Legionella*.

In the magnification shown in Figure 57 it seems to be clear that the faded blue reflection comes more from the beads itself than the *Legionella*. This makes it more likely that the small bright areas are also reflections from the debris.

NEG beads:

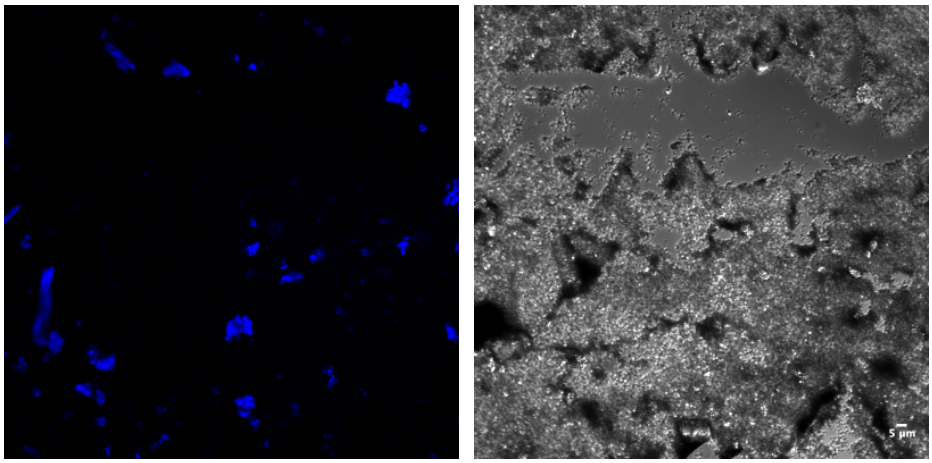


Figure 58: 500 Neg beads:60x objective. Left: Blue/Dapi. Right: DIC
The bead density on the surface is significantly less than in case of the positive controls, however there are still a lot of unspecific bound beads on the surface. Bead concentration has to be decreased and washing process has to be improved.

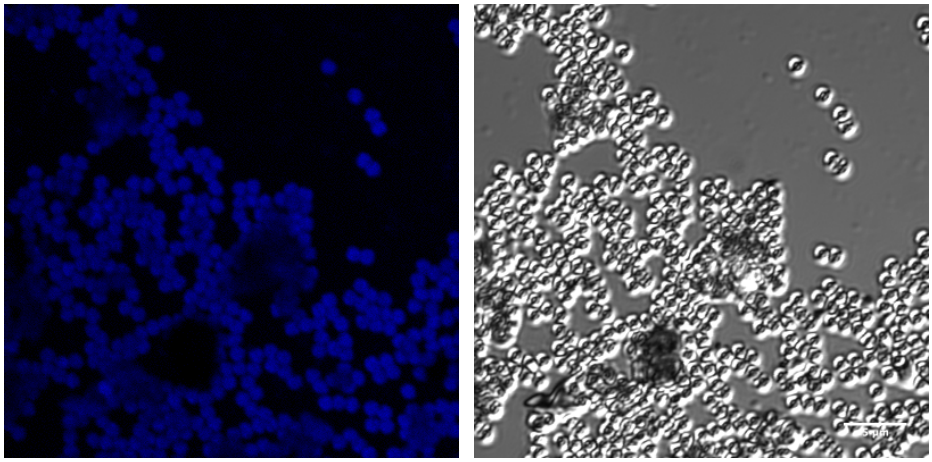


Figure 59: 1000 Neg beads: 40x objective / zoomed in. Left: Blue/Dapi. Right: DIC
It seams obvious that the blue is coming from the beads reflecting the laser because and we are seeing blue associated with the beads only there seems to be no blue from *Legionella*.

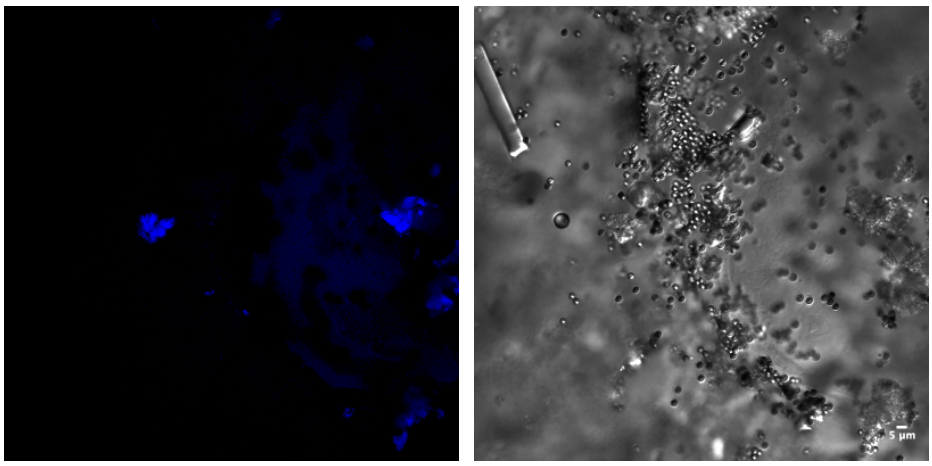


Figure 60: 2800 Neg beads: 20x objective. Left: Blue/Dapi. Right: DIC
In case of the larger 2800 nm beads there is significantly less unspecific binding, and this is more or less concentrated to the areas with debris. Washing needs to be further improved.

From above results it is clear that under real world conditions larger beads provide a significantly better signal to noise ratio. It is also clear that for industrial smaples the sample pre-processing and washing procedures have to be further improoved.

DYNAL beads (positive control)

Control measurements with commercial DYNAL beads under laboratory conditions.

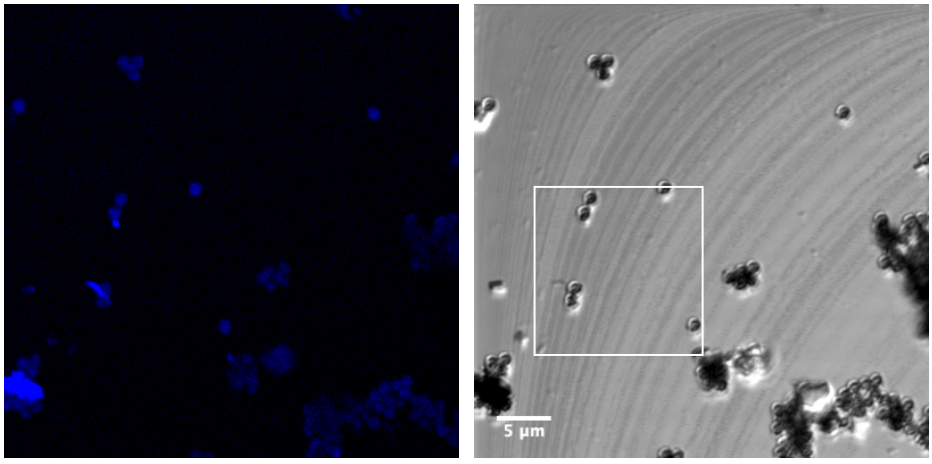


Figure 61: Dynal with Legionella beads: 40x objective / zoomed in. Left: Blue/Dapi. Right: DIC Legionella can be identified in the zoomed region, although there is a slight tendency for agglomeration.

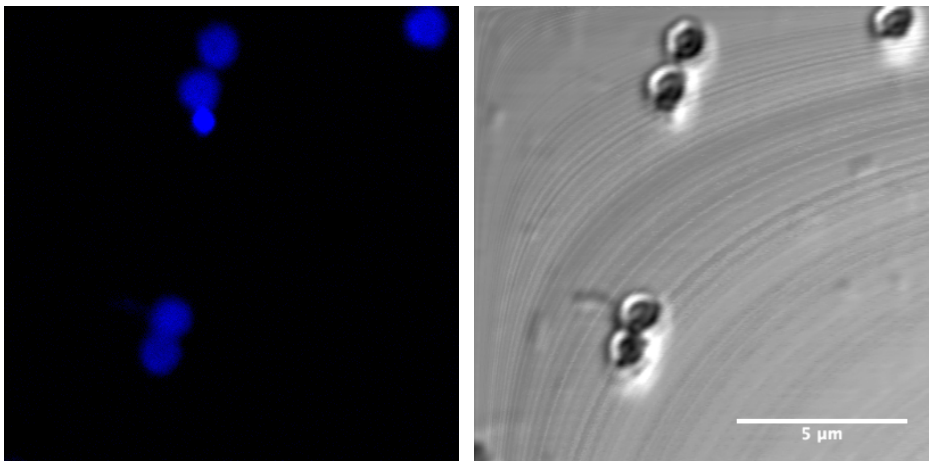


Figure 62: Dynal with Legionella beads: 40x objective / zoomed in more. Left: Blue/Dapi. Right: DIC This figure is the zoomed region from the above figure.

Chips (on sticks)

These results were obtained with the new Test Stick prototypes providing a cleaner surface and having significantly reduced unspecific binding.

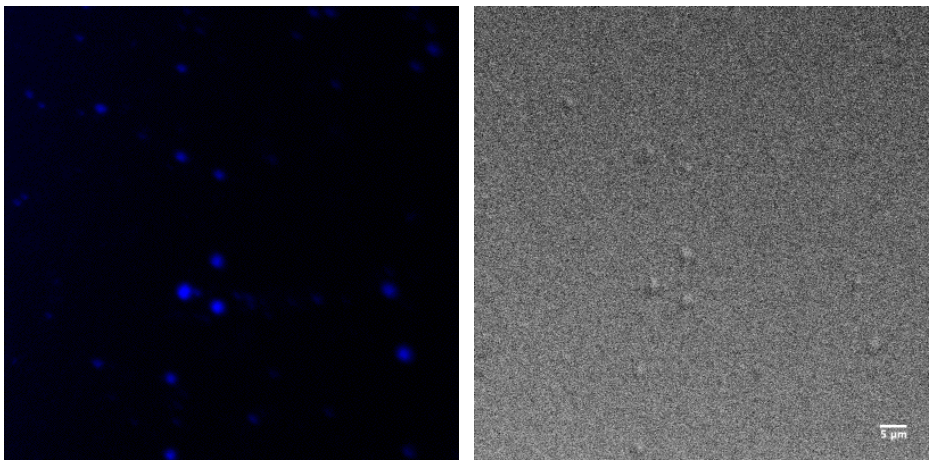


Figure 63: Dynal sticks 20x objective / zoomed in. Left: Blue/Dapi. Right: DIC
There is optical noise in the picture at this magnification, but can the outline of the beads can be clearly seen.

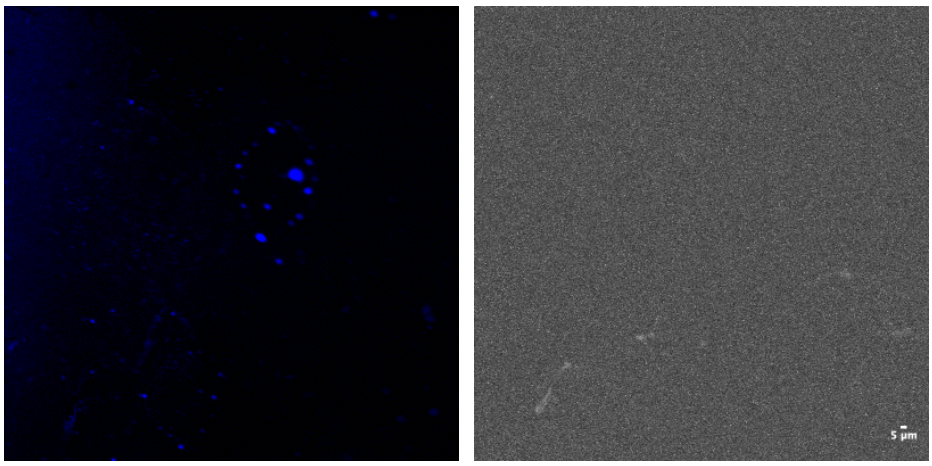


Figure 64: Dynal sticks 40x objective / zoomed in. Left: Blue/Dapi. Right: DIC
There is reduced noise with this magnification, but the outline of the beads is not visible in the white light DIC picture.

Confocal Microscopy is not suited to get a count of the beads, but it allows a good estimation about the effectiveness of the methods and protocols applied in IMMUNOLEGIO.

Conclusions for sample preparation:

- The water sample needs to be better cleaned of debris, especially for optical evaluation. Debris should not influence the magnetic read out, but it increases the amount of unspecific binding.
- The sample water should be needs to be filtered to remove most of the larger debris. Although in case of the above results contamination may also be introduce during the shipping the shipping from STL to UJ. Using a coverslip for the transport should prevent this problem.
- Beads should be further diluted, because the concentrations used in above experiments are obviusly too high. This observation is advantegous regarding the per sample testing costs, as the functionalized beads are the most expensive consumables in the IMMUNOLEGIO concept.

Second set of experiments

The beads used in following experiments were 1000 MAB and 2800 MAB. These produced a total of 18 functionalised chips that had been subjected to bead-bug complex.

Applied procedure for Experiment Set 1:

- One lenticule of *Legionella pneumophila* serogroup one reconstituted in 1 litre of sterile distilled water, filtered and the collected material collected in 20 ml ¼ ringers solution or MRD. This was further diluted prior to use to 120 ml.
- 10 ml aliquots were transferred to 15 ml sterile tubes.
- 10 ul of the beads were added. The beads have been diluted 1:10 for easier handling so this is the same as 1 ul of the undiluted beads.
- Using the fixed parameter of 30minute incubation time of the bug beads solution the samples were mixed by rotation at 20 rpm at room temperature (~28 °C) for 30 minutes.
- The beads were collected with the use of the DYNAL magnet particle condenser. The supernatant was discarded and 1 ml of PBS was added to the tube. The sample was transferred to a 2 ml screw cap tube. The beads were collected again by magnets. The beads were washed a total of three times with 1 ml PBS. The beads were resuspended in 1 ml of PBS for the next stage.
- The functionalised chips (on sticks) were added to the 2 ml tube containing the solution of bugs/beads. They were mixed by rotation 20 rpm, at room temperature (~28 °C).
- The time factor for the chip being in contact with the beads (bug complex) was the varied parameter for this experiment.
- The incubation times were 1 min, 5 min, 10 min, 20 min and 30 min.
- After the required time the chip (with stick) was removed from the tube and washed with PBS.
- The chip was allowed to dry and Methanol was added to the surface of the chip to inactivate the bacteria and fix the complex. The sticks were placed onto microscope slides for easier handling.

All Test Sticks being result of these experiments have been properly labelled and archived in a slide box and are transferred to the SMEs for reference and follow up measurements (i.e. by the final IMMUNOLEGIO sensor).

Experiment Set 2:

As above except that the varied parameter was the length of time that the beads were in contact with the *Legionella* solution. The incubation times were 1 min, 5 min, 10 min, 20 min and 30 min.

1000 MAB beads

Bead-bug incubation time	*Chip incubation time*
Set 1	
30 minutes	1 minute
30 minutes	5 minutes
30 minutes	10 minutes
30 minutes	20 minutes
30 minutes	30 minutes

The parameters of the last experiment (30 min – 30 min) overlaps with Set 2, thus it is not repeated in Set 2.

Bead-bug incubation time	*Chip incubation time*
Set 2	
1 minute	30 minutes
5 minutes	30 minutes
10 minutes	30 minutes
20 minutes	30 minutes

2800 MAB beads

Bead-bug incubation time	*Chip incubation time*
Set 1	
30 minutes	1 minute
30 minutes	5 minutes
30 minutes	10 minutes
30 minutes	20 minutes
30 minutes	30 minutes
Set 2	
1 minute	30 minutes
5 minutes	30 minutes
10 minutes	30 minutes
20 minutes	30 minutes

Using concentrate samples that had been processed by Legionella team.

The concentrates were measured prior to putting into a one litre bottle containing sterile distilled water. The samples were filtered and the membrane placed into a bag containing 10 ml of ¼ strength ringer or 10 ml MRD. They were stomached for 60 seconds on fast speed. The membrane surface was rubbed and the solution transferred to a 20 ml sterile tube.

Approximately half the sample was filtered for testing by the standard method. The remaining solution was filtered and kept for incubation with the Immunolegio beads.

Standard method as reference for the new IMMUNOLEGIO method

The concentrate obtained was tested by plating out 0.5 ml directly onto GVPC agar plates. 1 ml was put into an Eppendorf tube and incubated at 55 °C for 30 minutes before plating out 0.5 ml of this (Heat treated sample) onto GVPC agar plates. A third sample 0.5 ml was transferred to an Eppendorf tube containing 0.5 ml of Acid buffer. This was incubated for 5 minutes then a 0.5 ml sample was plated onto GVPC agar plates. The plates were incubated at 37 °C for 7 days.

Sample number	Sample description	Vol. of concn (ml)	Lp1 CFU/L	Volume filtered (ml)
11017553	Staffs sample	7.0	1260	473.5
11013975	Carillion bathroom 1-15 hot	6.7	1302	482.5
11013993	Carillion Cal 1	7.2	530	482.6
11017653	Kraft theo fld control room electric shower	6.4	2071	492.3
11016747	First environmental c63512 nct 20th male	6.6	1212	495.3

Results of the standard method on day 7

Sample number	Volume filtered (ml)	CFU/0.5 ml U	CFU/0.5 ml A x 2	CFU/0.5 ml H	CFU/L = $\frac{\text{CFU x 10 x 2000}}{\text{Vol. filtered}}$
11017553	473.5	222	28	68	2872 (H)
11013975	482.5	94	15	48	1990 (H)
11013993	482.6	264	68	80	3315 (H)
11017653	492.3	Contamin	27	21	853 (H)
11016747	495.3	19	3	13	525 (H)

Immunolegion beads 1000 MAB (using the concentrate samples)

The 10 ml solution obtained after filtration was transferred to a 15 ml sterile tube. 10 µl of 1:10 diluted 1000 MAB beads were added to the solution. The samples were mixed by rotation at 20 rpm, for 30 minutes at room temperature (~28 °C).

The beads were collected using magnets and were washed with 1 ml PBS three times. The bead complex was suspended in 1 ml of PBS. These were stored at 4 °C waiting to be incubated with functionalised chips.

Sample number	Sample description	Vol. of concn (ml)	Lp1 CFU/L	Volume filtered (ml)
11017553	Staffs sample	7.0	1260	485.5
11013975	Carillion bathroom 1-15 hot	6.7	1302	484.0
11013993	Carillion Cal 1	7.2	530	487.1
11017653	Kraft theo fld control room electric shower	6.4	2071	478.0
11016747	First environmental c63512 nct 20th male	6.6	1212	476.3

Approximately 900 µl of 1:10 diluted bead solution. Using 10 µl per experiment means about 90 more individual tests can be performed. All processed and remaining samples were transferred to the SME partners including their detailed documentation. The Test Sticks with immobilized *Legionella* resulting from the experiments were conerved safely to be used for later reference.

2.7 Knowladge management

Knowledge management included several actions through out the project run time. In the actual reporting period following main actions were performed: patent search, preparations for certification, training, creation of material for dissemination (i.e. flyer, multimedia guide), as well as the technology watch actions.

2.7.1 Patent Search

A thoughout patent and literature surch was performed to evaluate the novelty potential of the final IMMUNOLEGIO concept in respect to IPR protection, and to avoid infringing any existing patents

by the used technologies. Due to the device complexity the patent search has been divided into three areas, each covering a functional part of the device.

a) Micro-inductive sensor
b) Immunosensor
c) Automated sample preparation unit

The following tables include only the most relevant results found.

Micro-inductive sensor

Name	A sensor device for and a method of sensing particles		
Reference	http://www.wipo.int/pctdb/en/wo.jsp?wo=2008035252		
Date	20.09.2006		
Type	Patent	Risk	**High**
Abstract The invention relates to a sensor device for sensing particles with the help of a second type particles used as labels (e.g. magnetic labels). Presence of labels induces changes of magnetic property of a generated AC magnetic field and sensed by a magnetic sensor element on a substrate. Further relates to a method of sensing particles using Giant Magnetoresistance Effect (GMR) for biosensor applications, primarily targeting blood cell samples.			
Discussion WO 2008/035252 discloses a magnetic biosensor application using GMR effect and a sensing unit using mathematical correction of unbounded second type particles or labels. GMR requires precise control over the distance between the sensor element and the activated sample, which makes field application difficult. The mathematical correction demands high computational resources. Both of these factors result in increased cost of unit and per sample cost. IMMUNOLEGIO targets the detection of Legionella from water sample at very low bacteria concentrations. IMMUNOLEGIO uses a stick medium with an immobilized active surface to separate the unbounded second type particles. Only labeled particles are exposed to the magnetic sensor element, no further correction is required. The magnetic sensor element deposited on a Si substrate using thick film, metal-multilayer technology, eliminating the need of GMR excitement and avoids sticking problem. Further, absolute impedance measurement is replaced by a delta measurement, in which the differential signal is being processed. The reference element is placed in complemented configuration to the same substrate, improving accuracy.			

Name	Si technology based microinductive devices for biodetection applications		
Reference	http://ina.unizar.es/consolider/articulos/article%20sensors%20and%20actuators%202006%20corrected%20proofs.pdf		
Date	22.02.2006		
Type	Publication	Risk	**Medium**
Abstract The paper concerns the design and fabrication of inductive devices integrated in Si technology aimed at high sensitivity bio detection applications. The sensing principle is based on the changes of the coil inductance due to the presence of magnetic particles.			
Discussion IMMUNOLEGIO employs integrated coils forming an active magnetic sensor element. The technology is similar to the embodied, however to increase sensitivity, coils are place on multiple layers.			

Name	Inductive micro-sensor formed flat on an integrated circuit		
Reference	http://v3.espacenet.com/publicationDetails/biblio?adjacent=true&KC=A1&date=20030508&NR=2003085790A1&DB=EPODOC&locale=en_EP&CC=US&FT=D		
Date	08.05.2003		
Type	Patent	Risk	**Low**
Abstract The invention describes an inductive micro-sensor includes a flat-micro-coil formed on an integrated circuit by long segments Si with a high aspect ratio.			
Discussion IMMUNOLEGIO integrated coils use metallic conductive segments.			

Immunosensor

Name	Immunosensor and measuring method using the same		
Reference	http://v3.espacenet.com/publicationDetails/description?CC=US&NR=2009093068A1&KC=A1&FT=D&date=20090409&DB=EPODOC&locale=en_EP		
Date	09.04.2009		
Type	Patent	Risk	Low
Abstract The invention relates to an immunosensor, comprising of a base body having a sample-holding unit. Further relates to a measuring method with a simple configuration which suppresses the deterioration of the antibody and accurately measures the concentration of the material to measured, which is contained in the sample.			
Discussion IMMUNOLEGIO provides a sample preparation unit as a separate piece, thus enables easier maintenance and additional flexibility. The multistage process isolates the antibody marking phase and the cycle partially eliminates antibody deterioration.			

Name	Immunosensor and immunoassay method		
Reference	http://v3.espacenet.com/publicationDetails/biblio?adjacent=true&KC=A&date=20080410&NR=2008082729A&DB=EPODOC&locale=en_EP&CC=JP&FT=D		
Date	10.04.2008		
Type	Patent	Risk	Low
Abstract The invention relates to an immunosensor and an immunoassay method capable of determining accurately the concentration of a substance to be measured in a sample even under low-temperature environment.			
Discussion The submitted device focuses on optical recognition of concentration using non-magnetic bonding substrate. The immunosensor and the holding part are integrated into a single unit. IMMUNOLEGIO is not intended to be used in low-temperature environment and only water based samples have been targeted.			

Automated sample preparation unit

Name	Sample preparation system for processing of clinical specimens		
Reference	http://www.wipo.int/pctdb/en/wo.jsp?wo=2008123882		
Date	16.10.2008		
Type	Patent	Risk	Medium
Abstract The invention describes a system for automated handling of vials containing liquid medical specimens. The robotic system processing comprises automated vial cap removal, pipeting of the vial contents, and transfer of the vial contents to a destination tray such as a multiwall plate.			
Discussion Disclosed system intended for use with standard vial dimensions, for in-house laboratory applications. The design in well suited for multi sample analysis, executing as laboratory protocol. IMMUNOLEGIO two-axis preparation unit supports almost any size and shape sample containers, including larger pieces. Being robust, it can handle extended temperature range and suited for outside applications, field analysis.			

Name	System, method and computer product for automated high-throughput seed sample aliquot preparation, treatment, and disperal		
Reference	http://www.wipo.int/pctdb/en/wo.jsp?wo=2008006063		
Date	10.01.2008		
Type	Patent	Risk	Low
Abstract The invention relates to a system, method and computer program product for separating a seed aliquot from a bulk sample and preparing the seed aliquot for downstream research purposes.			
Discussion The system said, primary for agricultural research, specializing in plant breeding and plant product management. Main feature is segregating a seed aliquot from a bulk sample. Further treating it with selected treatment compounds and package for research or field identification. IMMUNOLEGIO measures the presence and the concentration/amount/quantity of first particles and recording the analytical data. No packaging and identification of the samples is			

necessary.

Name	Automated method for preparing blood sample analysis and automated device therefore		
Reference	http://www.wipo.int/pctdb/en/wo.jsp?wo=2007006903		
Date	18.01.2007		
Type	Patent	Risk	Medium
Abstract The invention concerns a method for preparing total blood sample analyses and a device. Samples being preserved in tubes, robotic gripping and displacing by a control automaton and transport in at least three directions XYZ between the storage zone and preparation zone.			
Discussion The device described is a large scale, mass blood sample preparation and analysis unit. IMMUNOLEGIO is moving the sample holding and displacing head, thus reducing the need of sophisticated multi dimensional gripping and control mechanism.			

Based on the results of the patent research the consortium considered other means of protection for the results, as none of the developed solutions are novel enough to result in a strong patent protection. The individual partners also performed patent searches in the fields they are interested in.

2.7.2 Preparations for certification

By obtaining the CE marking it is asserted that the item meets all the essential "Health and Safety" requirements of the relevant European Directives. In the case of IMMUNOLEGIO device the following regulations apply:

- Low Voltage Directive 2006/95/EC

The Directive applies to all electrical equipment designed for use with a voltage rating of between 50 and 1000 V alternating current and between 75 and 1500 V direct current Voltage ratings refer to the voltage of the electrical input or output, not to voltages, which may appear inside the equipment.

The Low Voltage Directive 2006/95/EC requires products to have protection against hazards that could arise from within the product itself or from external influences. The Directive covers all risks arising from the use of electrical equipment, including mechanical, chemical (such as, in particular, emission of aggressive substances) and all other risks. The Directive also covers noise and vibration, and ergonomic aspects which could cause hazards within the scope of the Directive.

- Machinery Directive 2006/42/EC

The Directive defines a machine as being: "an assembly of interconnected parts, at least one of which is able to move, serving a specific purpose". The definition also includes: "assemblies of several machines with connected functions, a removable attachment which alters the function of the machine, which is connected to the machine by the operator himself, and which is not a spare part or an engine" and "separately marketed safety components whose malfunction could endanger people or the environment".

- Electro-Magnetic Compatibility Directive 89/336/EC

The EMC-Directive includes all electric and electronic equipment as well as equipment and systems that contain electric and/or electronic components insofar as various aspects could cause electromagnetic interference or the operation thereof could be affected by this interference. The above applies, in fact, to all equipment in which use is made of electric energy, from battery-operated products to high-voltage installations.

The EMC-Directive simultaneously regulates the emission from electronic equipment and the degree of insusceptibility to electromagnetic interference in the immediate vicinity of this equipment. In other words, products that fall under this Directive may not cause impedimentary interference in other equipment and at the same time must be sufficiently immune to interference from other sources.

The criterion that equipment must be constructed in such a way that it neither causes interference in other devices nor can itself be affected by interference is referred to as an 'essential requirement'. In order to indicate that a product complies with the essential requirements of the EMC-Directive, a correlation can be made with the technical standards.

- Documentation

The IMMUNOLEGIO appliance has to be produced in a way to comply with all relevant requirements. The "Technical Construction File" which is the basis of the conformity assessment of the design. Also "Declaration of Conformity" shall be compiled; this document states that the product is in conformance with the approved design.

The File will identify the product and the requirements. It describes the assessment-activities, and contains the results of these activities in the format of photographs, brochures, technical construction drawings, material compositions, schematic diagrams, parts lists of components, descriptions of components.

Required information to be provided in the File:

- Copies of the users manual and service instructions,
- List of applicable EU-directives,
- List of normative technical documents or standards used for the conformity assessment,
- Design calculations,
- Hazard analysis,
- Description of measures to reduce or eliminate hazards,
- Evaluation- and test-reports, indicating:
- which evaluations and tests were performed,
    - methods of evaluations and tests,
    - evaluation- and test-equipment,

  - results of evaluations and tests,
  - acceptance criteria.
- Conclusion, indicating that the product complies with all relevant requirements.

Products with the CE marking are produced in accordance with the design that was found to comply with relevant requirements. The "Declaration of Conformity" is an official statement that a product is in conformance with an approved design. Essentially the declaration concerns an individual product. It states that the product is in accordance with "Essential Requirements" of applicable EU-directives.

In principle, a declaration shall be available for each individual product. The marking of the products (by serial-numbers, date-codes, etc.) and the registration-indications of the declarations shall clearly refer to each other.

In the Consortium none of the IPR owners has the expertise to compile all necessary documentation therefore certification institutes such as TÜV or SGS will be contracted to perform all activities necessary such as special evaluations and tests, as well as documentation, for CE marking. However, the deliverable and activity reports composed in IMMUNOLEGIO are of great contribution to the aforementioned documentations.

The automation concept for sample preparation is relevant for certification considering the hardware assembly of IMMUNOLEGIO appliance. According to existing standard EN ISO 11731 for Legionella detection, the method applied in IMMUNOLEGIO does not comply explicitly. For more information see Appendix.

2.7.3 Training

Two training sessions were organized to transfer the technological know how to the SME partners.

- 9th and 10th of February, 2009
- 27th of March, 2009 (combined with the final meeting)

Both times Barcelona was selected as location, because the all partners named this as best accessible locations from their home countries. The training covered following points.

- Legionella, the bacterium, its danger and associated regulations
- Overview of the State of the Art testing and detection methods
- The IMMUNOLEGIO technology and achieved results / specifications (in Training 1)
  Knowledge Transfer – Technical Summary of all developed technologies (in Training 2)
- (assimilation of achieved results & required actions for commercialization)
- How to operate the IMMUNOLEGIO device & Safety issues
- Demonstration of the IMMUNOLEGIO prototype
- Discussion of technological details

The most of the presented content is covered by other chapters of the current report, and will not be repeated here. The full minutes of the training sessions can be found in Deliverable D12 and D15.

2.7.4 Creation of material for dissemination

Several materials was created for dissemination proposes, flyers, posters, publications, as well as a multimedia guide demonstrating the use of IMMUNOLEGIO.

Multimedia guide

The IMMUNOLEGIO Multimedia Guide itself was realized as a Video User Guide to the developed IMMUNOLEGIO prototype. The video is available on the project website (http://immunolegio.mfkk.hu/) for download and is also distributed on CD. Multimedia Guide Video has a length of 10 minutes 20 seconds, and a file size of 2.3 GB. Note that the whole IMMUNOLEGIO process takes one and a half hours. The video demonstrates the whole process in a shortened way, concentrating mainly on the issues requiring user interaction. A low resolution version of the video was also created to allow distribution over the internet. The multimedia Guide is further described in deliverable D16.

Flyer

A Flyer was prepared for dissemination purposes, and was distributed at several conferences, the most important were:

- ESEAC 2008 conferecne in Prague (BVT)
- BIO 2008 (AROMICS)
- Published in Chemical News for EXPOQUIMIA 2008 (Barcelona)., exhibited at EXPOQUIMIA 2008 (Barcelona) (AQUALAB)
- (Prague)



Figure 65: Screen shoot of the flyer design (A PDF version with high resolution is available on the project web page).

A poster with similar content was also created.

Publications

In addition to above dissemination activities two journal and one conference publications were made by the RTD partners (UJ, MFKK) in agreement with the SME partners.

2.7.5 Technology Watch

Concurent products on the market

Biotica presented a new development on the last Legionella Conference in Spain. The Biotica technology has a similar concept to IMMUNOLEGIO as it uses magnetic beads to mark the sample, but it uses a different technology for the detection.

Biotica uses super-paramagnetic beads as immunolabels to selectively wash the sample, magnetically fixing the target bacteria and uses a secondary immunomarker resulting in a color change according to the legionella concentration. The Legionella concentration can be evaluated using a colour card. The promised detection limit is 2 * 10^3 CFU.

Biotica proposes its system:

- As a method for Legionella measurement: a) In lab b) In situ C) On-line: automatic biosensor
- As a method for Legionella concentration.
- As a method for sample preparation before PCR.

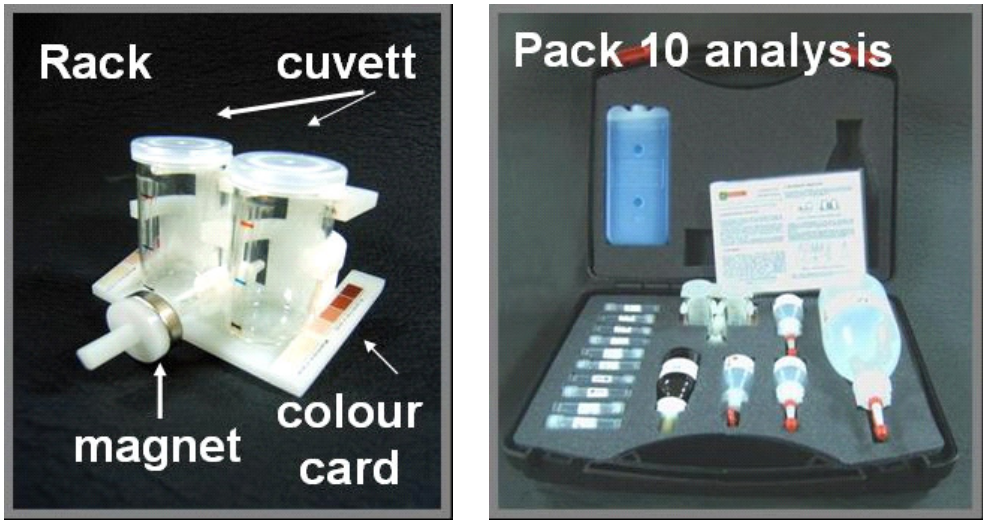


Fig. 1: *The hardware prototype consists of a semiautomatic washing and immunoreaction vessel and a test kit.*

Concurent technologies

Magnasense (http://magnasense.com) has developed in parallel to the IMMUNOLEGIO project a detection platform and instrumentation for sensitive and quantitative diagnostics at Point-of-Care locations. In the presented platform magnetic particles are used for detecting analytes of interest. The instrument reads several types of analyte-specific disposable test cartridges.



Fig. 2: *Magnasense standard reader instrument based on micro-coil technology is an assay reader for lateral flow tests.*

Magnasense technology is based on the use of magnetic nanospheres as labels for the analyte of interest. The device measures a change of magnetization in the capture zone of the test. This diverse instrument forms an easy to use quantitative biosensor when combined with disposable test cartridges. Either a traditional sandwich assay or a competitive assay format can be used to form the capture zone. The change of magnetization in the capture zone is measured with an independent measuring instrument, which uses an arrangement of coils and a fluctuating magnetic field. This measurement technology has a great potential outside of lateral flow tests as well, such as in the field of microfluidistics.

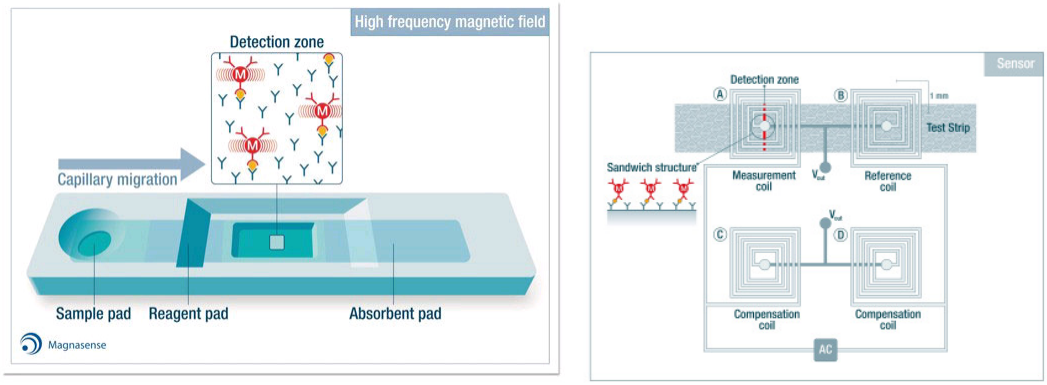


Fig. 3: *LEFT: Lateral flow test with antibody coated magnetic particles captured by detection zone antibodies. (Nanospheres are polystyrene beads with ferrite (Fe_3O_4) coating.) RIGHT: A set of planar micro coils symmetrically aligned in an impedance bridge. Two of the coils (A and B) are used for measurement and reference purposes, while the two other coils (C and D) function as floating ground potential with which the measurement is compared.*

A set of four planar micro coils symmetrically aligned in an impedance bridge is used for quantitative measurement of magnetic nanospheres. The sample is placed on one of the four identical coils and the control area of the lateral flow strip is placed on the other side of the symmetrical axis. The two other coils read the background from the surroundings. The inductance of the coils is changed due to the relative permeability of the nanospheres. This unbalances the bridge at high frequencies. An output signal is proportional to the amount of the particles.

3 Consortium management

3.1 Consortium Management Structure

The Co-ordinator is the responsible for supervision of the work programme, communication with the EC, reporting, delegation of work packages, motivation of the team, encouragement of creativity, creative problem solving, and corrective actions. Task leaders are appointed for specific tasks, and meet regularly under the supervision of the Project Co-ordinator. Informal communication between partners is encouraged at all times – with the responsibility to report via the task leader to the project co-ordinator on significant action/conclusions and immediately on experiencing problems.

The Project Manager of FKK acts as Chairman of the Management Board. It is his responsibility to ensure that correct procedures are carried out and that all deadlines and obligations are met. It is his responsibility to seek consensus on project steering, however, in case of dispute decisions are taken by majority voting, with representatives of all SME proposers having equal rights. RTD Performers have no voting rights.

The Management Board meets every 3 months to review the technical progress made by each partner, and to agree on the details of the actions for the next period. These meetings are likely to involve formal presentations from those partners who are at that time actively working on tasks, and the board has overall responsibility for the direction of the project. In addition to the formal 3 monthly meetings, the partners also set up a number of working parties to ensure delivery of specific tasks, involving those partners committed to specific tasks and objectives within the work programme.

The Consortium Agreement, signed by the partners during contract negotiations with the Commission, is reviewed periodically to allow for inclusion of actions to maximise exploitation potential or protection of intellectual property.

3.2 Communication strategy

Use of E-mail is encouraged. Agendas for all meetings are required (and found in the appropriated area of the project website). Discussions, decisions and actions are recorded and 3-monthly progress meetings are held at sites convenient for all partners. Special meetings may be arranged for urgent problem solving or creative thinking at the site most relevant to the subject in question.

All documentation is in compatible PDF format (other formats available on request). All reports are filed by the project co-ordinator in a master file, which are made available to all partners through the website of the project.

A specific website housed at FKK's Internet Service Provider is available to provide secure and restricted access to file documents, reports, minutes, etc., as well as to serve as a communication tool between partners and Commission Officers.

3.3 Monitoring and reporting progress and results

Monitoring and reporting progress is carried out through a series of reports and meetings, including regular visits by the Project Co-ordinator to Task leaders or Research locations.

A review and assessment form was prepared at the beginning of the project, and includes the work package identification, the duration, resource consumption, deliverables, as well as an assessment of the tasks involved. This form was prepared on a monthly basis, and sent to the Management Board. The work package leader (the partner with the highest contribution),

assessed by the coordinator (FKK), was responsible for collecting the information from the other partners, making the assessment, and preparing this form.

Every 3 months the partners make presentations in the Management Board meetings, covering progress on each of the active tasks and the actions to be carried out for the following three-month period. The content of these meetings will be recorded in minutes and circulated to all the partners, to ensure that there is a 3 monthly written record of progress.

Every 6 months a report is prepared and circulated to each of the partners, detailing progress on each of the tasks carried out in the previous six months, and performance against the project milestones. At month 12, this report is extended to detail progress during the first 12 months, the achievement of key milestones, and including a breakdown of the effort in each WorkPackage. At month 24, the report will cover the whole of the project, again including a breakdown of the various parts of the project.

To ensure that project results are accurate and reported correctly, the Co-ordinator asks all the partners to keep detailed records of input into the project using logbooks or a timecard system.

4 Other issues

Both SME and RTD partners have contributed in the different tasks carried out during the first year. The contribution from the RTDs has been higher than that of the SMEs. The overall contribution of the group of SMEs has been of 48,8 person-month in front of 93,4 person-month of the RTD Performers (see Table 1.3 in the Management Report). The reasons for explaining this balance are:

- The work of the RTDs is mainly focused on research and development tasks, which were perrformed during the first year of the project (WP2-WP5). In the case of the SMEs, their contribution is allocated mainly in the tasks dealing with the prototype test and validation, as well as to technology transfer activities (WP6-WP8). Such tasks are mainly distributed along the second year of the project.
- The person-month of management also correspond to an RTD performer (FKK)

On the other hand, the SMEs have notably benefit from the work carried out by the RTDs. Firstly, SMEs have been provided with advanced technology for handling superparamagnetic nanoparticles, antibody coating and magnetic sensor design. The use of such technology is usually restricted to university laboratories and large companies able to make large investments in R&D tasks. By collaborating in this joint project, the SMEs have gained access to this technology which may find other applications in their respective fields. And secondly, after the first year of the project the SMEs have already obtained a good understanding of the properties of magnetic sensors and magnetic nanoparticles as well as antibody coating, which constitutes the basis for developing an online Legionella monitoring system.

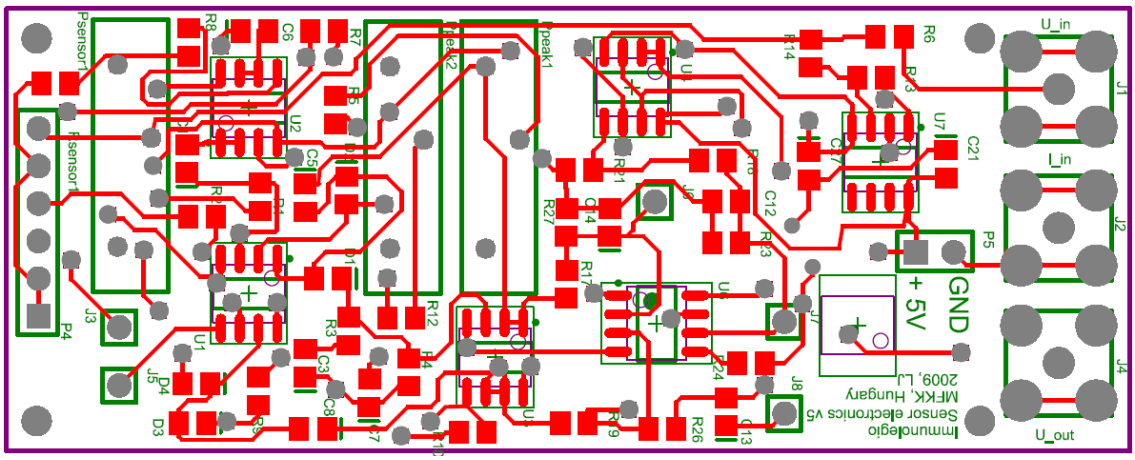
Appendix 1 – Detailed definition of the automated sample preparation steps

These process steps can be programmed and modified using setup software prowided for the IMMUNOLEGIO sample preparation device.

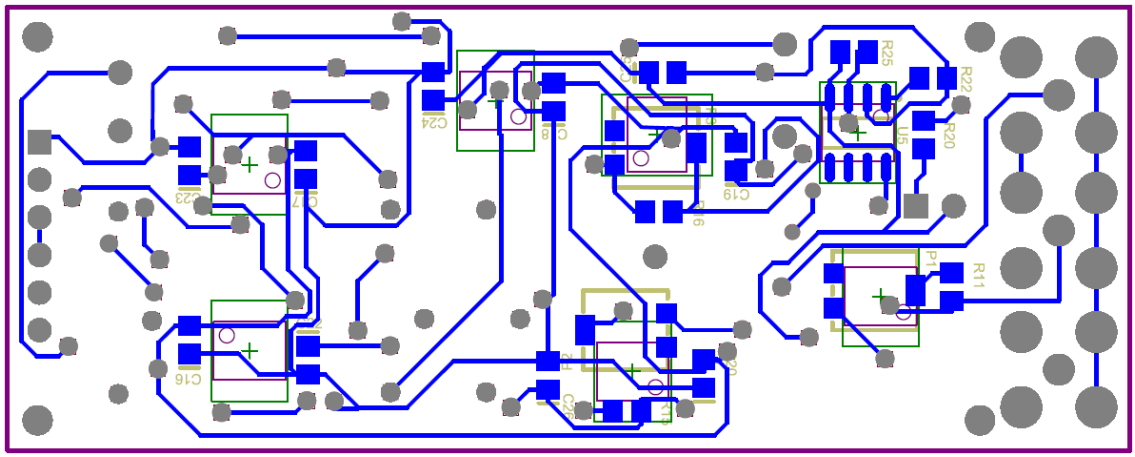
Process Steps			Controller	LEDs			Information to Display		Input requ.	Comment
No.	Main	Sub Steps	Function	1	2	3	Line 1	Line 2	from User	
Sample Preparation										
1		**Filtering**								
1	a	Get unique identifier	Get/Store to Memory							No. should be an incremantal unique number
1	b	Confirm preparation	Display/Input	x			*Preparation of*	*vessels ready?*	*Ok*	LED 1 marks user input required
1	c	Instruct user	Display/Input	x			*Fill funnel with*	*Sample! (pos.1)*	*Ok*	
1	d	Start Filterpump Pump	Pump 3 on/Display		x		*Refill funnel*	*continouosly!*		Display 3s / Pump 3 on / Display 5s / (LED 2 marks automated proces
1	e	Instruct user	Display/Input	x	x		*Press Ok when*	*funnel empty!*	*Ok*	
1	f	Stop Filterpump Pump	Pump 3 off/Display		x		*Starting Sample*	*preparation…*		
1	g	Calibrate XY-Axes	Calibrate XY 0-point		x					display unchanged / move Y first maximum up, before moving X !!
1	h	Move to Pos.1 - fill pos.	Move XY		x		*Resuspending*	*Sample…*		
1	i	Add 10ml fresh water (pos.1)	Pump 2 on-10ml-off		x					full speed, 10ml measured by pumping time
1	j	Move Pos.1 - pipetting pos.	Move XY		x					
1	k	Wash membrane (20 cycles)	Pump 1 alt. 3s bw/fw		x					alternating full speed backward 3s <-> full speed forward 3s / 20 cyle
					x					
2		**Pitpette sample from Pos.1 to Pos.2**								
2	a	Move to Pos.1 - pipetting pos.	Display / Move XY		x		*Pipetting Sample*	*to Pos.2 …*		
2	b	Pipette 2ml from Pos.1	Pump 1 bw.on-2ml-off		x					
2	c	Move to Pos.2 - drop pos.	Move XY		x					
2	d	Pipette 2ml to Pos.2	Pump 1 fw.on-2ml-off		x					
2	e	Repeat x 5 from 2a			x					
2	f	Change Pipette Tip	Display / Move XY		x		*Changing Pipette*	*tip…*		Complex movment combination, defined separatly
					x					
3		**Add Beads to Sample**								
3	a	Move to Pos.3 - pipetting pos.	Display / Move XY		x		*Adding beads to*	*Sample…*		
3	b	Pipette 1.1ml from Pos.3	Pump 1 on.bw-1ml-off		x					
3	c	Move to Pos.2 - drop pos.	Move XY		x					
3	d	Pipette 1.2ml to Pos.2	Pump 1 fw.on-2ml-off		x					
					x					
4		**Incubation (Beads)**								
4	a	Start Incubation Time countdown	Init Timer/Display		b		*Incubation Beads*	*Rem.time: 30 min*		Continously update remaining time display / LED 2 blinking
4	b	Move to Pos.2 - pipetting pos.	Move XY		b					
4	c	Air Mixing for 30s	Pump 1 fw.on-30s-off		x					
4	d	Repeat from 4c every 3 min			b					
					x					
5		**Washing**								
5	a	Move to Pos.2 - pipetting pos.	Display / Move XY		x		*Washing sample…*			
5	b	Air Mixing for 30s	Pump 1 fw.on-30s-off		x					full speed
5	c	Activate Magnet	Move XY		x					Complex movment combination, defined separatly
5	d	Move to Pos.2 - pipetting pos.	Move XY		x					
5	e	Air Mixing for 30s	Pump 1 fw.on-30s-off		x					full speed
5	f	Pump all water from Pos.2 (15ml)	Pump 1 bw.on-15ml-off		x					full speed / Tube must be pumped empty, so that air mixing is possibl
5	g	Deactivate Magnet	Move XY		x					Complex movment combination, defined separatly
5	h	Move to Pos.2 - fill pos.	Move XY		x					
5	i	Add X ml fresh water (pos.2)	Pump 2 on-Xml-off		x					X = 5 ml in first cyle / 1 ml in second cycle / 0 ml third cycle
5	j	Repeat x 3 from 5a			x					
					x					
6		**Resuspending and Pipetting Sample**								
6	a	Change Pipette Tip	Display / Move XY		x		*Changing Pipette*	*tip…*		Complex movment combination, defined separatly
6	b	Move to Pos.4 - fill pos.	Display / Move XY		x		*Resuspending*	*sample...(0.1ml)*		
6	c	Add 10ml fresh water (pos.1)	Pump 2 on-10ml-off		x					full speed, 10ml measured by pumping time
6	d	Move to Pos.4 - pipetting pos.	Move XY		x					
6	e	Pump 8ml from Pos.4	Pump 1 bw.on-8ml-off		x					full speed
6	f	Move to Pos.2 - special drop pos.	Move XY		x					
6	g	Pipette 0.1ml to Pos.2	Pump 1 fw.on-0.1ml-off		x					speed PWM 50% / controlled by time or encoder
6	h	Move to Pos.2 - top pos.	Move XY		x					
6	i	Pipette 1.2ml air	Pump 1 bw.on-1.2ml-off		x					
6	j	Move to Pos.2 - pipetting pos.	Move XY		x					
6	k	Pipette 1ml air to Pos.2 (air mix)	Pump 1 fw.on-1ml-off		x					speed PWM 70% / controlled by time or encoder
6	l	Pipette 0.2ml from Pos.2	Pump 1 bw.on-0.2ml-off		x					speed PWM 50% / controlled by time or encoder
6	m	Move to Pos.5 - drop pos.	Display / Move XY		x		*Pipetting Sample*	*to Pos.5 …*		
6	n	Pipette 0.3ml to Pos.5	Pump 1 fw.on-0.3ml-off		x					speed PWM 50% / controlled by time or encoder
					x					
7		**Incubation (Surface)**								
7	a	Start Incubation Time countdown	Init Timer/Display		b		*Incubation TS*	*Rem.time: 30 min*		Continously update remaining time display / LED 2 blinking
					x					
8		**Finishing Steps (desinfection)**								
8	a	Instruct user		x	x		*Fill Ethanol to*	*Pos.1 (15ml)!*	*Ok*	
8	b	Move to Pos.1 - pipetting pos.	Display / Move XY		x		*Desinfecting*	*Test-Stick (TS)*		

Appendix 2 – Schematic of the electronics developed and used in Immunolgio

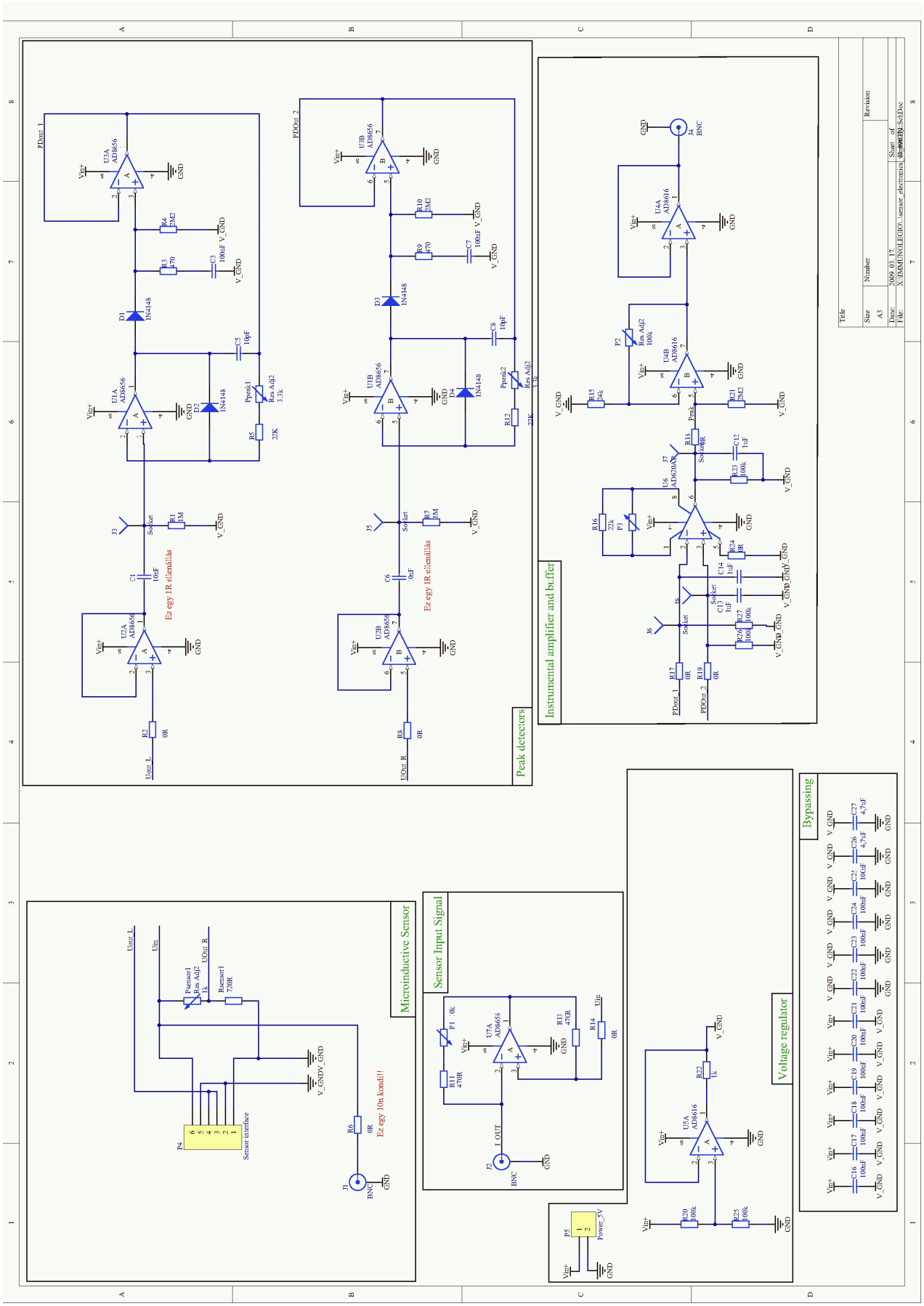
Sensor Electronics PCB layout top layer:



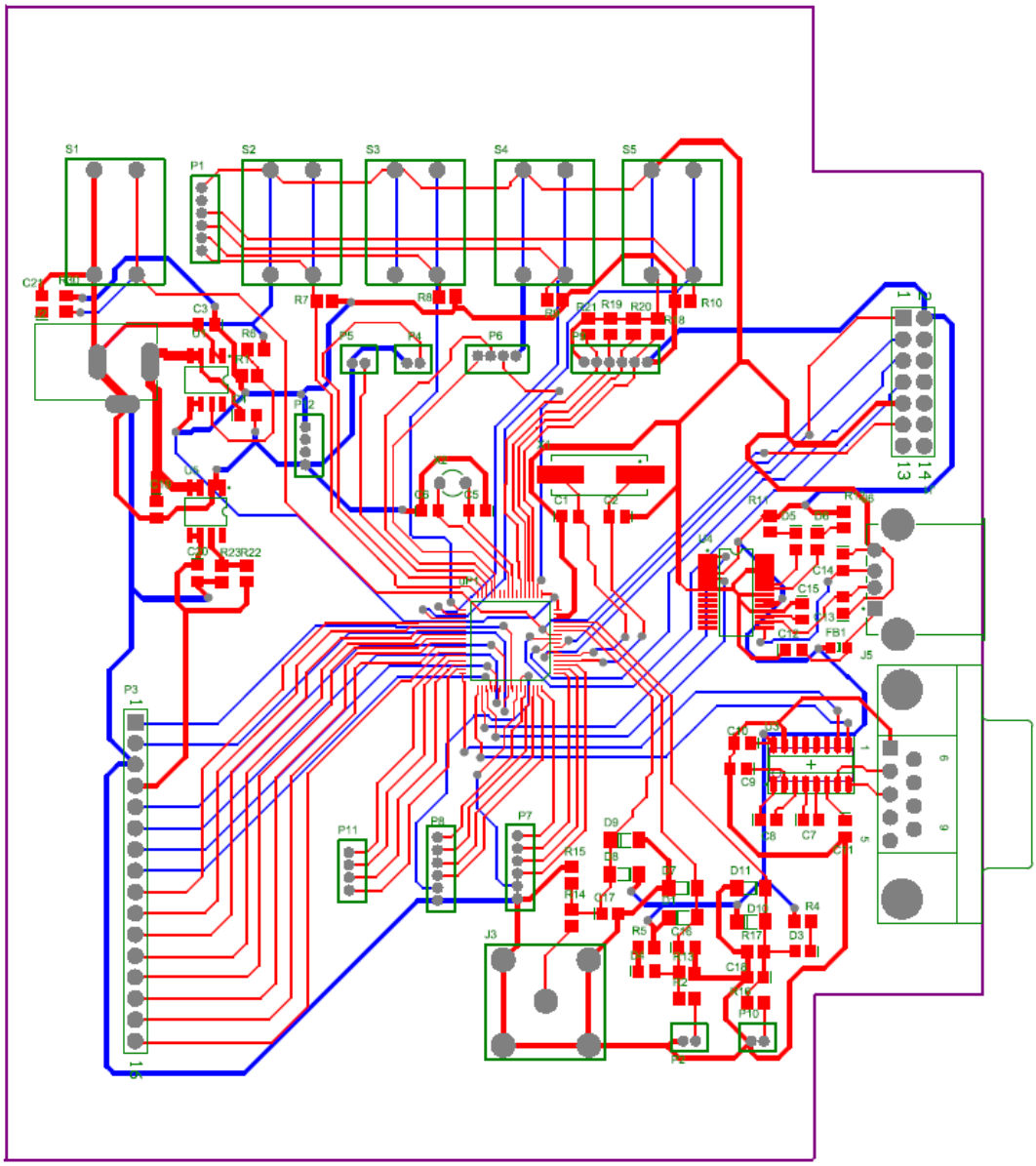
Sensor Electronics PCB layout bottom layer:



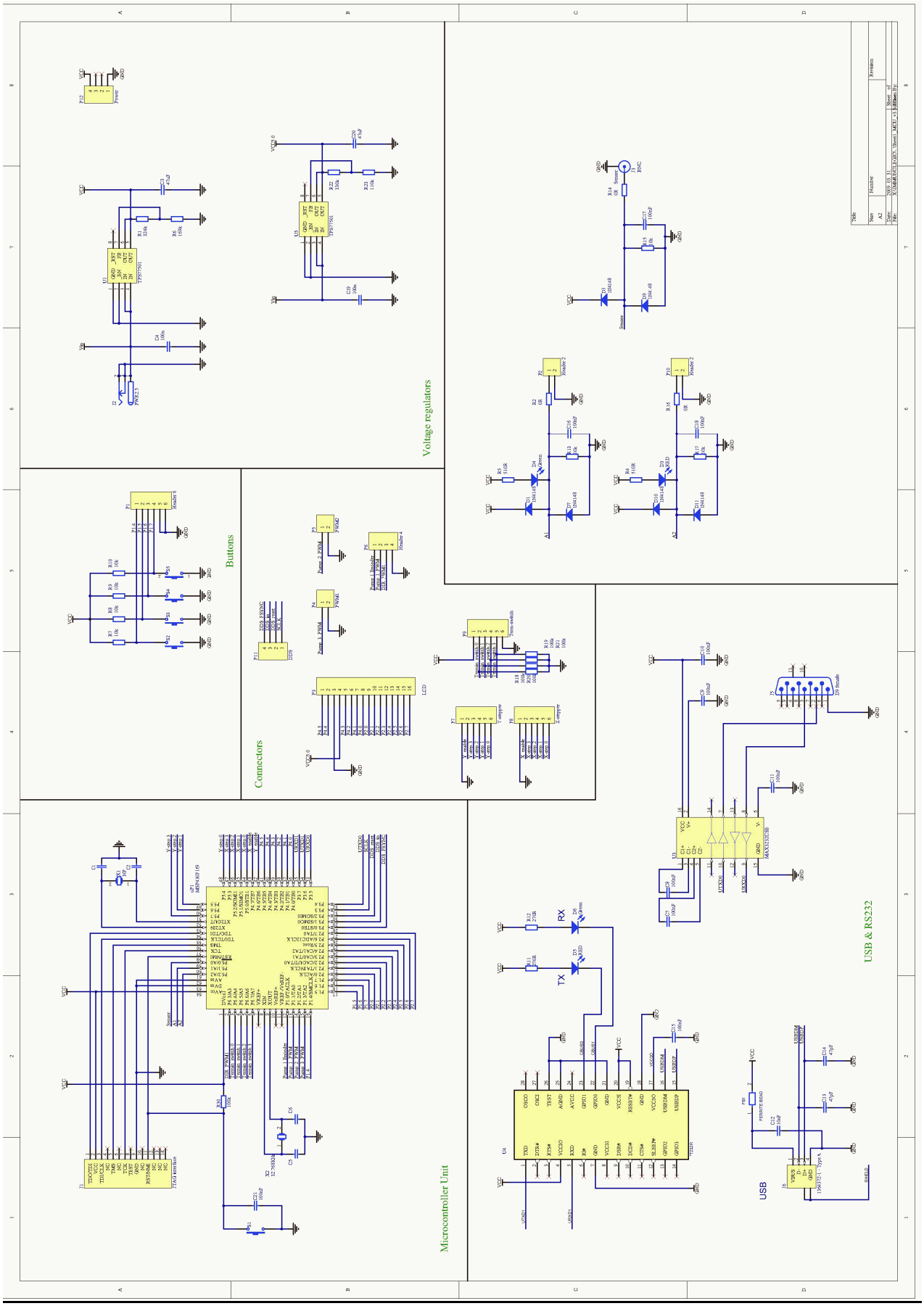
Sensor Electronics Schematics:



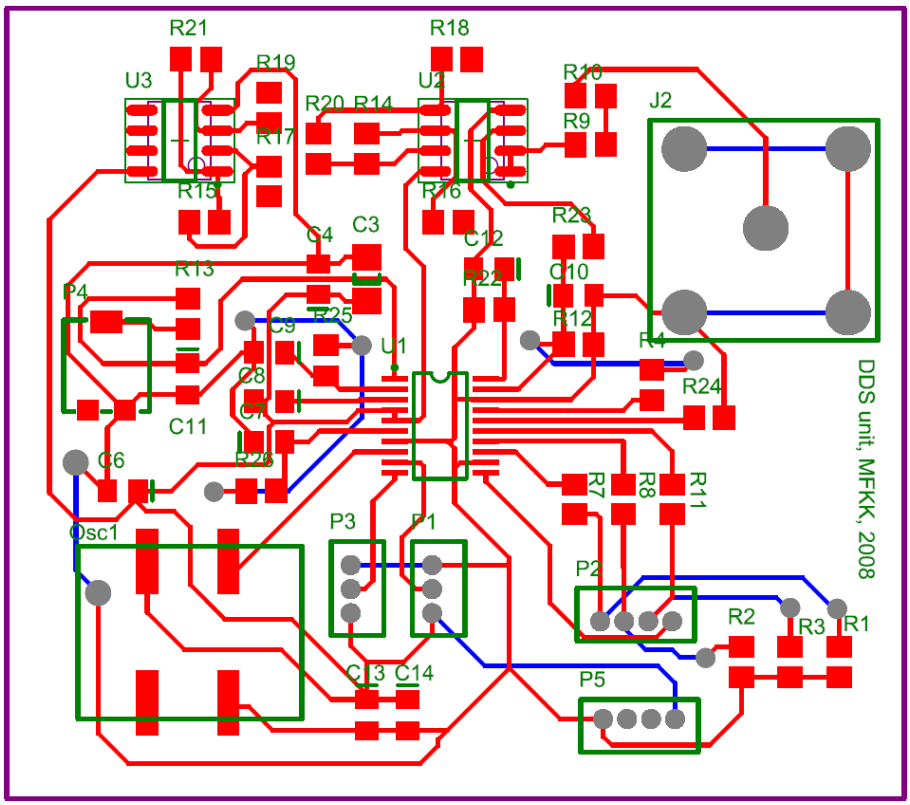
MCU Electronics PCB layout top and bottom layers:



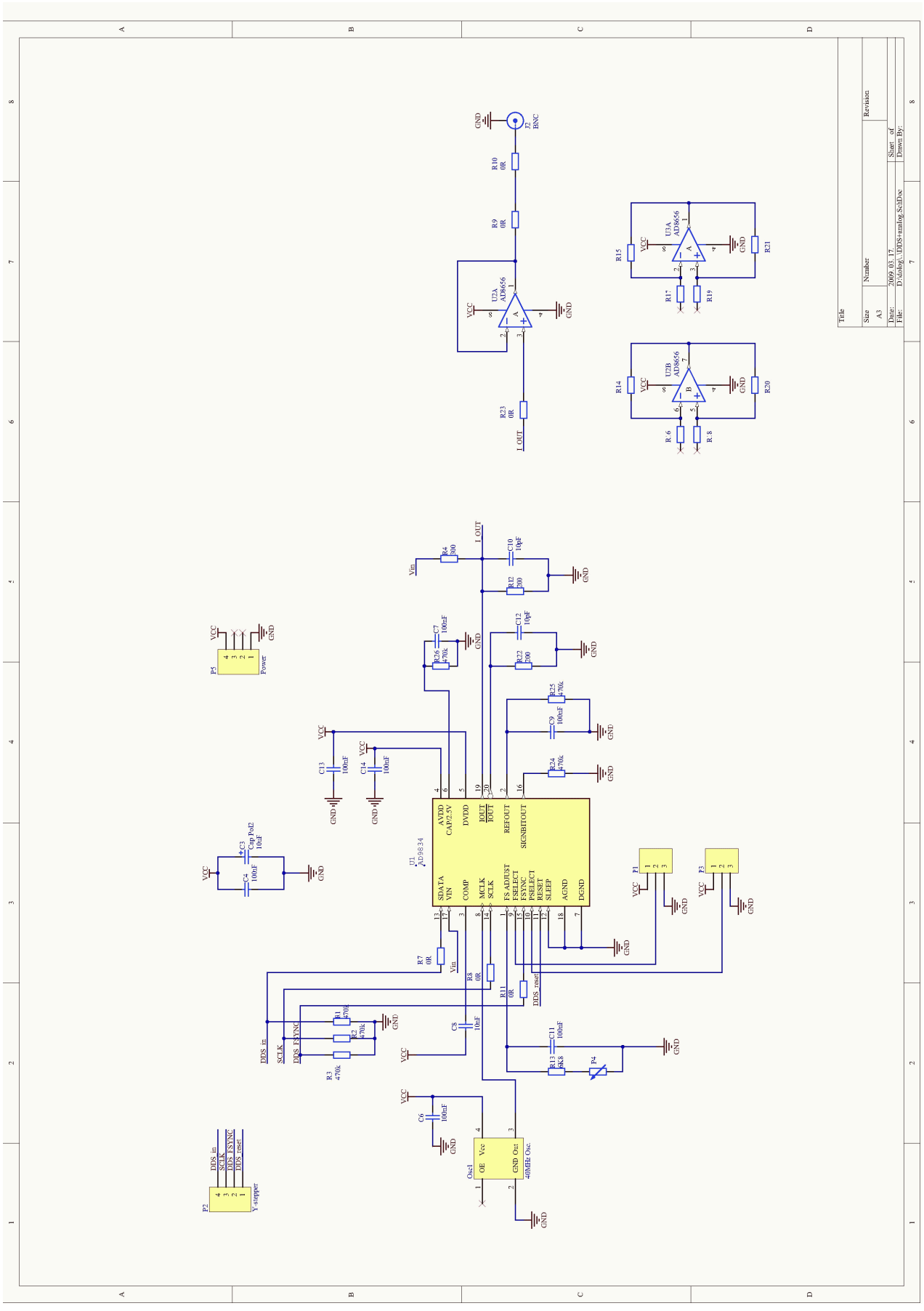
MCU Electronics Schematics:



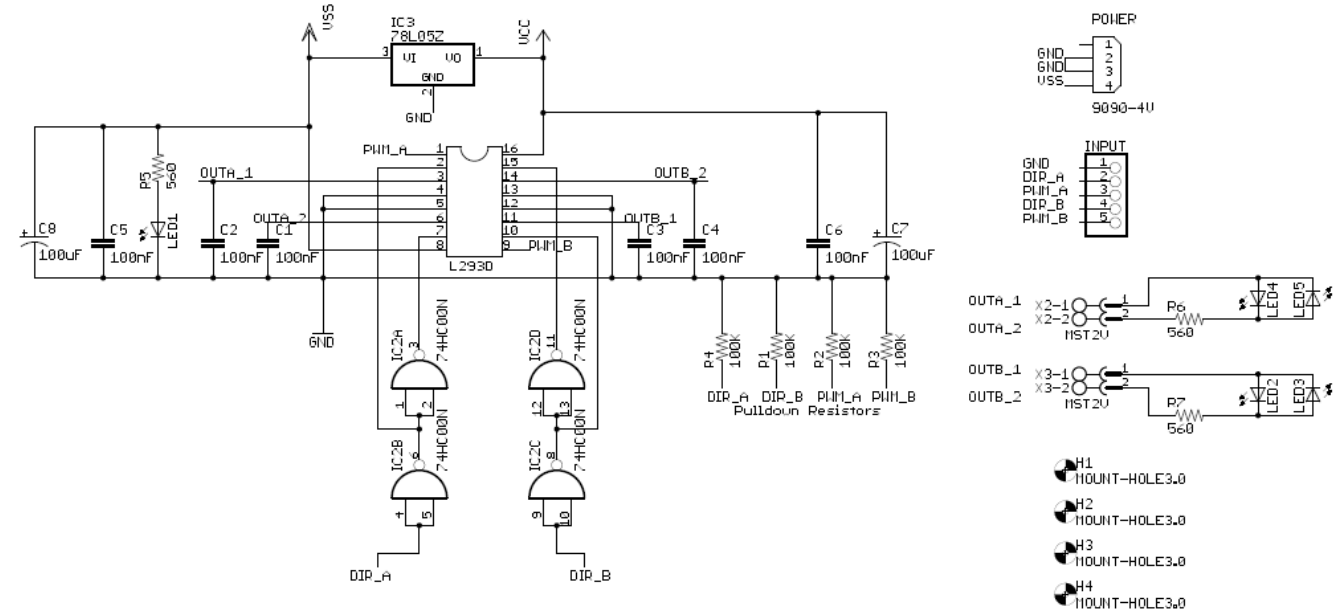
DSS Electronics PCB layout:



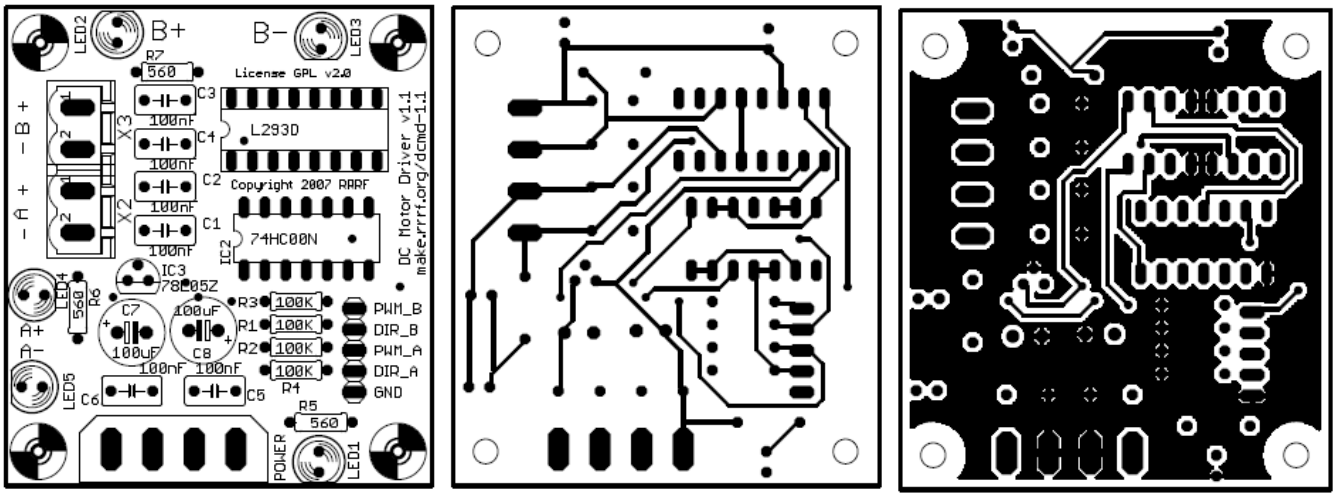
DSS Electronics Schematics:



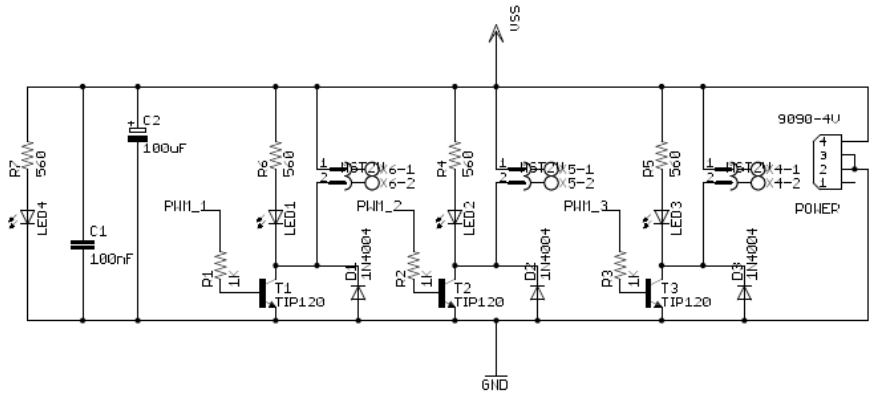
Two directional Motor Driver Electronics (for the Pumps) Schematics (source: make.rrrf.org/dcmd-1.1):



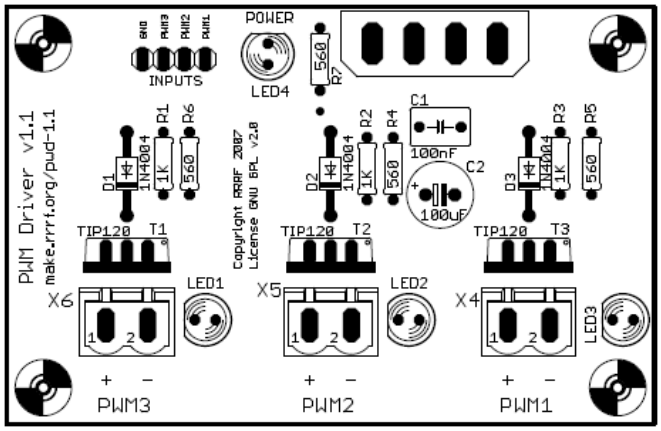
Two directional Motor Driver Electronics (for the Pumps) PCB layout (source: make.rrrf.org/dcmd-1.1):

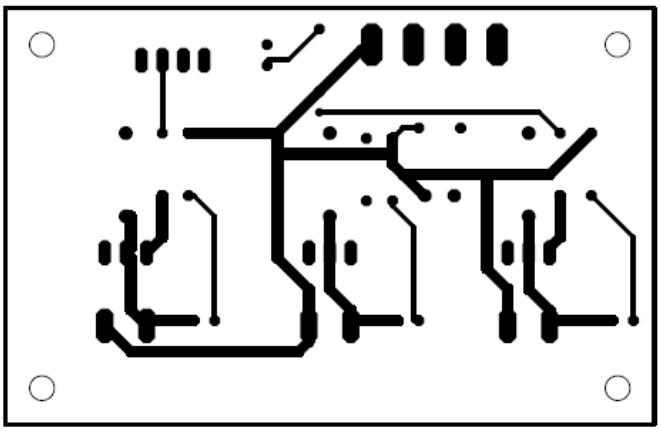


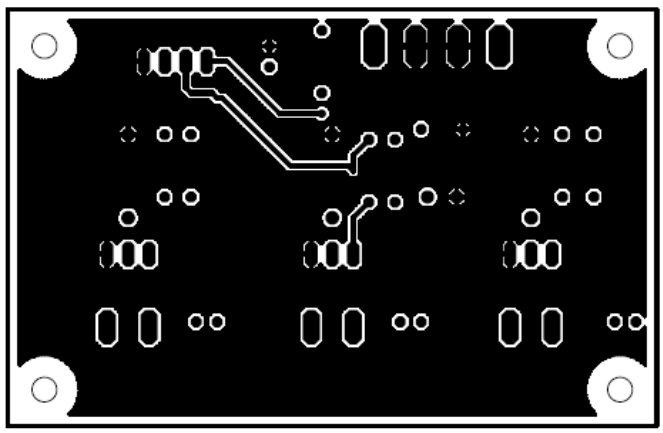
PWM Motor Driver Electronics (for the Pumps) Schematics (source: make.rrrf.org/pwd-1.1):



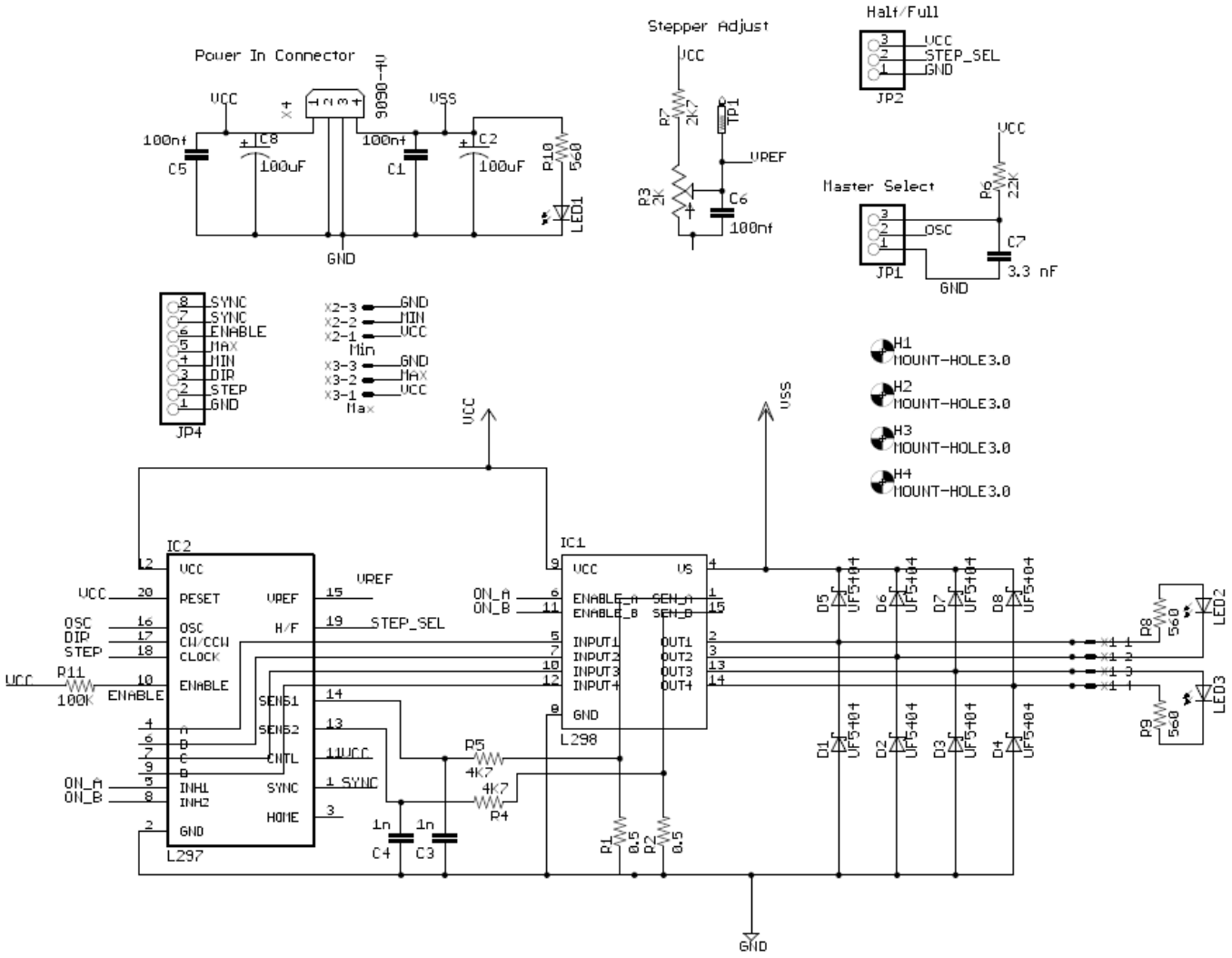
PWM Motor Driver Electronics (for the Pumps) Schematics (source: make.rrrf.org/pwd -1.1):







Step Motor Driver Electronics (for XY control) Schematics (source: make.rrrf.org/pwd-1.1):



Step Motor Driver Electronics (for XY control) PCB layout (source: make.rrrf.org/smd-1.1):

