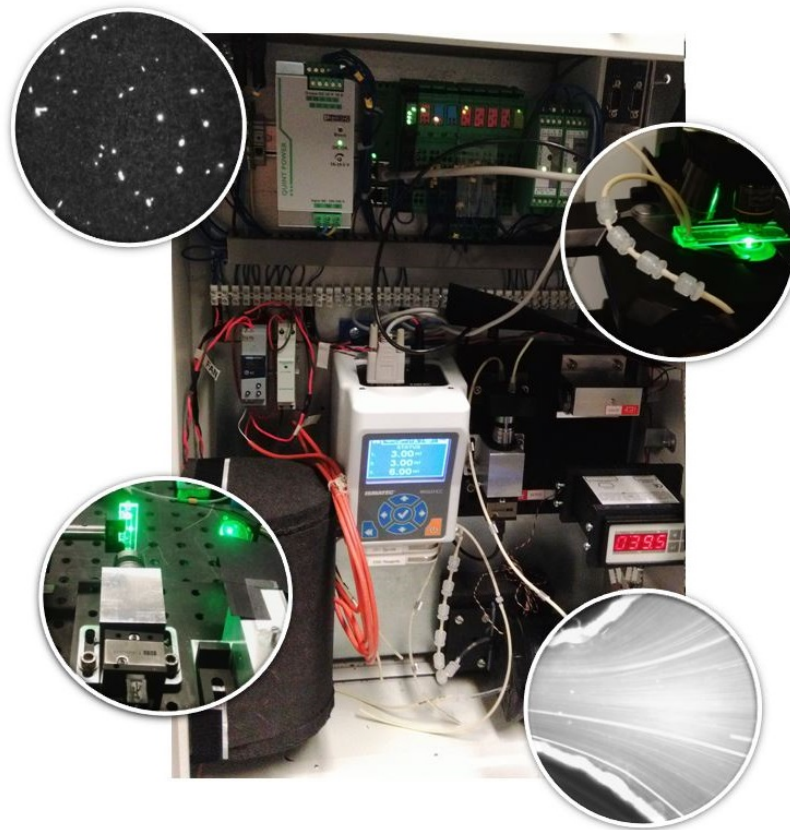




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# Water Quality Verification Through Optical Detection of Bacteria

A Study on the Application of Quantum Dots in a Flow  
Cytometric Immunoassay to Detect Escherichia coli in Water

Master's thesis in Nanotechnology

**PATRÍCIA FERNANDES BELLETATI**



MASTER'S THESIS 2016

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Immunoassay to Detect Escherichia coli in Water

PATRÍCIA FERNANDES BELLETATI



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Gothenburg, Sweden 2016

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to Detect Escherichia coli in Water  
PATRÍCIA FERNANDES BELLETATI

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Master's Thesis 2016  
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Cover: Picture of the developed sensor system, and illustrations of different experiments performed in this work. The picture in the top left shows an experiment with the immunoassay, and in the bottom left shows the set-up of the experiment with the flow cell. The top right shows the experiment performed with the mixing structure, and the bottom right shows a result of the mixing test.

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Department of Microtechnology and Nanoscience, MC2  
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## Abstract

Contamination in water supplies can occur and it results in the distribution of inappropriate water, affecting greatly the community well-being. Monitoring water utilities is key to avoiding possible health problems from this scenario. A conventional parameter to evaluate the quality of water is the concentration of *Escherichia coli*, a bacteria commonly present in sewage. Nowadays, the standard procedures for the counting of such micro-organisms are reliable but very laborious and time-consuming. Therefore they are not suitable for the demands of fast evaluation, which is necessary to circumvent any potential distribution of contaminated water. This masters thesis proposes to improve and automate the operation of a sensor system developed by Acreo ICT to monitor the water quality in water facilities. This sensor uses a quantum dot-based immunoassay for targeting the *E. coli* present in the water, and flow cytometry as a technique to detect and transform the obtained fluorescent signals into an estimated concentration value of the *E. coli*. First, immunoassay was performed by using IgY-conjugated quantum dots and IgY-conjugated organic fluorophore, which are used to label *E. coli* from lab strain and from sewage. The immunoassay was subjected to different solutions. Results of these immunoassay tests showed that the antibody-antigen reaction needs to take place in a saline environment for stronger binding. Furthermore, the combination of Triton X-100, BSA and EDTA in this system improved the specificity of the immunoassay and increased the signal strength. The increase of temperature up to 40°C accelerated the reaction rate, reducing the necessary time for the binding reaction. The sensor system was automated by the implementation of a proposed design of a mixing structure based on continuous flow. Furthermore, an improvement in the flow cytometry was made by replacing the former flow cell made of plastic to one made of quartz, which also has a smaller optical path. Lastly, tests using tap water and sewage showed that the final assembled sensor can be used to detect contamination of drinking water with sewage. With these improvements, the sensor system is, at the time of publication of this thesis, being subject to in situ tests in water facilities in Jerusalem and Zürich.

Keywords: Quantum Dots, Immunoassay, Flow Cytometry, Water Quality, *Escherichia Coli*, Faecal Contamination, Sensor System, On-line Monitoring.



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# List of Abbreviations

BSA	Bovine Serum Albumin
C <sub>H</sub>	Constant domain heavy chain
C <sub>L</sub>	Constant domain light chain
click reaction	Cu(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition reaction
CMOS camera	Complementary Metal Oxide Silicon camera
dH <sub>2</sub> O	Deionized Water
DIBO	dibenzocyclooctyne
DNA	deoxyribonucleic acid
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
EU	European Union
F(ab') <sub>2</sub>	Fragment antigen-binding
Fc region	Fragment crystallizable region
FISH	Fluorescence in situ hybridisation
FP7	7 <sup>th</sup> Framework Programme for Research and Technological Development
fps	frames per second
Gal-T	$\beta$ -1,4-galactosyltransferase
GalNAz	N-azidoacetyl-galactosamine
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
LED	Light Emission Diodes
LP filter	Longpass filter
LPS	lipopolysaccharide
M	molar
N-glycan	N-linked glycosylation
PB	phosphate buffer
PBS	Phosphate Buffer Saline
pH	potential of Hydrogen
PMMA	Polymethyl methacrylate
PMT	Photon Multiplier Tube
QD	Quantum dots
SDS	sodium dodecyl sulfate
UDP	uridine diphosphate
V <sub>H</sub>	Variable domain heavy chain
V <sub>L</sub>	Variable domain light chain

## List of Abbreviations

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VC	Viable Count
WVZ	Wasserversorgung Zürich



# 1

## Introduction

It is of utmost importance that the population has access to clean water to avoid water-borne diseases. However, due to accidents such as sewage leakage or even acts of bioterrorism, water supplies can get contaminated, resulting in the distribution of inappropriate water. Studies estimate that approximately 1.8 billion people in the world consume water with faecal contamination [1]. This number is alarming since several diseases such as cholera, diarrhoea and dysentery are caused by the consumption of infected water, and that approximately half a million deaths each year are caused solely by diarrhoea [1]. Therefore, in order to track and minimize these health issues and death episodes, verification of water quality in water facilities is of major importance. SafeWater is an EU FP7 project with a focus on safeguarding drinking water from different contaminations. Acreo takes part in the project by developing a system for quantification of the quality of water.

The common way to verify water quality is by checking for the presence of microbial contaminants that causes diseases, such as the presence of faecal bacteria. Currently, the standard procedures for bacteria detection are laboratory tests based on culture methods, which the Viable Count (VC) method is an example of a typical procedure. Even though these procedures quantify the bacteria very precisely, they usually consist of several steps, being very laborious and time consuming [2, 3]. Therefore they are not suitable for the demands placed on early warning systems in order to avoid potential water-borne diseases.

The foundation of these early warning systems is the prevention of catastrophes by giving notice of a threat with sufficient time, hence allowing for the proper treatment measures to be taken. A common approach for the implementation of such systems is by on-line monitoring. For the studied case, on-line monitoring means the monitoring in nearly real-time of the quality of the water in water utilities. Therefore if any contamination is taking place, an immediate notice is made and the proper care is taken to avoid that contaminated water is distributed to the community.

To automate the on-line monitoring for this case, a combination of biological recognition methods with detection technology must be used. One of the available techniques for biological recognition is the so called immunoassay. The immunoassay is based on the use of antibodies to identify specific organisms and generate a measurable signal. It is very common for this signal to be fluorescence emitted from organic fluorophores that are conjugated to the antibodies. On the other hand, with the advancements in nanotechnology, nanomaterials can be developed to present properties

similar to that of the organic fluorophores but with greater benefits to the performance of the immunoassay. Thus, these nanoparticles can assist in the detections and diagnosis present in biological recognition. An example of such nanoparticles are the quantum dots. Quantum dots are synthesized with such properties that enables them to be used as fluorescent dyes for immunoassay.

For the detection system needed, flow cytometry is the technology used by Acreo. Flow cytometry uses a laser system to count organisms in a flow. Traditionally, this technology is capable of detecting one cell at a time. However, one important aspect of the system developed by Acreo is that it is capable of detecting several bacteria simultaneously, thus speeding up the detection process. Therefore, this system could be used for early warning of raw water quality problems, which would be very useful for the water facility to adjust the treatment on demand, i.e. as input for process control.

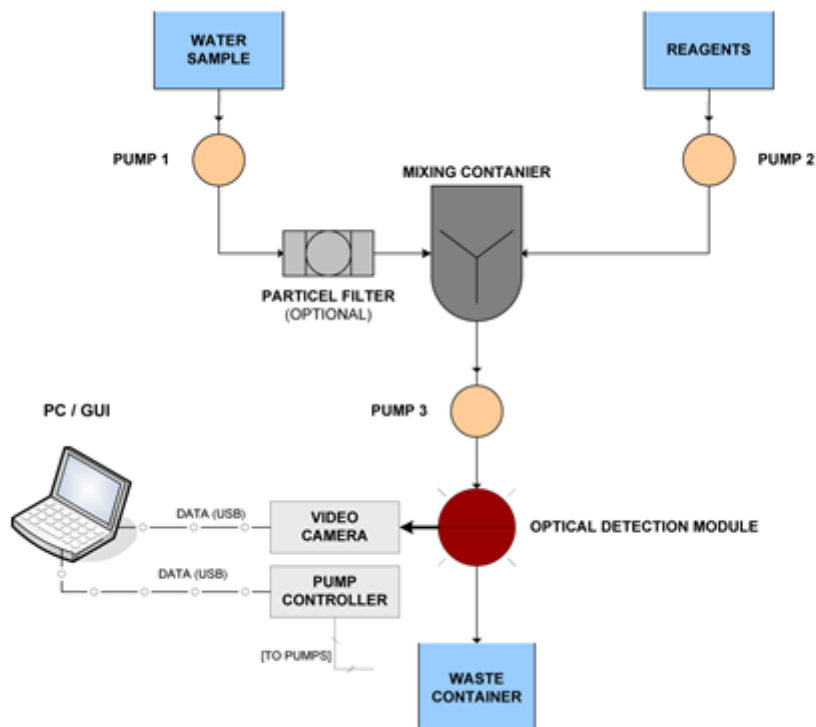
With the solution of uniting the immunoassay method with the flow cytometry technique, a sensor system is being developed by Acreo to achieve the desired automated on-line monitoring. This system is thus used to detect and quantify the “faeces bacteria” *Escherichia coli* in drinking water. This is the bacteria recommended to be used as an indicator for sewage infiltration/contamination in water, and is therefore used here for this purpose.

In the ongoing project, Acreo has already designed and tested a first version of the sensor system in the water utility in Zürich, Wasserversorgung Zürich (WVZ). Based on the experiences from this field test, a new improved system was designed. The functioning of the first version is explained in the next section.

### 1.1 Background

In this section, a brief explanation of the first version of the sensor system, as well as the starting point for this Master thesis work is briefly presented.

In the first version of the sensor, the sample water is submitted to a quantum dot-based immunoassay, to label each *E. coli* bacterium present in the sample. This was done by pumping the water and the antibody-conjugated quantum dots into a mixing container, where they were mixed with a magnetic stirrer during a determined incubation time. After this period, the solution was consequently pumped to the optical detection module. There, the water with the quantum dots passed through a plastic (PMMA) flow chip, with a green laser beam focused perpendicular to the flow. Through the use of a camera, the system recorded the fluorescence signals of each bacterium, and, after data processing, returned the bacteria concentration. Figure 1.1 illustrates the functioning of the sensor.



**Figure 1.1:** Schematic view of the functioning of the previous sensor system developed by Acreo. Picture taken from reference [4].

Acreo has already developed two different variants of flow cytometers, one video-based and one PMT based (Photon Multiplier Tube). These flow cytometers were designed in earlier projects and had as a requirement the ability to perform diagnostics at a very low cost. The focus on low cost was important to make the system affordable in all locations, and is also relevant for the quality monitoring of distributed water.

This master thesis project involves work in different functional parts that build up the sensor. The focus of these individual works is the improvement of the performance of the system as a whole. Hence, results in this thesis are of the highest importance for the SAFEWATER project. The aims for the improvements of each specific functional part are described in the next section.

## 1.2 Aim

The main objective of this thesis work is to improve two different functional parts that build up the whole sensor system. One of these parts is oriented towards the biochemical aspects of the sensor system and the other part is oriented to the optical/physical aspects.

For the biochemical aspects, the aim is to obtain a high degree of specificity for the

recognition of the *E. coli* by immunoassay, without harming the generated signals from them. Thus, studies on different buffer solutions which hold the reagents to be mixed with the sample water are performed. Variations on the conditions used for the reaction between antigens and reagents are studied to acquire stronger binding between them. Different chemical solutions are also studied with the desire to avoid cross-reactivity. Lastly, the parameters used for the incubation of the solution are studied to obtain an optimal relation between signal strength and time.

The second point aims to improve the system operation. This involves the automation of the procedure followed by the sensor, and the improvement of the performance from the flow cytometry to collect the signals from the stained bacteria. For this, a new concept to be used for the mixing between antigen and reagent is proposed, along with investigations on the aspects of this new concept. A study on the flow cell through which the sensor system obtained the measured signal is also performed. This study varies the material and design of this component in order to improve the optical performance from the flow cytometer.

With the unification of the results from all the proposed studies, a final evaluation of the sensor system as a whole is performed. Thus, with changes to improve the generation of the measured signal (in the biochemical part) and to improve the collection of this signal (in the optical/physical part), the main purpose to achieve a more autonomous system with high performance is obtained.

This report is organized in the following way: Chapter 2 presents the fundamental theory related to this work, as well as a deeper view of the earlier releases of the sensor system developed by Acreo. Chapter 3 describes the preparatory tests performed in this work and the main experimental procedure used. Chapter 4 presents the result for all the tests which focused on the optimization of the immunoassay. Chapter 5 describes the results of the experiments that acted upon the physical/optical parts of the sensor system. Chapter 6 has the description of the current assembly of the sensor and an evaluation of its final performance. And lastly, chapter 7 presents the overall conclusions of the work and possible future works related to this thesis.

# 2

## Theory

On-line monitoring of drinking water is based on tracking samples from the water utilities during different stages of the urban water cycle, ranging from water collected from the source to the end product ready for distribution to the consumers. For each stage, different properties of the water should be investigated [3, 6]. Hence, the sensor system used to monitor the water quality differs according to the indicator chosen for each of these steps.

The stage of interest in this thesis work, which is the stage Acreo has been developing its on-line sensor system for, is the final stage of the water supply system. Thus, the goal of the sensor system is to detect contamination of sewage in drinking water from water utilities. The first system developed used samples collected from WVZ.

There are many different microorganisms in sewage, and direct testing for each of these microorganisms would be inappropriate when considering the necessity of an effective, cheap and fast evaluation of their manifestation. Therefore, the determination of the indicator for contamination used in the studied case is very important. A common indicator for faeces, which is present in sewage [3, 5, 7], is the bacterium *Escherichia coli*. Since the natural habitat of the *E. coli* is the intestines of warm-blooded animals, these bacteria are considered to have high specificity when searching for presence of faeces. Due to this fact, EPA recommends using this pathogen as faeces indicator, and it is also the one chosen to be detected in this thesis work [7, 8].

For the development of the on-line sensor, Acreo selected a technique called flow cytometry. Generally, flow cytometry detects targets fluorescently labelled in a fluid stream. The main reason for this choice made by Acreo is that this technique has great potential to be close to an autonomous method, demanding little or no manual work provided that the labelling procedure can be simplified. In this project, the *E. coli* bacteria in the water sample are stained with fluorescent labels, thus allowing for their detection through flow cytometry. The bacteria labeling done here relies on a fluorescent based immunoassay.

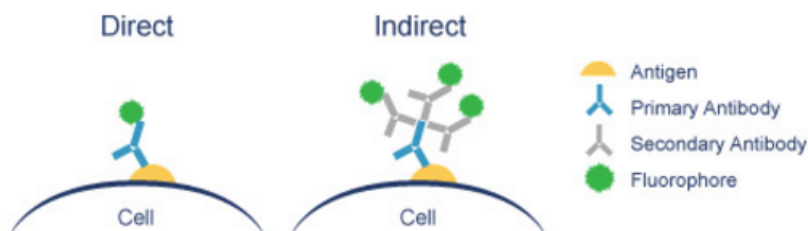
In this chapter, the relevant theory regarding immunoassay, fluorescence and fluorophores, antibodies and their functionalization, and the flow cytometry technique are presented. This is then followed by a section that describes in more details the function of the earlier releases of the sensor made by Acreo, as well as the weak points it presented and which were meant to be improved in this research work.

## 2.1 Fundamentals applied in this work

### 2.1.1 Immunoassay

Immunoassay is a detection method consisting mainly of immobilized antibodies that specifically bind the analyte (the target), and a detection system to generate the signal. In immunoassay the analyte can be another antibody or an antigen. Since the purpose of this work is to detect *E. coli* bacteria, here the analyte used are the antigens of the *E. coli* cell wall [10, 11]. For the detection system, there are several detection reagents which can be used to generate the signal, the one used in this work is the fluorophore [10], and a particle that has similar properties to that of a fluorophore called quantum dots. Fluorophores are fluorescent compounds, i.e., materials that absorb and emit energy in the form of light at specific wavelengths. More about this type of compound is explained later in this chapter.

Two different detection methods for fluorescent based immunoassay can be performed, the direct method and the indirect method. The direct method consists of an antibody conjugated with a fluorescent reagent, which binds into the analyte. The indirect detection consists of two antibodies; the primary antibody, a unconjugated antibody that binds into the target, and a secondary antibody, which is fluorescently labelled and binds to the primary antibody. Figure 2.1 illustrates both detection methods [11].



**Figure 2.1:** Illustration of both the direct and indirect detection modes. Picture taken from reference [12].

Both of these detection techniques were used in this work to label the *E. coli*, however the sensor system is based on the direct detection only. Some disadvantages of indirect detection when compared to the direct labelling are the needed time, since it consists of additional steps due to the presence of both a primary and a secondary antibody, and the specificity, where cross-reactivity of the primary and the secondary antibody can occur [12]. In both these methods, specificity and signal intensity can be enhanced by improving the conditions for the antibody-antigen reaction [13]. It is important to note that the stability of the fluorophore and quantum dot also depends greatly on the buffer media.

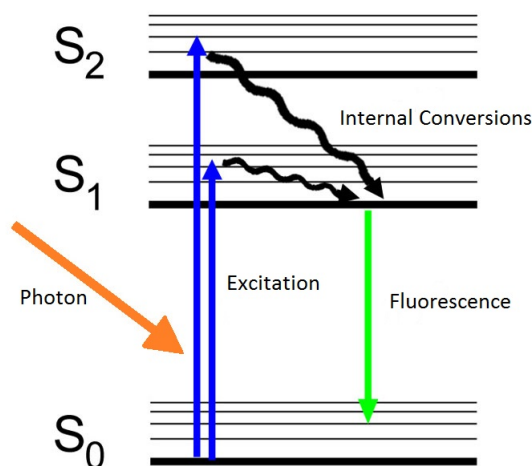
In this thesis, a deep investigation upon the best buffer media was made, as well

as a study upon assay optimization for the specific compounds present in the immunoassay and detection. This is further discussed in the next chapter.

### 2.1.2 Fluorescence and fluorophores

Certain materials and compounds present a peculiar characteristic in which, when excited by an electromagnetic radiation with a certain wavelength, they emit electromagnetic radiation back at different wavelength. This emitted light is known as fluorescence and the materials as fluorophores [14, 15]. As aforementioned, the immunoassay performed to detect the bacteria in the water sample relies on using this type of material as the detection reagent. In this subsection, the fluorescence phenomenon for two types of fluorescent compounds studied in this work, the organic fluorophores and the quantum dots, will be described.

For the case of the organic fluorophore, when reached by a photon, there is a possibility that this photon is absorbed by the fluorophore. This possibility relies on the energy of the photon as well as the electronic state of the molecules within the fluorophore. This concept is illustrated by the Jablonski diagram, presented in figure 2.2. As shown in the diagram, the necessary energy for the absorption to take place is equal to the difference in energy between two of the sublevels of the compound's electronic states: the ground ( $S_0$ ) and one of the excited states ( $S_1$  and  $S_2$  are the examples given in the diagram). These electronic states of the material are related to the electron energy and spin orientation, and its sublevels correspond to the vibrational energy states [14].



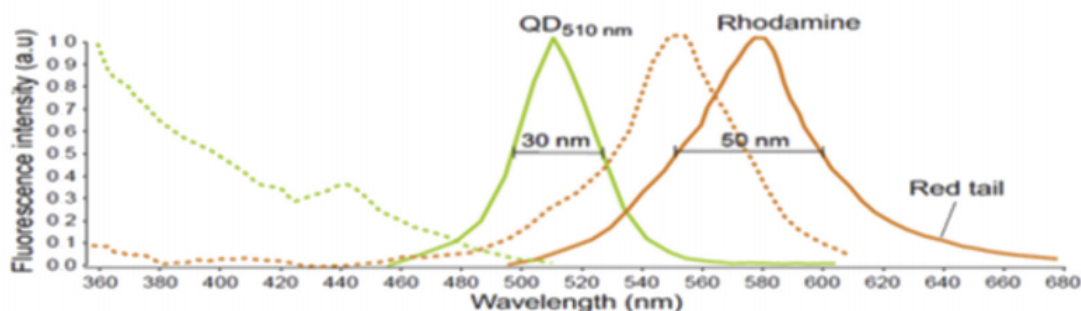
**Figure 2.2:** Jablonski diagram illustrating the fluorescence phenomenon. In the picture the blue arrows corresponds to the excitation of the electron and the green arrow is the return of the electron to the ground state by emitting fluorescence.

The black arrows corresponds to energy lost due to internal conversion. The orange arrow represents the incoming photon. Picture taken from reference [16].

When absorption occurs, the electron jumps to one of the excited states but rapidly loses the energy due to internal conversion, and goes to the lowest vibrational sub-

level of the  $S_1$ . From there, the electron loses energy and goes back to the initial sublevel of  $S_0$ . The energy is lost by several different processes, where one of these processes is the emission of photons; henceforth referred to as fluorescence [14, 15].

It is important to highlight that the photons emitted have energy smaller than the initial energy of the absorbed photons, therefore having longer wavelengths as well [15]. The absorbance and corresponding emission spectra of a generic organic fluorophore has the form shown in figure 2.3.



**Figure 2.3:** Excitation and emission spectra for an organic fluorophore (Rhodamine) and for quantum dots. The green dotted line is the excitation for the quantum dot(QD) and the solid green is the emission spectrum of the QD. The orange dotted line is the excitation spectrum of the organic fluorophore and the solid orange line corresponds to the emission spectrum of the organic fluorophore. Picture taken from reference [17].

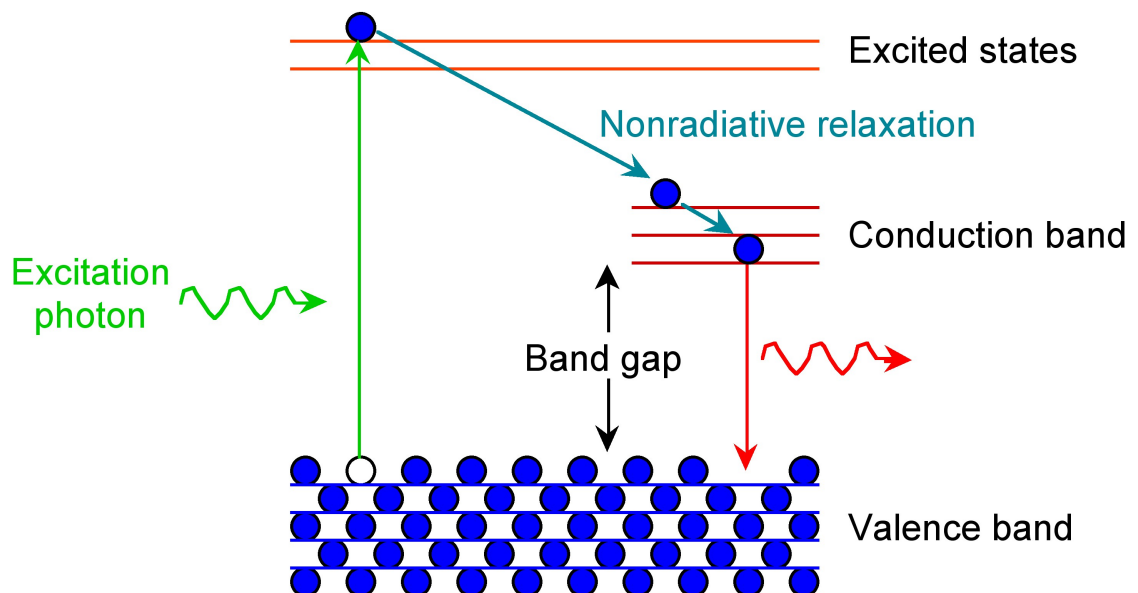
Some characteristics of organic fluorophore are the broad emission spectra, with small Stokes shift. This shift is the distance between the peaks of the excited and emitted curves. Therefore the emission and excitation peaks for this class of fluorophores have similar wavelength. This is a disadvantage for signal collection in applications using several fluorophores because spectral overlap can occur. In addition, these compounds suffer photobleaching: this is when the intensity of the emitted fluorescence decreases continuously until it is irreversibly lost [17, 18, 19].

Quantum dots (QD) are particles that are engineered to emit fluorescence. They are semiconductor particles, therefore their optical properties are very different from that of the organic fluorophores. Moreover, their sizes are in the nano range, thus being called nanoparticles. The range of the QD size is what makes this nanoparticle have different properties from semiconductor bulk. A semiconductor in the bulk range has continuous energy in the valence and conductive bands, which are the allowed energies for the electrons. These two bands are separated by an energy range which no electron can exist. This energy band is known as the band gap, and its energy range is intrinsic to the material. When an electron from the valence band is excited by a photon with energy equal or higher than of the band gap, it jumps to the conductive band, leaving a hole in the valence band. This hole behaves as having positive charge hence attracting the electron at the conductive band, which are then bound by Coulomb force. The bound electron and

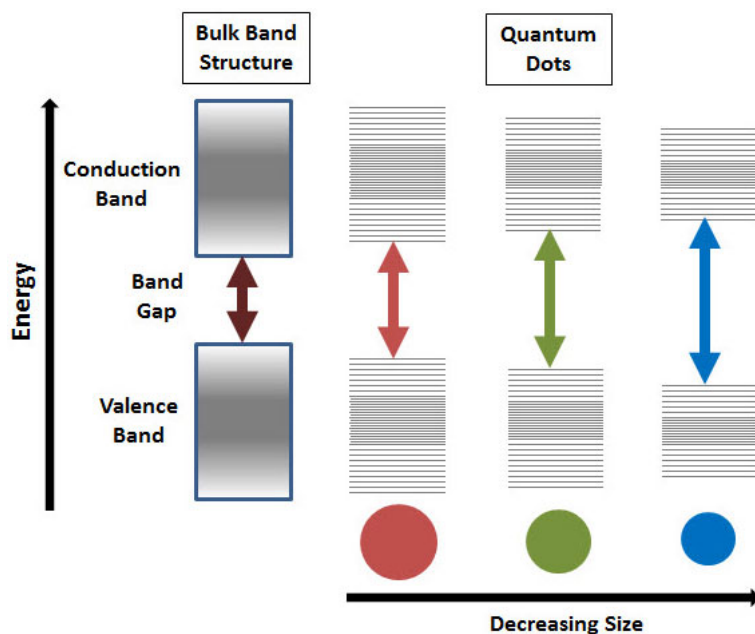


hole are known as exciton. The probable distance between electron and hole in an exciton is related to the Bohr radius of the material, which depends of the band gap.

For the case of the quantum dots, its dimension is smaller than that of the Bohr radius, which in result confines the exciton. This confinement is responsible for the discretization of the allowed energy states in the energy bands. Furthermore, the band gap energy is related to the nanoparticle size. Therefore, for these cases, when an electron is excited by a photon, it goes to an excited state in the conductive band and creates the exciton. The exciton then loses energy through non-radioactive relaxation reaching the bottom level of the conduction band. Finally, recombination of the exciton occur by dissipation of energy, released in the form of fluorescence, and the electron returns to the top of the valence band. The photon is only absorbed when its energy is bigger than that of the band gap [20]. This is illustrated in figure 2.4. Hence, the energy of the dissipated photon is related to the band gap energy. As aforementioned, the band gap is related to the QD size, therefore the fluorescence wavelength changes accordingly to the QD size. This is an important feature of the QD which enables the manipulation of the fluorescence wavelength. Figure 2.5 illustrates the relation between the band gap and the nanoparticle size. It is interesting to say that the smaller a QD is, the bigger its respective band energy will be [17].



**Figure 2.4:** Scheme of the energy levels for a quantum dot. After being excited, the electron leaves a hole in the top of the valence band. The green arrow corresponds to the absorbed photon, the red arrow corresponds to the dissipated energy in radioactive form, i.e. fluorescence. The blue arrows correspond to the dissipation of energy in nonradioactive form. The band gap size is illustrated with the black arrow. Picture taken from reference [21].



**Figure 2.5:** Comparison between the energy diagram of the bulk and nanoparticle semiconductor. It is also illustrated how the size of the quantum dot affects the band gap. Picture taken from reference [22].

The quantum dots pass through many of these excitation and emissions cycles, and sometimes the excited electrons do not return to the ground state. They go into another state in which they do not emit fluorescence. For the QD to emit fluorescence again, these electrons need to leave this state and return to the ground state in order to be excited again. This period in which no emission occurs is called blinking. This inherent characteristic of the QD is a drawback in application of single molecule analysis. However, this is irrelevant for the application present in this thesis work. [18, 19]

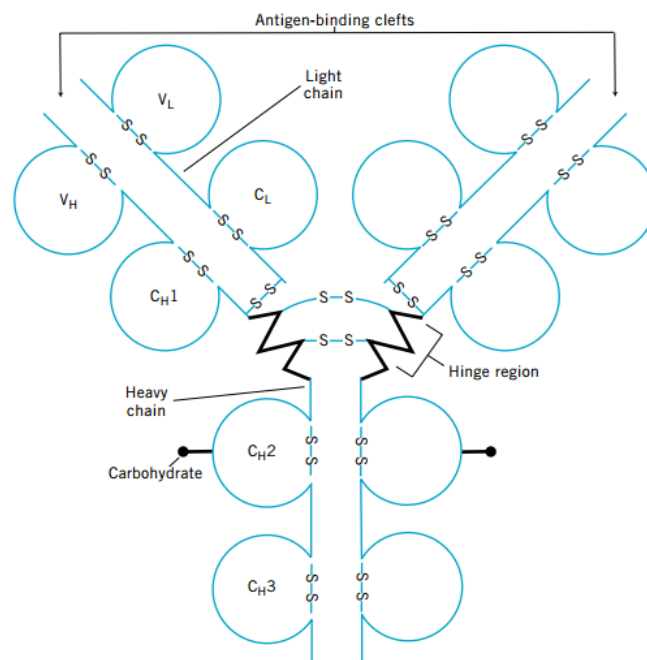
Quantum dots have long Stoke shifts due to a broad absorption spectra with narrow emission spectra, as illustrated in figure 2.3. This property is advantageous when detecting the fluorescence because it facilitates the distinction of the excitation and emission signals. In addition, it is possible to use several quantum dots with different emission wavelengths but at the same excitation wavelength, allowing for different organisms to be detected simultaneously in the same system [18, 19].

The lifetime of the QD depends directly on the size of the quantum dot. This increases greatly its usability, since it can be a part of the design of the quantum dot. Also noteworthy is that it has been estimated that the fluorescence lifetime of the quantum dots can get from 10 to 100 times more stable than some traditional organic fluorophores [19, 23]. This presents an advantage for applications where it is necessary to distinguish the signal from autofluorescence of cells [19].

### 2.1.3 Antibody structure and its functionalization

As stated previously, a fluorescent based immunoassay method is used to detect the bacteria in this thesis work. This section first presents an explanation about the structure of a typical prototype class of antibody, the IgG. This is done to understand how the antibody is functionalized with the fluorophore. Then a brief discussion about immobilization and functionalization of antibodies in immunoassay, and the steps followed in this thesis work, is presented.

Antibodies are Y-shaped proteins produced by and constituting an important part of the immune system. Their main purpose is to neutralize foreign substances such as pathogens. The antibody has several parts in its structure but can be separated into two major parts, as shown in figure 2.6: the bifurcated part, which is called  $F(ab')_2$ , and the stalk, which is called Fc. The  $F(ab')_2$  region has the binding sites for specific pathogens and the Fc interacts with the surface of the cell. [24]



**Figure 2.6:** Sketch of the IgG structure. Picture taken from reference [24].

Similarly to all proteins, the antibodies are formed by aminoacid chains. As indicated in figure 2.6, the antibody contains two types of aminoacid chains named as Light Chain (L) and Heavy Chain (H), where the names refers to the weight of the aminoacid chains. Besides that, antibodies also have the so called constant chain, which always presents the same aminoacids, and the variable chain, where the composition of the aminoacids changes. Figure 2.6 also shows the sites of the variable and constant domains, indicated by the letters V and C respectively. As further displayed in this figure, there is one variable domain ( $V_L$ ) and one constant domain ( $C_L$ ) in the light chain; and one variable domain ( $V_H$ ) and three constant domain

in the heavy chain ( $C_{H1}$ ,  $C_{H2}$  and  $C_{H3}$ ) [24]. There is a wide range of possible amino acid chains in the antibodies' variable domain. Therefore, a great diversity of antibodies exist. This is also responsible for the specificity of the antibody towards the pathogen [24, 25].

Due to the high specificity to various antigens, immobilized antibodies have been gaining more space in immunoassay for diagnosis and sensing. Here in this work, antibodies are immobilized onto fluorescent particles, to later bind onto the analyte to be detected. One drawback that needs to be considered when immobilizing the antibodies is that random orientation of the antibody on the surface will decrease the binding capacity [26]. To be fully functional, the antibody needs to be immobilized via its Fc region. Otherwise, the variable chains that carry the antigen-binding sites would not be available to bind. This is easier to understand by the illustration in figure 2.7. Therefore, orientation of the immobilization to maintain the antibody fully active is very important.



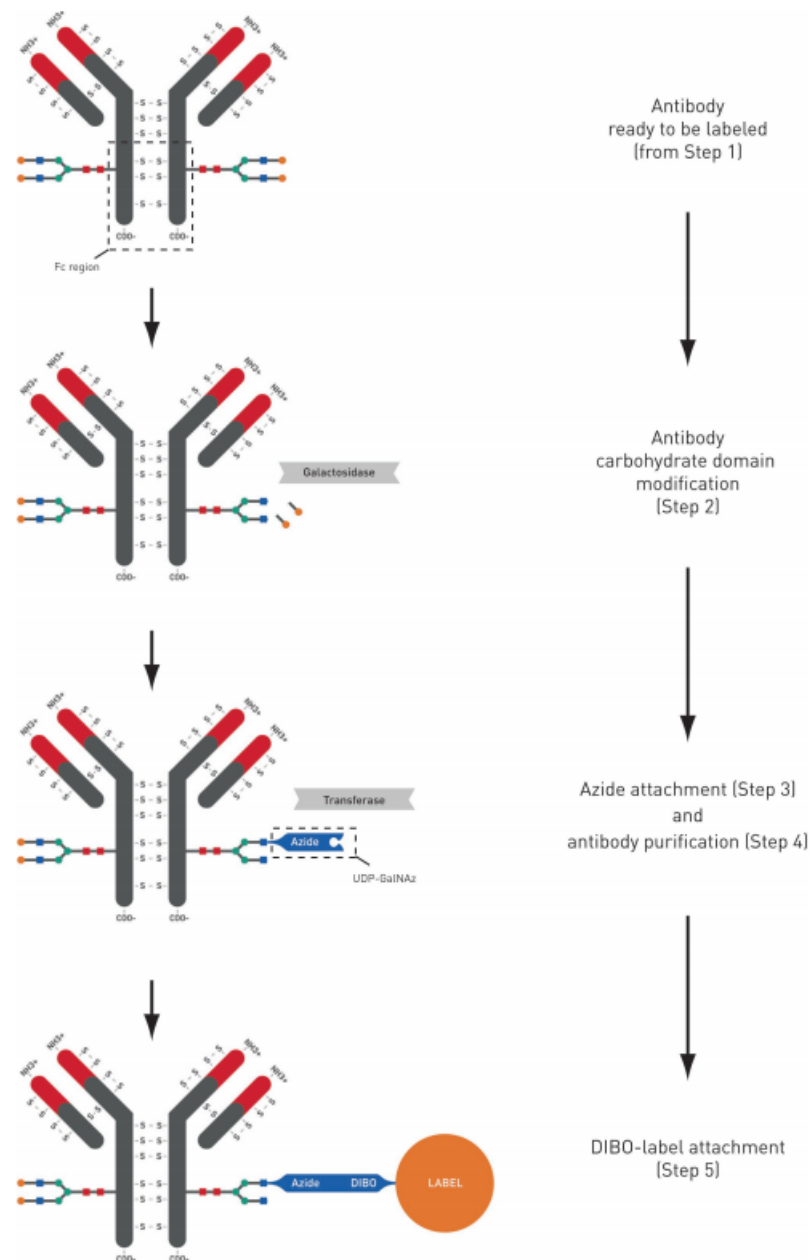
**Figure 2.7:** Possible orientations of the binding site of the antibody. Picture taken from reference [26].

There are several site-specific modifications of antibodies to immobilize them in an oriented fashion. One of these makes use of the heavy glycosylation of the Fc region. The sugar chains can be specifically modified to incorporate an azide group. This reactive group can subsequently be used to form covalent cross-linking to another reactive group located on a surface, or as in the case of this project, a quantum dot in the preferred orientation. Since the Fc region has no antigen binding site, the antibody is left fully active [26, 27]. This technique is the one used in this thesis work for binding IgY antibodies into quantum dots, where the sugar chains for this class of immunoglobulin are the N-glycan. [27, 28]

In the functionalization done in this thesis work a "click" reaction is used. The procedure for this consists of first removing the N-glycan, followed by azide (GaINAz) attachment in the Fc modified region by the  $\beta$ -1,4-galactosyltransferase (Gal-T)-catalyzed reaction. In the final step, DIBO-label attaches the azide moiety by a copper-free click reaction. This result in IgY antibodies conjugated with the desired organic fluorophore or quantum dot to later be used for detection in immunoassay [29]. Figure 2.8 illustrates these steps taken to functionalize the antibody.

This process allows for the use of a fluorescently tagged antibody to label E. coli, which then becomes detectable by the use of flow cytometry to identify the fluores-

cent signals.



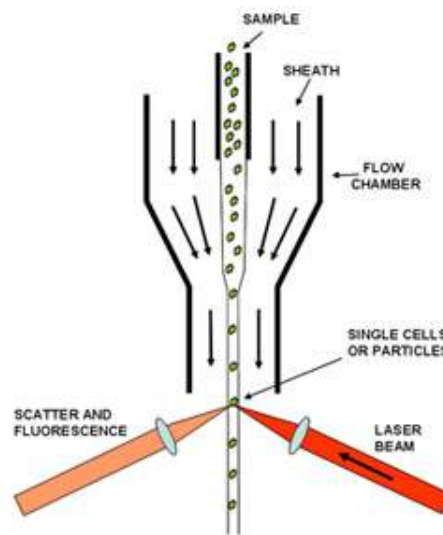
**Figure 2.8:** Diagram of the step-by-step procedure used to functionalize the antibodies with quantum dots used in this work. Picture taken from reference [29].

### 2.1.4 Flow cytometry

The sensor system developed uses the flow cytometry to detect the signal of the labelled E.coli. In this section, the most typical way of using this technique is described, followed by a brief discussions on why this system should be modified to better suit the main purposes of this work. Later, in another section, the modifications are better illustrated, clarifying how this technique is being used in the sensor

system developed at Acreo.

In flow cytometry, the chemical and physical properties of organisms in a fluid are measured. Three main components comprise the flow cytometry; the fluidic, the optical and the electronic systems. Generally, the fluidic system is based in hydrodynamic focusing, which is a method to focus the fluid containing the particles by means of a sheath fluid. The focused fluid, which contains the microorganisms to be detected, is known as the sample core and it is modelled by microfluidic physics due to its magnitude. The sheath fluid and the sample core are in laminar regime; therefore the adjacent fluids don't mix. This enables for the cells to be ordered, and due to the small extent of the stream, they are actually aligned in single file. In this way, single cells pass through the optical system [30]. Figure 2.9 illustrates a scheme of this fluidic system.



**Figure 2.9:** Scheme of the fluidic system in a traditional flow cytometry which is based on hydrodynamic focusing. Picture taken from reference [31].

Traditionally, the optical system is composed by a laser or a LED, and by emission filters. In this manner, each particle is excited by the light beam, and the fluorescence to be detected is selected into specific wavelength band. The bandwidth is chosen in a manner to isolate the excitation signals from the emission signals, since the fluorophore can have a broad emission and excitation wavelength spectrum [32]. Lastly, the fluorescence signals are converted into electrical signals, which are stored in a computer. Usually, PMT or photodiode are chosen to transduce the photons detected into electron current, to analyze the signal obtained in the flow cytometry [32].

The conventional flow cytometry is very reliable, with high sensitivity and can even be used for analysing different organisms at the same time, however it is very expensive. In this project, fundamental changes were made to the traditional flow cytometry described above. In the next section, the earlier versions of the sensor

are explained. Most of the alterations were made due to the need to design a cheap, fast and reliable device to be used in large scale.

## 2.2 Earlier releases of the Sensor System

Two variants of the sensor system were earlier developed at Acreo, one PMT based and one video based. Originally they intended to diagnose tuberculosis from sputum samples, aiming to obtain a cheap diagnostic system. In the SAFEWATER project, this technology is adapted to analyse water samples for presence of *E. coli* bacteria as indication of contamination of drinking water with sewage. The system not only returns data about the quality of the water, but also measures the concentration of the bacteria in it. It was previously shown that the video based is more suitable for this new purpose of the project.

The sensor system can be separated into three parts, the biochemical part which concerns mainly the target labelling, the instrument implementation regarding the flow cytometry and target detection, and the data analysis software, in which are all further discussed.

### 2.2.1 Biochemical part

As aforementioned, the labelling of the *E. coli* is done through antibody-based fluorescence. In this work, the antibody IgY was the one chosen to be used to recognize and bind into specific antigens of the *E. coli* bacteria. This immunoglobulin is uncommon in the research field, however it was chosen due to some relevant aspects: it has heavy glycosilation in the Fc region, which is an advantage when performing the click chemistry to immobilize these antibodies to the quantum dots; it is more stable than other antibodies, e.g. IgG; and lastly IgY with a specificity towards *E. coli* are found naturally in chicken eggs, an attribute that allows for the antibody to be harvested without harming an animals' well-being.

By click chemistry, the antibodies are first fluorescently labelled with quantum dots which are then later mixed with the water sample. In this manner, the antibodies will bind into the bacteria's antigens, which are present all over the bacteria's surface. Therefore, several fluorescent antibodies will be immobilized on each bacteria, making its surface fluorescent as well. Figure 2.10 shows a preliminary result of successfully labelled *E. coli*.

Even though the signal is quite strong, it does not show if cross reactivity with different bacteria other than the *E. coli* occurs. For that, this first release needed an optimization of the assay. The adaptations proposed in this work focused on achieving faster labelling, with high specificity and better signal-to-noise ratio.



**Figure 2.10:** Fluorescently labelled *E. coli* by immunoassay taken from fluorescent microscopy.

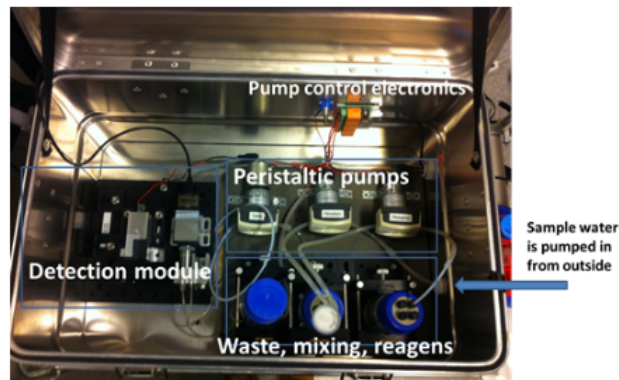
### 2.2.2 Hardware

The hardware of the first release of the instrument was composed of an optical detection module (the flow cytometer), three electronically controlled peristaltic pumps, the pump's control electronics and three vessels: one to store the reagents, one to perform mixing and one to store the waste. Figure 2.11a presents a photograph of the overview of this first release of the sensor's hardware.

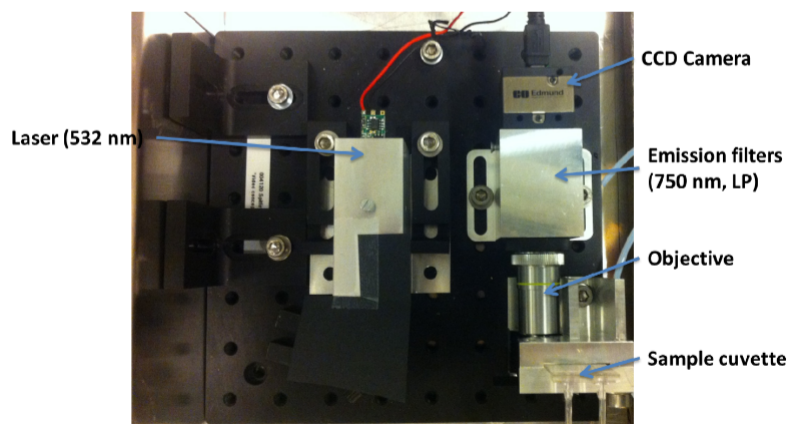
The sample water with the labelled *E. coli* is pumped into the flow cytometer, with a flow rate of 1 mL/min. As shown in 2.11b the flow cytometer was built containing a laser source of 532 nm (standard green laser light). The light from the laser is reflected by a mirror in a manner that the beam is directed into the lateral side of the sample cuvette (in this picture the mirror is covered). The sample cuvette is custom designed, with optical depth of 0.2 mm and made of PMMA, a transparent and rigid plastic. Figure 2.12 shows a scheme of this cuvette. In contrast to the traditional flow cytometry, the optical module developed did not focus the flow of the sample in order to detect one organism at a time. Thus, a sheath fluid was not used and there was no need to implement microfluidic technology in the fluidic system.

The cuvette stands in front of a CMOS camera coupled with longpass (LP) emission filter of 750 nm, and with a microscope objective of 10x magnification. The camera was used to record the signals from the labelled bacteria flowing through the cuvette. The volume of the flow was much higher in comparison to the used in the conventional flow cytometry, with the recorded volume in this set up being 1.25  $\mu\text{L}$  at each frame. This high volume enabled several organisms to be simultaneously detected, as opposed to the one-by-one cell counting of a traditional flow cytometry .



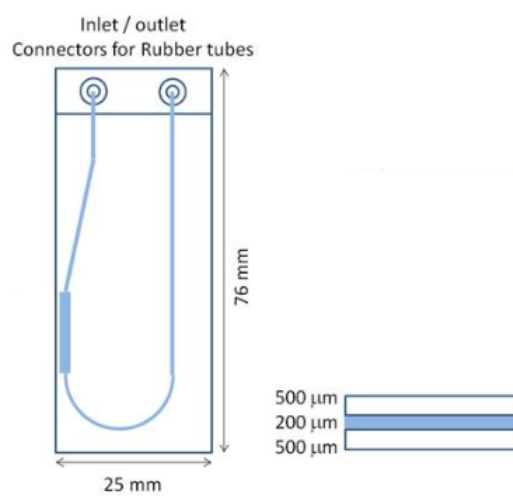


(a) Hardware



(b) Optical detection module

**Figure 2.11:** Pictures of the hardware components and the optical module from the first release of the sensor system [4].



**Figure 2.12:** Scheme of the cuvette used in the first sensor system.

### 2.2.3 Data collection

The continuous flow of labelled sample was recorded with a CMOS camera at a high frame rate (at around 12 fps), and was later analysed with the Line Counter software. This software was written in a manner that it recognizes the signal of the immobilized quantum dots in the *E. coli*, and differentiates it from the free quantum dots by setting an optimal threshold for the signal strength. Since the camera records the movement of the particles, several frames from the same particles are recorded as a video stream. Therefore, the software recognizes successive images from the same particles and concatenates it as one image from a single organism. Two different instances need to be considered when identifying each individual organism since they are flowing with a significant high flow rate (at around 1 mL/min). First, objects formed by adjacent pixels in the concatenated image are identified as a single bacterium, since their size is bigger than one pixel, and also their brightness can be spread in several pixels around the particle. Second, consecutive objects in the same direction as the flow are also considered as one bacterium, since it is necessary to account for the laminar movement of the particles.

The flow channel where the sample passes can get some fluorescent particles stuck in its surface. These trapped particles can interfere in the data processing since they could be counted several times. In this manner, when the sequential images are concatenated by the software, the set of images from the stationary particles are subtracted. This reduces substantially the background noise. Therefore, the software actively improves the signal-to-noise ratio by two means: by reducing the pixels' signals when performing image concatenation, and by deducting the background noise.

## 2.3 Weak points to be improved

This system was tested at Acreo with samples collected from WVZ. In the tests, pre-treated sewage, drinking water and more purified sewage were evaluated by the sensor system. According to what was expected, the sensor returned highest concentration values of the *E. coli* bacteria in the pre-treated sewage, and the lowest concentration in the drinking water. However, the measured concentration value for the drinking water was higher than expected. This result suggests that the quantum dots were not very stable after some time and aggregated together even before being mixed with the sample water to be analysed. The aggregated quantum dots were then also counted as bacteria, elevating the concentration value. This indicates that efforts should be put to increase the labelling agents' stability, therefore an assay optimization and blocking procedure are indispensable for improving the conditions for reaction.

With the preliminary tests, it was also clear that the choice of the peristaltic pumps also influence the data collection, in which there is a necessity for a smooth flow. Furthermore, the mixing between the reagents and the sample water by means of

magnetic stirring within a vessel makes the system slow and difficult to automate its cleaning. Therefore, better flow components and functions should be implemented, considering the necessity of automatic cleaning, which is fundamental to avoid biofilm growth, and climate control to increase reagents stability. In addition, improvements should be made at the optical detection module, and in the choice of the cuvette material. Even though PMMA is transparent, there is also some level of autofluorescence that can increase the noise, therefore it is necessary to test different materials to obtain an ideal cuvette.

The next chapter will present the preparations done before performing the main experiments in the work.



# 3

## Test Preparations

In this chapter, the preparations needed for the work performed in this thesis are described. These preparations involved the conjugation of the quantum dots to the antibodies, the conjugation of the organic fluorophores to the antibodies, the experimental procedure followed to perform direct and indirect immunoassay, and lastly, the selection of the *E. coli* strain to be studied in this work based on preliminary tests which will be also described here.

### 3.1 Conjugation of the quantum dots

The labelling of chicken IgY  $\alpha$ -*E. coli* with the quantum dots was done by Click Chemistry, following the SiteClick™ Antibody Labelling Kits protocol. This protocol is in the Appendix A. To modify the carbohydrate domain of the IgY, 10  $\mu\text{L}$  of beta-galactosidase were added to 125  $\mu\text{g}$  of IgY stock solution with concentration at around 2 to 2.5 mg/mL, and incubated for 4h at 37°C. To attach the azide onto the antibodies, an azide modification solution was first prepared by mixing 75  $\mu\text{L}$  of dH<sub>2</sub>O (MilliQ), 10  $\mu\text{L}$  of 20x Tris buffer (pH 7.0), 25  $\mu\text{L}$  of buffer additive present in the labelling kit and 80  $\mu\text{L}$  of GalT enzyme into the vial containing the nucleotide azide-sugar donor (UDP-GalNAz). The modified IgY solution (60  $\mu\text{L}$ ) was added to the azide modification solution and incubated overnight at 30°C. This solution is later purified by adding 1.75 mL of 1x Tris, pH 7.0 and centrifuged at 1200 x g over a spin-filter, and later concentrated by adding 1.8 mL of 1x Tris, pH 7.0 and centrifuged at 1400 x g until the final volume is less than 100  $\mu\text{L}$ .

Lastly, 50  $\mu\text{L}$  of DIBO-modified Qdot® nanocrystal is added to the azide-modified antibody, briefly centrifuged and incubated overnight at 25°C, resulting in a click-reaction where the azide-modified antibody is conjugated with optimal orientation onto the Qdot®. The labelled IgY can be stored at 2–8°C.

### 3.2 Conjugation of the organic fluorophore

To obtain a fluorescent secondary antibody to detect chicken-IgY, first it was prepared a direct labelling of the secondary Rabbit  $\alpha$ -chicken antibody onto the organic fluorophore. The labelling was performed by following the protocol of Alexa Fluor® 488 Labelling kit, this protocol is presented in Appendix B. This kit provides the necessary equipment to perform labelling reactions for small amounts of antibodies

(100  $\mu\text{g}$ ) of maxima excitation and emission wavelength values of 494 nm and 519 nm respectively. The dye has molecular weight of 855 g/mol.

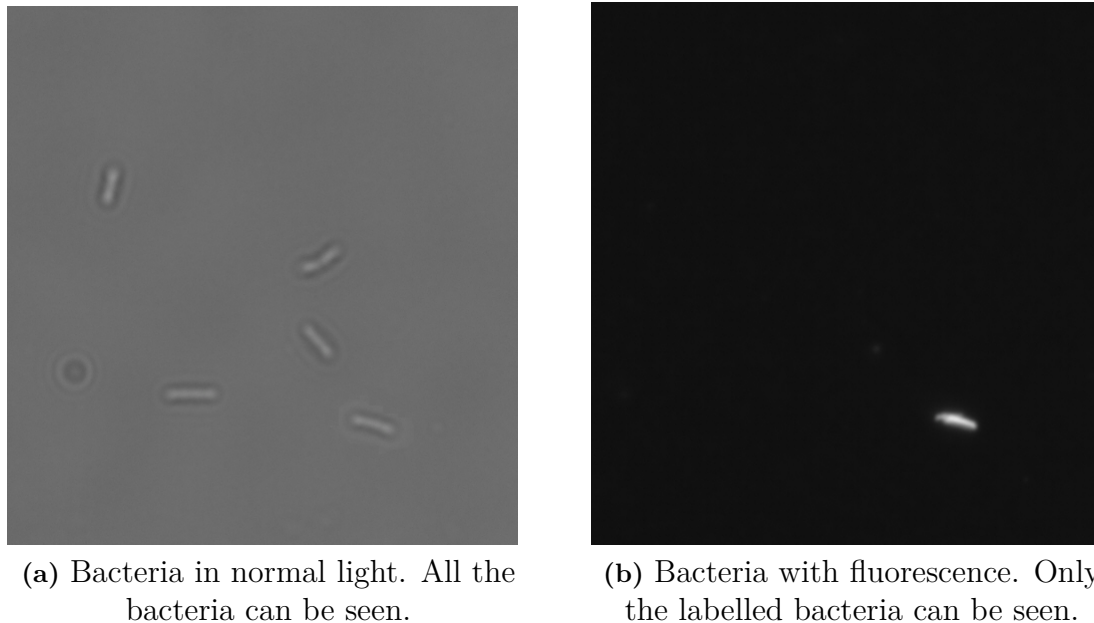
The brief steps followed from the protocol were: 100  $\mu\text{L}$  of 1 M sodium bicarbonate solution was mixed with antibodies with concentration of 1 mg/mL. 100 $\mu\text{L}$  of this solution was added to the vial of Alexa Fluor<sup>®</sup> dye present in the kit, mixed and incubated for 1 hour at room temperature. To purify the labelled antibodies, it was followed the size-exclusion chromatography method: first a purification is assembled by filling a spin column with 1.5 mL of the purification resin (both present in the kit) and centrifuging it at 1100 x g for 3 minutes, then the labelled antibodies are added in this spin column and centrifuged at 1100 x g for 5 minutes for collection. The labelled IgY can be stored at at 2–8°C. This last step of purification is important to remove the unbounded fluorophores, leaving in the solution only the conjugated antibodies.

### 3.3 Experimental procedures for assay optimization

In this thesis work, several experimental tests of direct and indirect immunoassay in different media and conditions were performed. These tests enabled the study of the best conditions for the reaction between the antibodies and antigens to take place in the sensor system.

The standard procedure followed for the tests when direct immunoassay was executed was as followed: the *E. coli* bacteria strain was first suspended in PBS (phosphate buffer saline). This solution is centrifuged for washing at 6000 x g for 4 minutes. The bacteria pellet is resuspended in a specific buffer, and mixed with 1:10000 antibody functionalized quantum dots. Subsequently, it is left for 30 minutes at room temperature for incubation. This is followed by a second washing procedure by centrifugation at 6000  $\times$  g for 4 minutes, and finally the suspension of the final pellet in PBS. Samples of this final solution were used for the evaluation of the immunoassay through the analyses of the images collected by fluorescent microscope coupled with mercury arc lamp and band pass filters. The microscope was equipped with a camera, used to collect images from the bacteria. The evaluation is done by comparing the microscope images of the bacteria before and after they are excited, in this manner it is possible to know which bacteria were stained. Picture in figure 3.1 exemplifies the analyses of the immunoassay's performance. In this example it is possible to compare the same sample submitted to normal light where all the *E. coli* can be seen in figure 3.1a, with when the sample is submitted to the light source that excites the fluorescent particles, shown in 3.1b. Figure 3.1b shows that only one *E. coli* is fluorescently labelled out of the five *E. coli* in the sample. Therefore this is a result of an unsatisfactory performance of the immunoassay. The performance would be considered adequate in this sample if all the five bacteria emitted a fluorescent signal upon excitation, indicating that all the *E. coli* in the

sample were labelled.



**Figure 3.1:** Example of how the immunoassay is evaluated through fluorescent microscope. In this example the performance of the immunoassay is not as good as expected because only one out of five bacteria was stained.

For the next results of the immunoassay performance in this thesis work, only the pictures of the fluorescent bacteria will be presented, therefore the pictures are similar to the one shown in figure 3.1b. More information about the steps followed in the tests and the materials used are presented in Appendix C.1. Table 3.1 presents a summary of the materials used in this procedure.

**Table 3.1:** Materials and its functions used for direct immunoassay

Material used	Function
Chicken $\alpha$ -E. coli	Immobilized antibody on quantum dot
Buffer	Tested media in which the reaction takes place
MilliQ water	To adjust molarities when necessary
PBS	To wash the bacteria
Fluorescent microscope equipped with camera	Used to take pictures for analyses of the binding performance

The procedure for the indirect immunoassay was similarly executed, being as follows: the E. coli bacteria strain was first suspended in PBS. This solution is centrifuged for washing at  $6000 \times g$  for 4 minutes. The bacteria pellet is resuspended in a specific buffer, mixed with 1:500 primary antibody. The mixture was left for 30 minutes at

room temperature for incubation. This is followed by a second washing procedure by centrifugation at 6000 x g for 4 minutes, and by suspension of the pellet in the same buffer used previously. The solution is then mixed with 1:500 secondary antibody and left again for 30 minutes at room temperature for incubation. Finally a last washing procedure is executed followed by suspension of the final pellet in PBS. The immunoassay is then ready to be evaluated in the same fashion as for the direct immunoassay. More information about the steps followed in the tests and the materials used are presented in Appendix C.2. Table 3.2 presents a summary of the material used in this procedure.

**Table 3.2:** Materials and its functions used for indirect immunoassay

Material used	Function
Chicken $\alpha$ -E. coli	Primary antibody
Rabbit $\alpha$ -chicken	Secondary antibody conjugated with organic fluorophore
Buffer	Tested media in which the reaction takes place
MilliQ water	To adjust molarities when necessary
PBS	To wash the bacteria
Fluorescent microscope equipped with camera	Used to take pictures for analyses of the binding performance

Washing procedures must be done for both indirect and direct immunoassay. This is because it discards the non bound antibodies, minimizing the background noise when verification is done by using the fluorescent microscope.

Moreover, the aforementioned buffer is composed of different solutions, varying accordingly to the goal of the test. Therefore, the procedure and/or materials used might have some adjustments depending on the investigation taking place. These adjustments will be described in the respective sections.

## 3.4 Definition of the studied bacteria strain

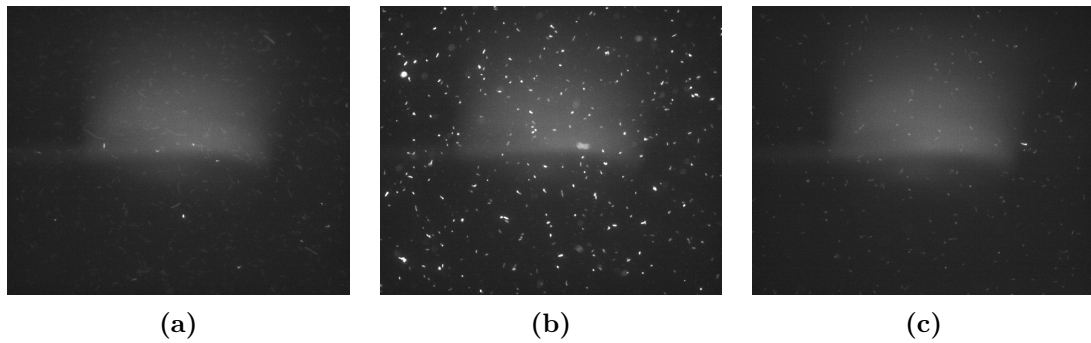
In this thesis work, several tests of the buffer conditions were made by using cultured bacteria in immunoassay. Therefore the bacteria strain used is very important for further experimental performances. For this reason, a preliminary test was executed aiming to choose the best bacteria strain for these studies.

In this manner, different strains of E. coli were subjected to indirect immunoassay. The procedure for this was similar to the one described in section 3.3. The bacteria strain selected for this study were: K-12, XL1-Blue and XL10-Gold.



The buffer chosen was PBS with 150 mM NaCl/PBS, since it is known to be compatible with antibody reactions. The specific steps and materials used for these tests are presented in Appendix D. In figure 3.2 are images taken of the fluorescent signal of the three different *E. coli* strain, resulting from the indirect immunoassay previously mentioned. Interesting to note is that it was used similar bacteria concentrations for all the three strains in the tests.

As exhibited in figure 3.2, the *E. coli* K-12 has a much stronger signal than the others. This was expected since this is the closest strain to the wild type of the *E. coli* of the three strains. Hence, this strain was selected to be used in the further assay experiments. The K-12 strain was then cultured in agar plate overnight, in a incubator chamber with controlled temperature of 37°C and 5% carbon dioxide. All subsequent presented experiments were performed using the K-12 strain of the *E. coli* bacteria.



**Figure 3.2:** Result of the labelled bacteria for different *E. coli* strains. From left to right: XL1-Blue (a), K-12 (b) and XL10-Gold (c)



# 4

## Assay Optimization

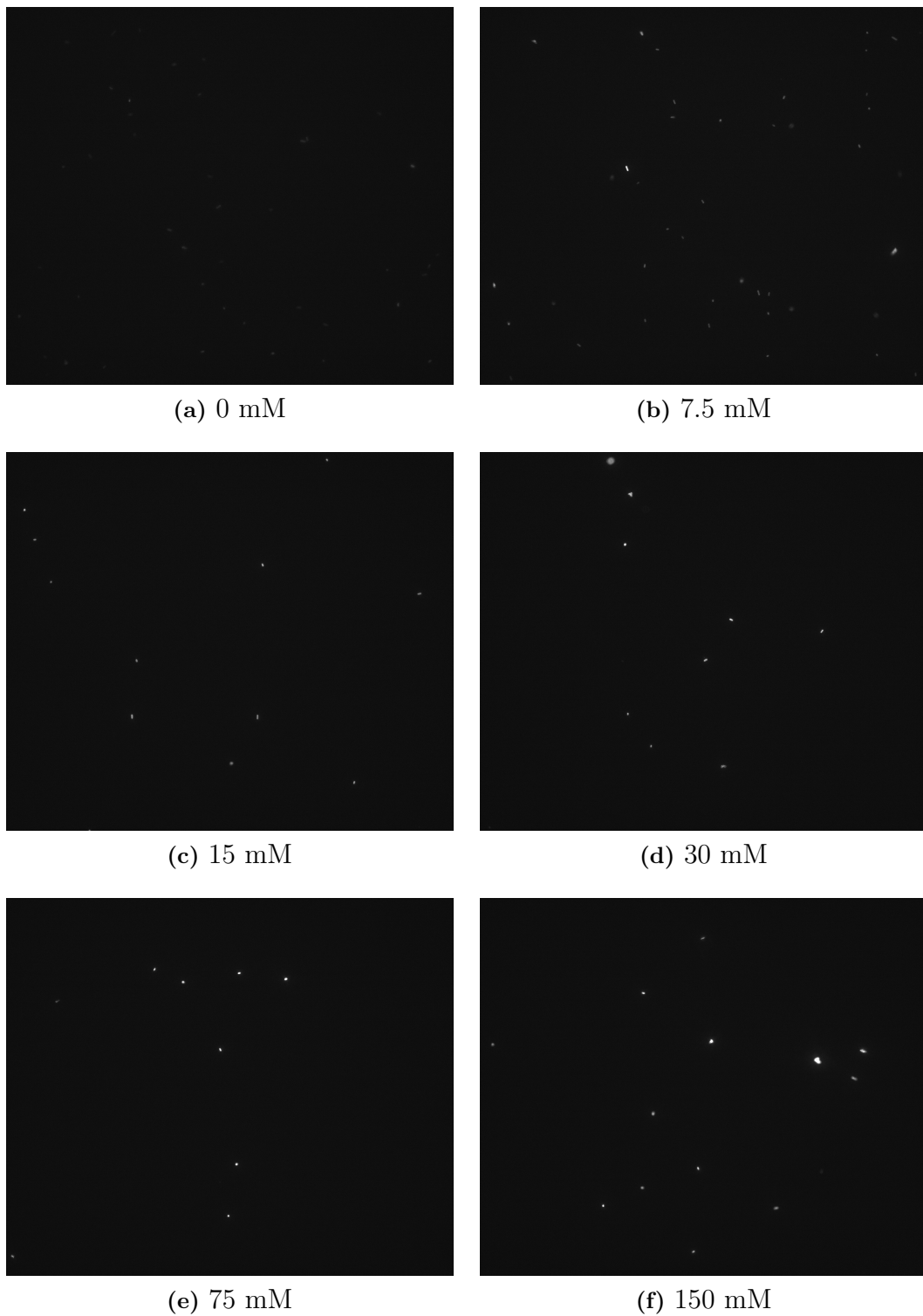
Since each reaction system is unique, there are no standard procedure to determine the conditions to be used in the immunoassay. Thus, several empirical tests with the appropriate theoretical background were necessary in order to obtain the desired improvements for this case. The main goal of each of these empirical tests will be described in this chapter.

### 4.1 Buffer salt molarity

Reactions involving antibodies are generally performed in a saline environment, with pH at physiologic strength. However, in the studied case, the antibodies will react with *E. coli* in water. Therefore, a verification of the viability of the labelling in pure water was needed to improve the reaction conditions. These conditions directly affect the quality of the bacteria staining. In this manner, a study on how the saline environment affects the performance of the binding of the antibodies was conducted.

The procedure selected for this study was the direct immunoassay (described in section 3.3). In general, PBS is chosen as the buffer media for antibodies reaction. This is because it contains NaCl at physiologic strength and the phosphate buffer (PB), which sets the pH at physiological conditions. Therefore, PBS and phosphate buffer with different salt molarities were used as the buffer media in this study. The salt molarities chosen were 0, 7.5, 15, 30, 75 and 150 mM of NaCl in phosphate buffer. It is relevant to point out that the salt molarity of 0 mM represents pure MilliQ water.

The materials used are described in table 3.1, and more information upon the experimental steps followed in these tests are described in Appendix E. Pictures taken of each of the final experiment were selected to illustrate the binding efficiency found. These are presented in figure 4.1. Table 4.1 presents the results of the salt molarity tests. The results are quantified by numbers ranging from 1 to 5, in which 1 indicates worst staining, and 5 best staining.



**Figure 4.1:** Result of the labelled bacteria for indirect immunoassay at different buffer. The buffers differ in their NaCl molarities in phosphate buffer: 0 mM NaCl/PB (a), 7.5 mM NaCl/PB (b) 15 mM NaCl/PB (c) 30 mM NaCl/PB (d) 75 mM NaCl/PB (e) and 150 mM NaCl/PB (f)

**Table 4.1:** Summary of the result for the salt molarity tests

Ion concentration (mM NaCl/PB)	0	7.5	15	30	75	150
Binding efficiency	1	2	4	4	5	5

It was found that a certain degree of sodium chloride is required for the antibodies to function properly. This can be visually evaluated in figure 4.1. This result is likely because the antigen-antibody bond relies on intermolecular forces, which are greatly affected by salt and pH [33]. If the media has insufficient salt concentration, the electrostatic interactions and hydrogen bonds that occur between them may be weakened, harming the antigen-antibody affinity.

Since 30 mM NaCl in phosphate buffer showed a satisfactory binding, this molarity was chosen to be used in the subsequent tests of this thesis work.

## 4.2 Blocking Agents

It is a goal to keep the degree of specificity of the labelling high, avoiding false counting of bacteria. To increase the specificity of the labelling and decreasing the probability of cross-reactions, it is common to block unoccupied sites of the binding surface. Quantum dots conjugated to the antibodies still might have free sites in their surface and to avoid unspecific binding in these sites during immunoassay, proteins known as protein blockers, are used. These proteins permanently occupy the free sites of the QD without interfering with the QD purpose or performance. As a result, only the specific immobilized antibodies are bound into the surface of the QD [34]. To investigate the best blocking agent, BSA, a widely used protein blocker in immunoassay [34], and two commercialized blocking agents, BBG and SuperBlock™, were tested and compared.

Along with the protein blocker, it was chosen to use a chelator. The chelator blocks metal ions, and in the case of the *E. coli*, the presence of some divalent cations can interfere with the reaction. In the presence of divalent metals such as  $Mg^{2+}$  and  $Ca^{2+}$ , the lipopolysaccharide (LPS) chains bind together. This occurs because the metals neutralize the negative charge of the phosphate groups existent in the bacteria's cell envelope. This forms strongly bound stacks between the LPS and the membrane, protecting the bacteria membrane from external chemical reactions. When the chelator removes these cations, the adverse repulsive forces act by harming or extracting the LPS. The disruption of the interactions between the LPS chains gives better access for the IgY  $\alpha$ -*E. coli* conjugated quantum dots to interact with the antigens in the bacteria membrane, which before was hidden by the bound LPS chains [35, 36, 37].

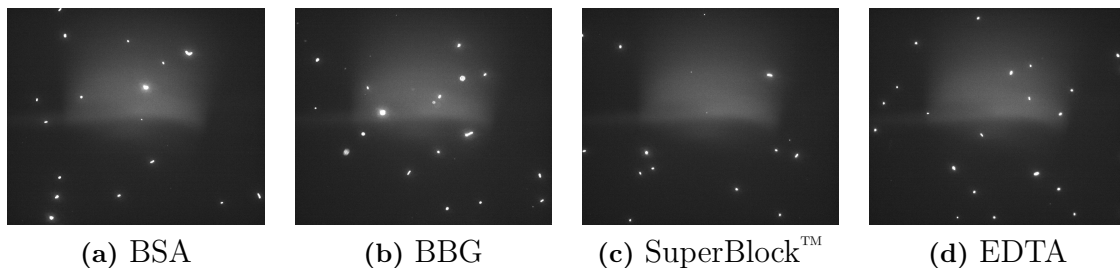
Therefore, it was investigated if a chelator could be used in this work to modify the *E. coli* membrane increasing the access of the antigens to the conjugated antibodies, without harming the antigen-antibody binding. The chelator chosen is the EDTA,

which is generally used for this purpose in immunoassay [38].

For these experiments, direct immunoassay is performed, following the steps discussed in section 3.3. The different buffers used for each test is presented in table 4.2, and pictures illustrating the binding efficiency result for each buffer are presented in figure 4.2. More information upon the steps followed in these tests are in Appendix F.

**Table 4.2:** Buffers used in each different test for different blocking agents

Buffer	Description
BSA (0.5%) dilluted in 30 mM NaCl/PB	standard protein used as blocking agent
BBG	commercial blocking agent
SuperBlock <sup>TM</sup>	commercial blocking agent
EDTA (5 mM) in 30 mM PB	chelator used to increase accessability to antigens in the bacteria membrane for antibody-conjugated quantum dots



**Figure 4.2:** Result of the labelled bacteria for different blocking agents used in the buffer.

The tests show that all the blockers used result in a good labelling, indicating that they are all compatible with the antibody-antigen binding. Therefore these blockers achieved the expected, not interfering with the antibody binding and increasing its specificity. Since the composition of the two commercial blockers are not available, it can be difficult to predict and understand any possible interference in any aspects of the immunoassay. On the other hand, there is extended documentation on BSA performance as a blocker agent, and it is possible to store it as an aqueous solution at 4°C [38]. Thus, it is easier to administrate this protein, and therefore, BSA was added in the assay and was used in all subsequent tests.

In addition, the tests with EDTA showed that its presence did not disturb the protein binding, which can be inferred by the strong signal obtained. Therefore this component was also added in the media to increase the immunoassay performance when conducting the subsequent tests with detergents.

### 4.3 Detergents

Another type of substances that has been used to saturate the unoccupied sites are detergents. Detergents are considered good for this application since they can block the unoccupied areas in the surface of the QD while being cheap and stable in diluted form. In addition, they can also improve the accessibility of the antigen towards the antibodies by dislocating molecules that might be detained in the surface, and improve the detection. It has also been shown that the effect of the EDTA chelator can be increased when used in combination with detergents [39]. However, one important characteristic of detergents is that they are not permanent blocker, given that their attachment with the surface can be washed with water or buffer in a solution.

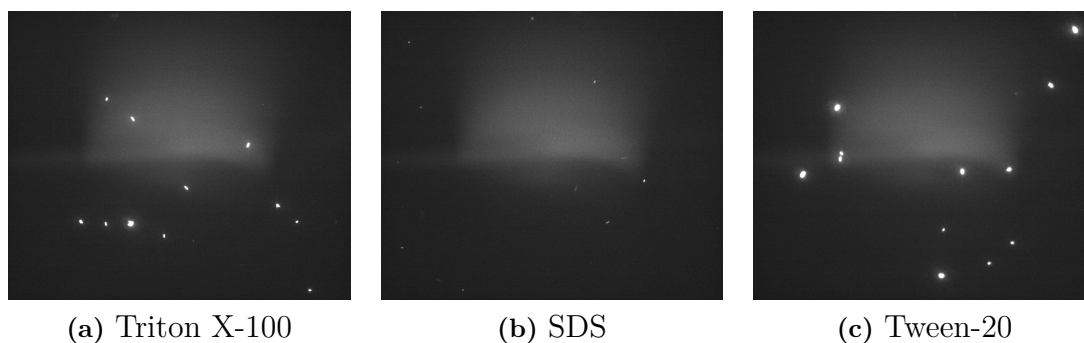
There are three main types of detergents; ionic, non-ionic and zwitterionic. It has already been shown that zwitterionic is not very good for the desired blocking, therefore only the ionic and non-ionic detergents are explored for this purpose in this work [40]. Ionic detergents have the property of breaking protein-protein binding, which can denature protein molecule structure, therefore altering the antibody and antigen structure. This property can worsen the signal strength obtained from the immunoassay since it can fragilize the bacteria cell wall, which is where the antigens are present [41]. The non-ionic detergents wash unbound units of molecules in the surface and can break unwanted lipid-lipid and lipid-protein interactions, without denaturing protein molecule structures [41].

In this section, tests were performed to verify the best detergent that improves the specificity without harming the signals. For that, direct immunoassay, as described in section 3.3, with buffers containing different non-ionic detergents were used. For control purposes, one ionic detergent was also tested with the intention of checking if the expected worsening of the signal is obtained. Therefore two different non-ionic detergents with applications in immunoassay were tested, the Triton X-100 and Tween-20, as well as one ionic detergent, the SDS (Sodium dodecyl sulfate).

Together with the detergents, the buffer also contained 30 mM of NaCl in phosphate buffer, as mentioned in section 4.1, and BSA as the blocking agent, as mentioned in section 4.2, however the EDTA was not yet added. Table 4.3 describes the detergents and their used concentrations in the tests, and figure 4.3 shows the their results. More information upon the steps followed for these tests are in Appendix G

**Table 4.3:** Detergents tested and their used concentrations.

Detergent	Concentration
Tween-20 (non-ionic)	0.05%
Triton X-100 (non-ionic)	0.1%
SDS (ionic)	0.1%



**Figure 4.3:** Result of the labelled bacteria for different detergents in the buffer. From left to right: Triton X-100(a), SDS (b) and Tween-20 (c)

It is clear by analysing the pictures in figure 4.3 that SDS had the worst immunoassay signal as expected. For both, the non-ionic detergents the signals were stronger than the SDS.

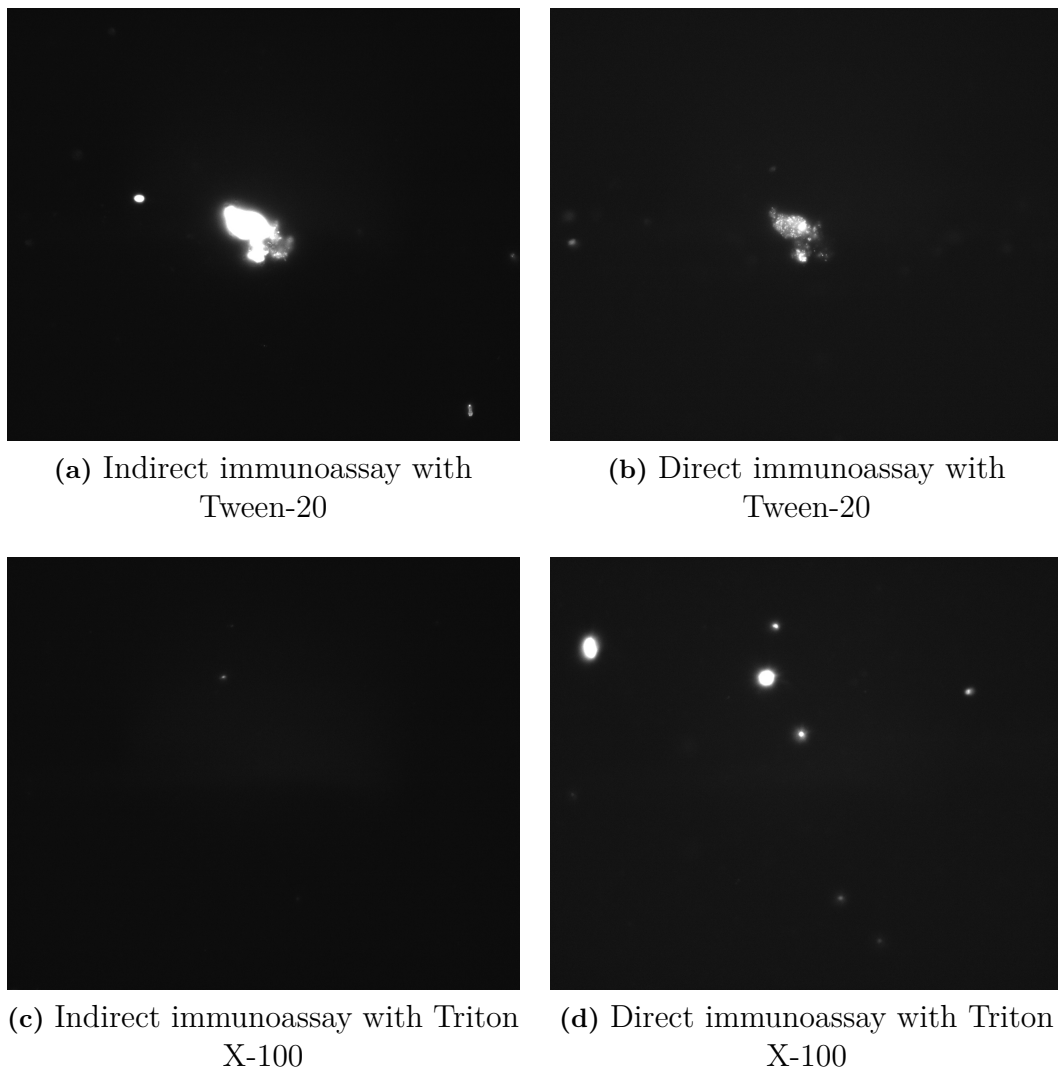
Further investigations upon both non-ionic detergents were made, which also considered the chelator EDTA. Studies have shown that EDTA together with non-ionic detergents can better achieve the desired cell membrane modification of the *E. coli*, as previously discussed [37].

For these experiments, direct and indirect immunoassay were performed simultaneously. The steps followed were similar to the ones described for the indirect immunoassay in section 3.3. However, the antibodies functionalized with QD were added with the primary antibody in the same step. Hence, the final solution had both the QD and the organic fluorophores used for detection. Since the QD and the organic fluorophores present different excitation spectra, it was possible to excite them separately. This allowed for a comparison of the direct and indirect immunoassay in the same buffer conditions.

The concentrations of the detergents were the same as the ones presented in table 4.3, with SDS not being evaluated in this test, and the EDTA used was as described in section 4.2. More information upon the concentrations used of all chemical solutions, and the specific steps followed for these tests are in Appendix H. Figure 4.4 presents the results obtained for these tests.

Comparing the performance of the direct and indirect immunoassay through figure 4.4, it is possible to note that Tween-20 results in better performance for the indirect immunoassay when compared to using Triton X-100. However, for the direct immunoassay the use of Triton X-100 caused an improvement in the signal when compared to using Tween-20. Since the sensor system developed by Acreo performs direct immunoassay with quantum dots to detect *E. coli*, Triton X-100 is the most suitable to improve the specificity in this case. Therefore also improving the signal-to-noise ratio in the sensor system.



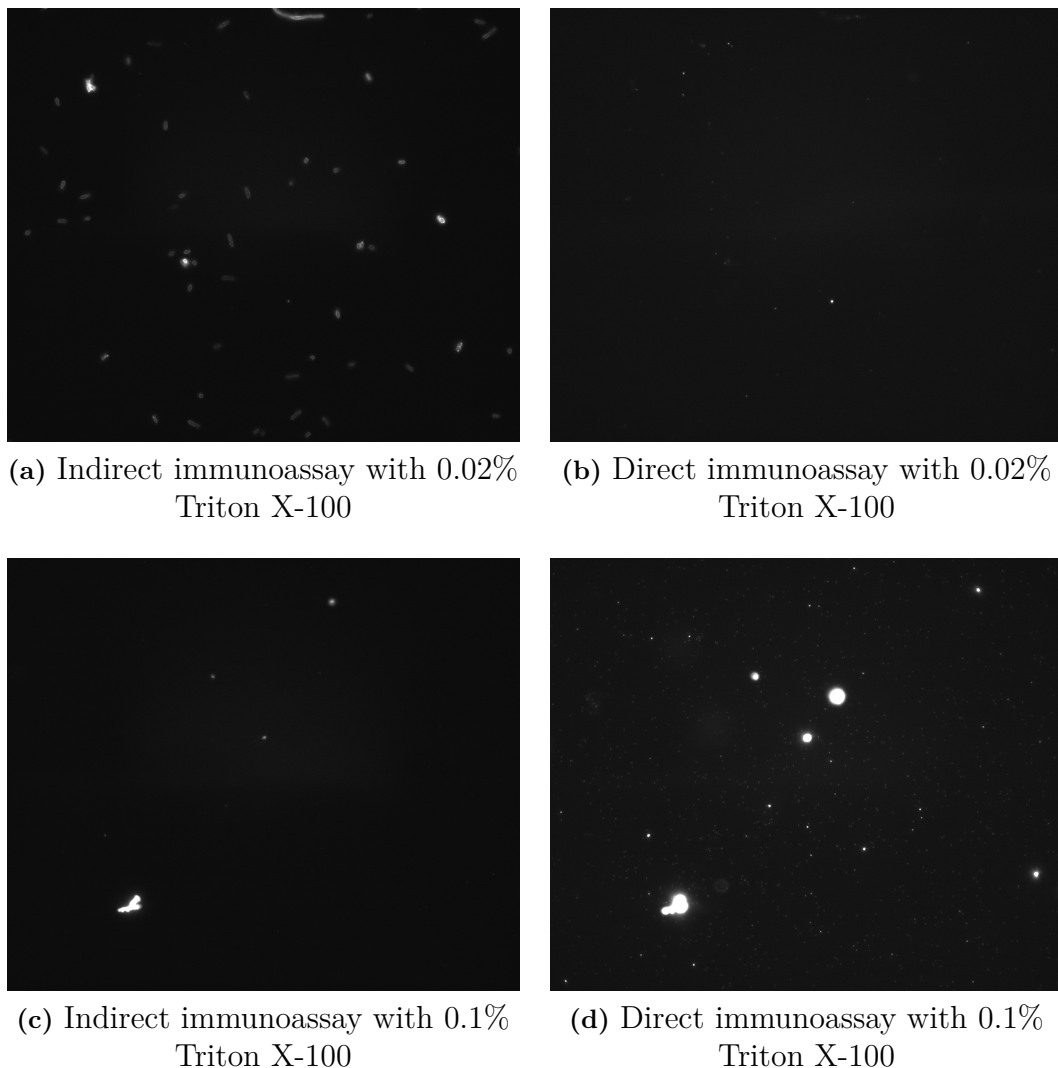


**Figure 4.4:** Result of the labelled bacteria for different non-ionic detergents in the buffer with EDTA and different immunoassay detection methods.

After the aforementioned result, an in-depth investigation upon the best concentration of Triton X-100 in the assay was made. The same procedure of the simultaneous indirect and direct immunoassay was followed, but in this case Triton X-100 concentrations were varied in each test. Four concentration points were selected to be studied, these were 0.02%, 0.04%, 0.06% and 0.1%. More information about the materials used and the steps followed to perform the immunoassay are presented in Appendix I. Figure 4.5 presents the results obtained for two of these tests, the one with the lowest detergent concentration and the one with the highest, illustrating how the immunoassay is affected by this variable.

As exemplified in figure 4.5, it was noticed with these tests that the signals of the indirect immunoassay were not much influenced by the presence of the Triton X-100, in which the variation of this detergent resulted in similar fluorescent signals for all the tests. A high specificity was obtained, in which all the *E. coli* present

were labelled. However, the desired signal strength was not achieved.



**Figure 4.5:** Result of the labelled bacteria for different Triton X-100 concentrations and immunoassay detection methods.

For the direct immunoassay, it was clear that the immunoassay was strongly influenced with the presence of the Triton X-100. For very low concentrations of this detergent, the direct immunoassay signal was very weak with low specificity. However, stronger signals were obtained when the detergent concentration increased up to 0.1%. For the case of using Triton X-100 with concentration of 0.1%, the signals had high specificity and achieved a desired signal strength. Therefore this concentration value of Triton X-100 is the optimal one for the evaluated case, since it fulfils the desired performance for this work. Hence this was the detergent and concentration value chosen to be used in this project.

It was seen that the greatest benefit from Triton X-100 came with in combination with quantum dots, which can be due to a better reduction of sterical hindrance.

The summary of the performance of the immunoassay for all the tests discussed in this section are presented in table 4.4. The results are all quantified by numbers ranging from 1 to 5, in which 1 indicates worst staining, and 5 best staining.

**Table 4.4:** Results for all tests performed in this section.

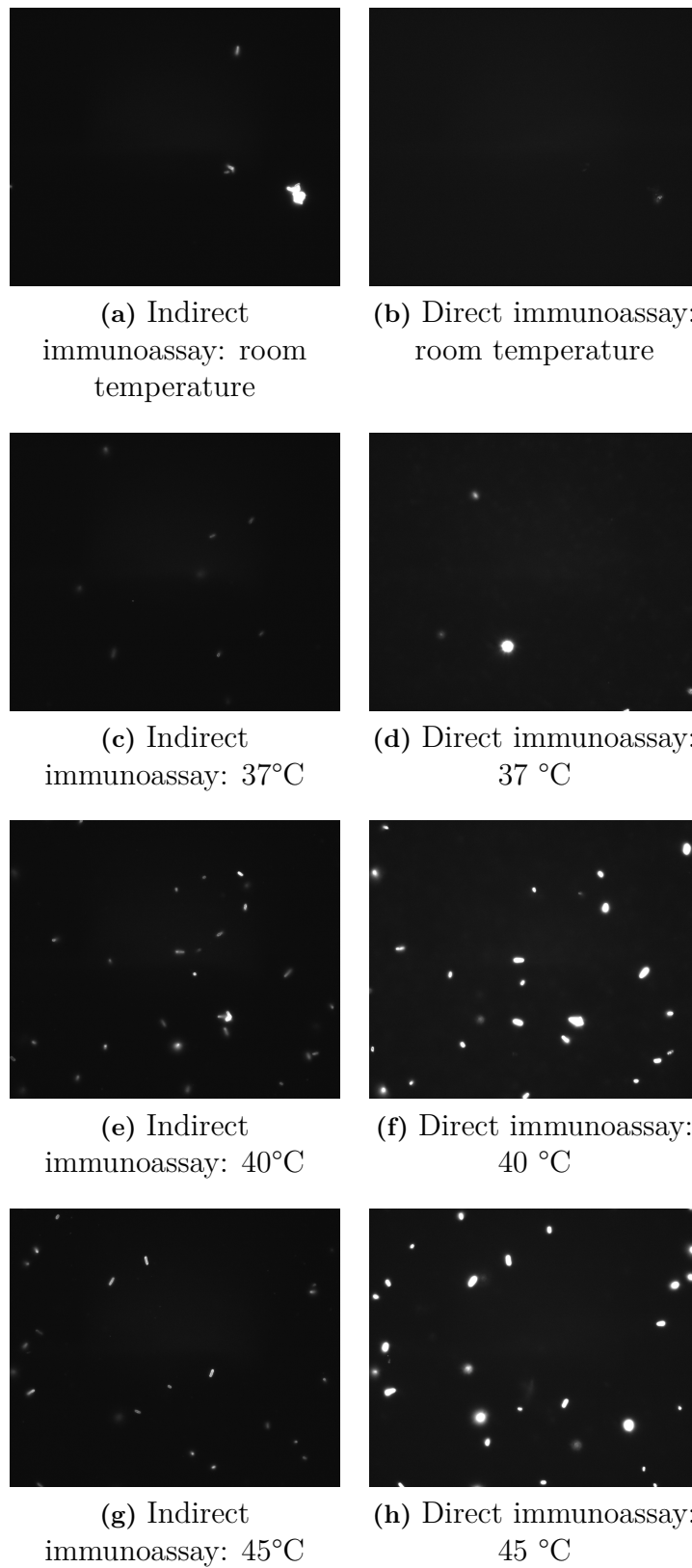
Detergents	Concentrations	Immunoassay binding efficiency		EDTA (mM)
		direct	indirect	
SDS	0.1%	1	1	0
Tween-20	0.05%	3	5	0
Triton X-100	0.02%	1	3	5
Triton X-100	0.04%	3	3	5
Triton X-100	0.06%	3	3	5
Triton X-100	0.1%	5	3	5

## 4.4 Incubation parameters

One important point of interest in the improvement of the sensor system performance is the shortening of the incubation time needed for the antibodies. This incubation time directly affects the time needed for sensor to return data and provide an evaluation of the water sample. Hence, this incubation time was also selected as one of the studied parameters for the tests performed in this thesis.

The intention of the incubation time is to allow the antibody to react with the antigens in the bacteria. Thus, to speed this reaction time, it is important to increase the kinetics of the conjugated antibodies, where the higher mobility of antibodies would result in a higher probability for them to meet the antigens in the bacteria. One way to increase the kinetic energy of the reagents is by increasing the temperatures during the incubation. However, it is important to consider that the proteins can denature at too elevated temperatures, which would in contrast harm the signals. Therefore, a study with the purpose of finding the optimal assay temperature was performed.

For these experiments, direct and indirect immunoassay were performed simultaneously, as done in section 4.3, but the incubation time selected was reduced to only fifteen minutes. All results found in the previous sections of this chapter were applied to these experiments. The materials used and the experimental steps followed are presented in Appendix J. Four different temperature values were selected to be studied, these were room temperature (approximately 23°C), 37°C, 40°C and 45°C. Figure 4.6 presents all the results obtained for these experiments.



**Figure 4.6:** Result of the labelled bacteria for different incubation temperatures and immunoassay detection methods.

In figure 4.6 it is easy to interpret the beneficent effect of higher temperatures during the indirect immunoassay when analysing the obtained results. At room temperature, both the direct and indirect immunoassay were unsatisfactory, because many bacteria were not labelled. It is important to highlight that what can be seen in the indirect immunoassay with incubation at room temperature is an aggregation of several bacteria, which leads to an apparently strong signal. This can be misleading but does not represent a good quality labelling, which can be observed at the higher temperatures.

It is also interesting to note that when comparing the indirect and direct immunoassay methods, the first had better results at room temperature than the latter. This is probably due to the fact that the organic fluorophores have much less molecular weight and their molecular size are much smaller than the quantum dots. This can influence their kinetic energy and their distribution throughout the sample volume [42].

For the direct immunoassay, even though the signal was strong at 37°C, some bacteria were not fluorescently labelled, whereas for the indirect labelling, the affinity is high but the signal strength is low at this temperature. Direct immunoassay had good fluorescent signals for the cases of 40°C and 45°C as incubation temperatures. At these temperatures, the direct immunoassay method also presented a better stain than for the indirect immunoassay. In those cases the direct immunoassay resulted in satisfactory high affinity and signal strength. Thus, it was chosen to perform more thorough studies for incubation time at 40°C, with the interest of better understanding the improvement found.

An explanation of different optimal temperature for the direct and indirect immunoassay might be that the indirect labelling involves one single primary antibody bound onto the antigen. In this case, high temperatures might disrupt the antigen-antibody binding, losing the fluorescent signal. However for the case of the direct immunoassay, several antibodies are conjugated onto one quantum dot, therefore several parallel binding between the QD and bacteria can occur, obtaining stronger combined binding strength. Thus in the case of a disruption of the binding between the antigen and antibody for high temperatures, adjacent antibodies onto the same QD would still be bound to the bacteria. Therefore direct immunoassay might have less temperature dependency at high temperatures (from around 45°C).

The summary of the performance of the immunoassay tested for different incubation temperatures are in table 4.5. The results are quantified by numbers ranging from 1 to 5, in which 1 indicates worst staining, and 5 best staining.

Deeper investigations were made seeking the optimal combination between temperature and time for the incubation stage. Thus, tests were made comparing the signal performance of the direct immunoassay for different incubation times, at 40°C. For this, the solution in incubation was analysed at a fixed interval of 5 minutes through a fluorescent microscope. This was performed up to 25 minutes, thus generating 5

data points. Due to the short period between each test, the washing procedure generally performed after the incubation in the immunoassay was not followed in these tests. More information on the experimental steps followed are presented in Appendix K. Figure 4.7 shows the result of the binding during different incubation times.

**Table 4.5:** Results for the binding efficiency at different temperatures during incubation and for different immunoassay methods. The incubation time was 15 minutes for all the tests.

Temperatures	Immunoassay binding efficiency	
	indirect	direct
Room temperature (23°C)	2	1
37°C	3	3
40°C	3	4
45°C	4	5

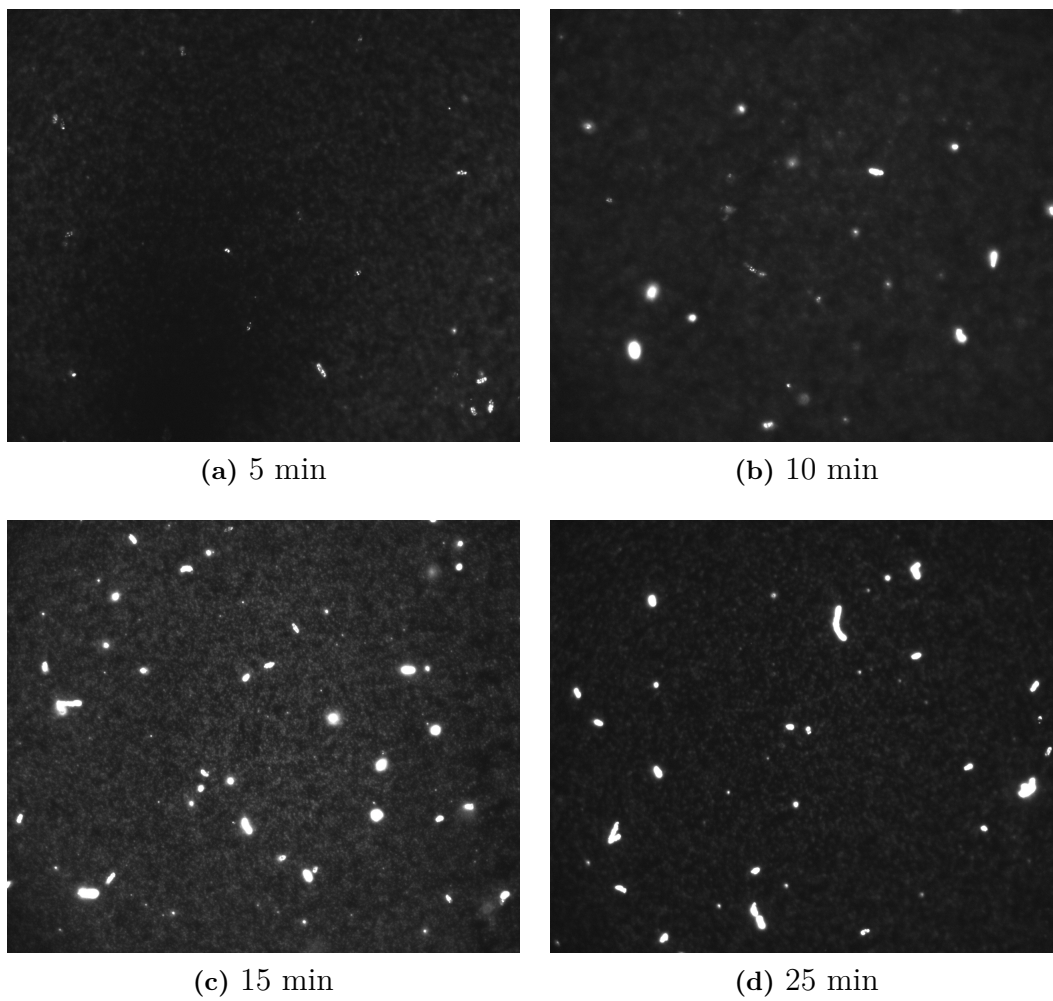
The pictures in figure 4.7 show that after 5 minutes, antigen-antibody binding already had taken place, but not all the bacteria were stained. After 10 minutes of incubation, all the bacteria were detected and with strong signals. For incubation time of 15 minutes and beyond, the signal strength and the specificity of the bacteria detection achieved the desired performance. It is interesting to note that by not performing the washing procedure after the incubation, it is possible to see the unbound antibodies conjugated QD in the background.

The summary of the performance of the immunoassay tested for different incubation times at an incubation temperature of 40°C are presented in table 4.6, illustrated by numbers ranging from 1 to 5, in which 1 indicates worst staining, and 5 best staining.

**Table 4.6:** Binding efficiency of the direct immunoassay for an incubation temperature of 40°C and different incubation intervals

Incubation time (minutes)	Binding efficiency
5	2
10	3
15	4
20	5
25	5

In this chapter, it was seen that the buffer that contributes with stronger binding and higher specificity for the case at hand is composed of 30 mM NaCl in phosphate buffer, 5 mM EDTA, 0.5% BSA and 0.1% Triton X-100, while the incubation should be taken at 40°C during 15 minutes. Therefore these are the parameters used at the sensor system.



**Figure 4.7:** Result of the labelled bacteria for different incubation times with an incubation temperature of 40 °C.





# 5

## New Components

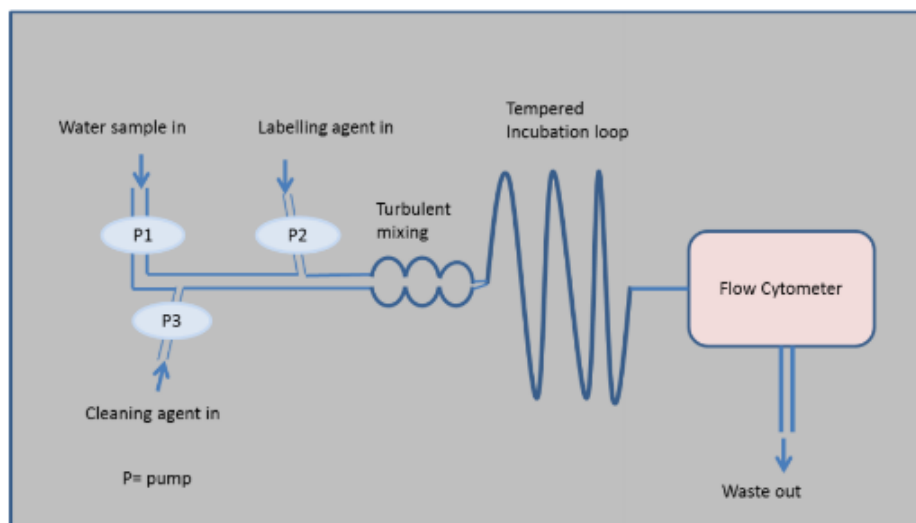
The tests previously presented in this thesis dealt mainly with biochemical improvements to the developed sensor system. This chapter presents the approach taken to improve some of the mechanical and physical aspects of the system. Hence, here two main areas are explored: first, the enhancement of the mixing system, and second, the revision of the flow cell present in the optical module of the sensor.

### 5.1 Mixing

As discussed in section 2.2.2, the previous sensor system developed by Acreo mixed the water sample with the reagents (fluorescent-labelled antibodies) by means of a magnetic stirrer in a vessel. After a determined time period, usually set between 20 and 30 minutes, the reagents were bound in the bacteria's antigens located in the bacteria's cell wall. The disadvantages of using this system is the need of, at least, three vessels. This results in a set up which requires space, is time consuming and not as cheap as desired to be. In addition, the vessels need to be regularly washed to avoid biofilm growth, and this washing needs to be done manually.

For a faster and more automatic sensor than that of the earlier generation, the mixing system should avoid the need of a vessel for mixing the sample water and the reagents. With that in mind, mixing through continuous flow in a tube was proposed. This is where the reagents and the water sample are injected into the same flow, thus generating one output flow from two separate flow sources. The mixing in this scenario occurs through fluid-mechanical characteristics. This makes the necessity of using a vessel obsolete and it is also possible to inject washing agents. This enables an automation of the cleaning of the tubes and the flow cell in the optical module, improving the washing process.

Therefore, a new fluidic layout, based on continuous flow of the sample together with the labelling agents was proposed. In this scenario, the two fluids would flow alongside in a laminar regime until the mixing takes place by passing through a designed structure that creates turbulence. The binding reaction between the reagents and bacteria is completed in a temperature controlled incubation loop, which then flows through the optical detector for enumeration. The proposed layout is depicted in figure 5.1.



**Figure 5.1:** Sketch of the layout for the sensor system with the new proposed mixing method.

The simplest form to create this turbulence needed for the mixing is through the use of a T connector. To study if this connector would suffice for the case evaluated in this thesis, fluorescent particles were mixed with tap water and the final mixture was analysed through a fluorescent microscope with a camera. The choice of both of these fluids was based on the fact that they present similar properties to that of the fluids which will be used in the sensor system. It is important to note that this procedure is not similar to the ones presented in chapter 4 because the intention here was to evaluate the final mixture between the fluids and not the bacteria labelling (which was not performed here). More information about the materials used and the steps followed in this test are present in Appendix L.

Figure 5.2 presents the result obtained from the T connector mixing. It is possible to see in this figure that the sole use of a T connector to join the studied fluids is not enough for them to be mixed with each other in this system.

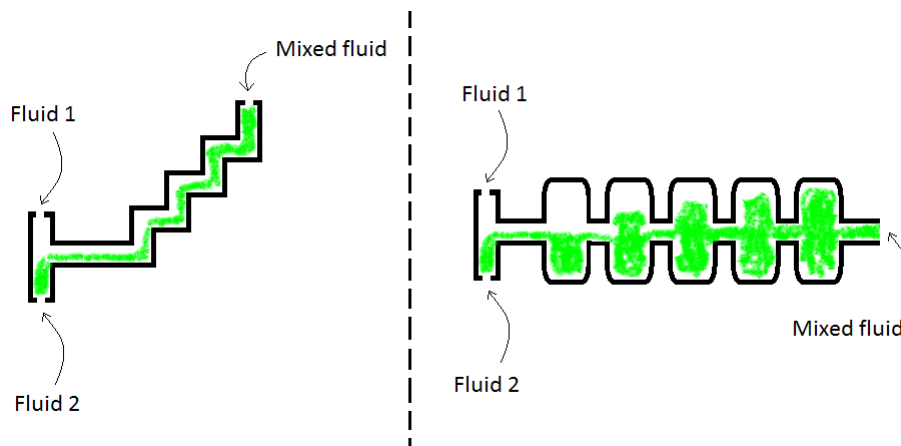


**Figure 5.2:** Result for the mixing through the use of a T connector to generate turbulence.

It is clear from figure 5.2 that the majority of the fluorescent particles remained in the upper part of the image. The bottom part of the image is dark and corresponds

to the tap water. Therefore, the fluids did not mix. This means that the flow was still laminar and not enough turbulence was generated. For this new fluidic layout, it is important that the mixing structure generates enough turbulence between the adjacent laminar fluids, until proper mixing between them is achieved. Hence, it is necessary to inject energy into the system, which can be done by directing the flow through obstacles. In this thesis work, different geometries were designed and empirically tested, checking the optimal pattern for creating turbulence.

The first design was based on injecting energy into the system by changing the course of the fluids with perpendicular angles, forming a stair-like structure. The second structure designed was based on changing the course of the fluids through enlargement and constrictions, made with rectangular-like barriers. The scheme of these two structures are presented in figure 5.3. Both structures varied on their length and quantity of the periodic barriers.



**Figure 5.3:** Illustration of the two proposed designs for the mixing structure. On the left is the stair-like structure and on the right is the one with enlargements and constrictions.

Both designs use the same concept to create turbulence: laminar recirculation when the laminar flow passes through each perpendicular barrier, whether it is a step of the “stair” or an angle of the “rectangle”, laminar recirculation takes place. This means that when the fluids pass through each of the perpendicular barriers, the inertial effect causes a fluid separation, creating an opposite pressure gradient. This creates recirculation, in which eddies and swirls of the fluid appear around the obstacles. Due to the formation of these eddies, the adjacent fluids mix [43, 44].

The standard steps followed in the tests were: Tap water and fluorescent particles are pumped into a T-connection, flowing alongside in a tube and consequently sent through the mixing structure to be evaluated. The mixture then flows into a flow cell, which is placed in a fluorescent microscope equipped with mercury arc lamp and bandpass filters, the same used for the assay optimization tests. The microscope was coupled with camera which was used to take pictures of the final mixture. The

pictures were used to analyse the efficiency of the structures.

The flow rate of the tap water was always 10 times higher than the fluorescent particles' flow rate, simulating the procedure followed in the Sensor system. The flow rate of the final fluid varied from 1 to 1.5 ml/min. These two fluids were chosen once their physical properties, such as Reynolds number, were similar to the fluids to be used with the Sensor system. Aiming to obtain the best mixing layout, these tests used variations of the two structure designs in figure 5.3. These variations were made upon the length and number of barriers in the structures. The materials used are presented in table 5.1, and a picture of the experiment with one of the structures tested is shown in figure 5.4.

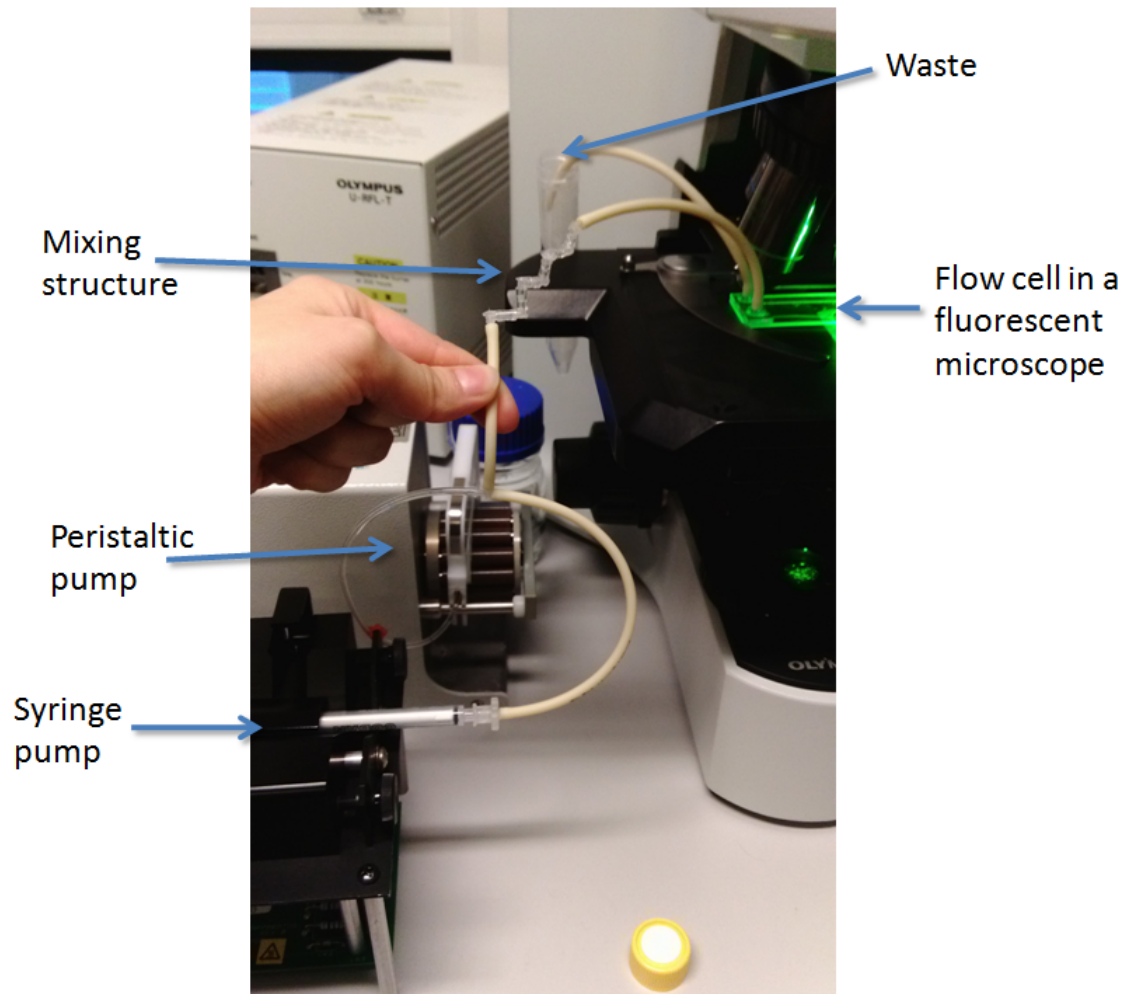
**Table 5.1:** Materials used for the mixing experiments and their functions.

Material used	Function
Fluorescent particles	Fluid to be mixed
Tap water	Fluid to be mixed
Tubes	Where the fluids flow through
T- connector	Used to join both fluids before the mixing structure
Peristaltic pump	Pumps the tap water into the system
Syringe pump	Pumps the fluorescent particles into the system
Mixing structure	To mix the fluids in laminar regime
Plastic flow cell	Where the final mixture passes and can be seen with a microscope
Fluorescent microscope equipped with camera	Used to take pictures to analyse the mixing performance

Pictures of the final mixing of some of the tests are shown in figure 5.5. They illustrate the results obtained when using different mixing structure designs, and the effect of the variation of the length and number of barriers in these structures.

The picture in figure 5.5a was the resultant mixing when using the structure design of the stair-like barriers. It was clear that using this structure the two fluids were mixed, but not in an effective manner. The concentration of the particles was much higher in the bottom than in the top of the flow. Even when the amount of barriers was increased and when the length was shortened, this structure design continued to present ineffective results. Thus, the mixing with this design idea was determined to be unsatisfactory.

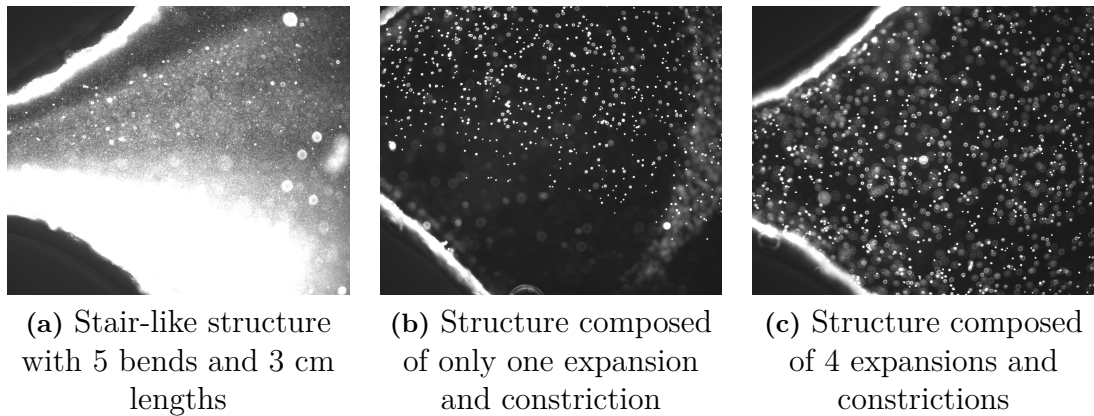
Pictures in figure 5.5b and 5.5c are the results of mixing with the use of the structure design with constrictions and enlargements. Picture 5.5b had only one barrier, whereas picture 5.5c had 4 barriers. With only one barrier, the mixing was not effective. The bottom volume is mainly just water with no presence of the fluorescent



**Figure 5.4:** Picture of the experiment set up with the stair-like structure coupled to the system.

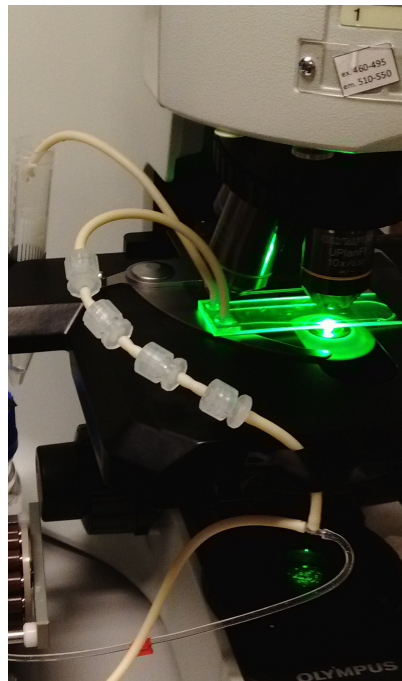
particles, whereas the top portion presents a large quantity of these particles. However, when increasing the number of barriers, a satisfactory mixture was obtained, which is seen in figure 5.5c. Hence, this was determined to be the optimal design for this work.

As an overall perception of the structures' performances, the rectangular-like design presented better results than the stair-like design. This can be related to the fact that the recirculation formed around the obstacles reaches only a small region, where after this region the velocity profile is redirected and the fluid shortly returns to laminar regime [43]. In the rectangular-like structure, the distance between the barriers could be easily designed within the recirculation region, avoiding an integral redirection of the fluids' velocity, and thus achieving a higher level of turbulence. However, the stair-like barriers were designed in a manner that it was hard to make consecutive barriers within this region in a simple way. Therefore, for smaller lengths between the obstacles the mixing is considered to be better for the mixing performed in this work.



**Figure 5.5:** Results for three of the mixing structures studied in this work.

The design used to obtain the mixture in figure 5.5c demonstrated an adequate performance. It was therefore chosen to be used in the sensor system with the objective of mixing the quantum dots conjugated antibodies with the water sample to be analysed. The picture of the rectangular-like structure is presented in figure 5.6, which also demonstrates the simplicity of the final design. However, when this mixing structure was implemented in the sensor system, it had an increase in the numbers of barriers to 7, instead of the 4 showed in figure 5.6. This was not necessary however it was done to establish high degree of reliability upon this design.



**Figure 5.6:** Picture of the rectangular-like mixing structure used in this thesis work.

## 5.2 Flow cell

An important component of the optical detection module is the flow cell. This is where the labelled *E. coli* are excited and their fluorescence signals are recorded with a camera. In this manner, the optical performance of the flow cell directly affects the images obtained. Therefore, the flow cell should be made of a material that has high optical performance, where reflection is minimal. In addition, it is important that the material has good chemical resistance. This property would allow for the implementation of an automatic periodic washing procedure through the use of detergents, avoiding the build up of biofilm on the surface of the tubes and on the flow cell itself. One material that meets these requirements is quartz.

The performance of the optical detection module is also influenced by the sharpness of the images of the bacteria recorded by the camera. It is known that smaller objective magnifications present a larger depth of field, which means that the range of distance where the camera could capture sharp images is bigger. Therefore, it is important to have the depth of the channel within the depth of field of the objective to its possible extent, thus allowing for better detection of bacteria in the entire flow.

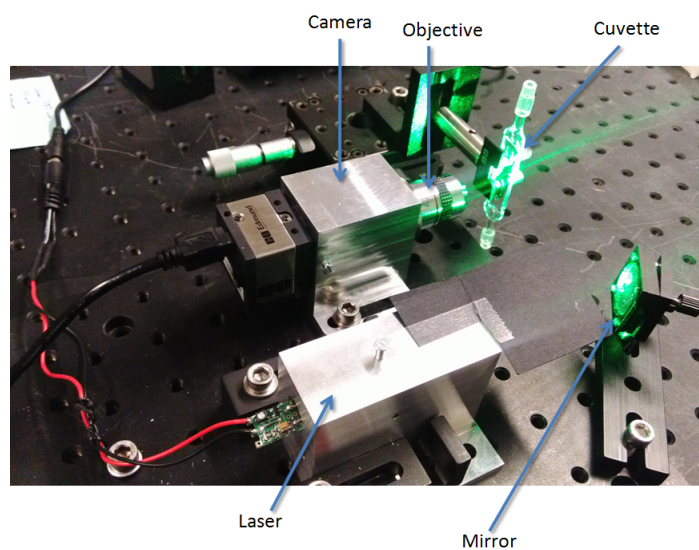
Different flow cells made of quartz with different optical path sizes were tested. These tests aimed to check for the best combination of quartz cuvette, optical path size and objective magnification.

The standard procedure followed to test the performance of different quartz cuvettes was the following: fluorescent particles diluted in water were pumped with a peristaltic pump into the optical detection module. There, the flow was directed into the cuvette being tested and the laser beam brightened up the particles. The images of the fluorescent particles passed through the LP emission filter and were then recorded by a camera coupled with a microscope objective of different magnifications (4 x, 6 x and 10 x magnification). The recorded video was then used for the analysis.

The materials used in these tests and their respective functions are presented in table 5.2, and a picture of the set up of one of the tests is shown in figure 5.7. Two main types of cuvette design were studied. Figure 5.8 shows the schemes and pictures of these two types of cuvettes.

**Table 5.2:** Materials used to analyse the performance of different quartz flow cells and their functions

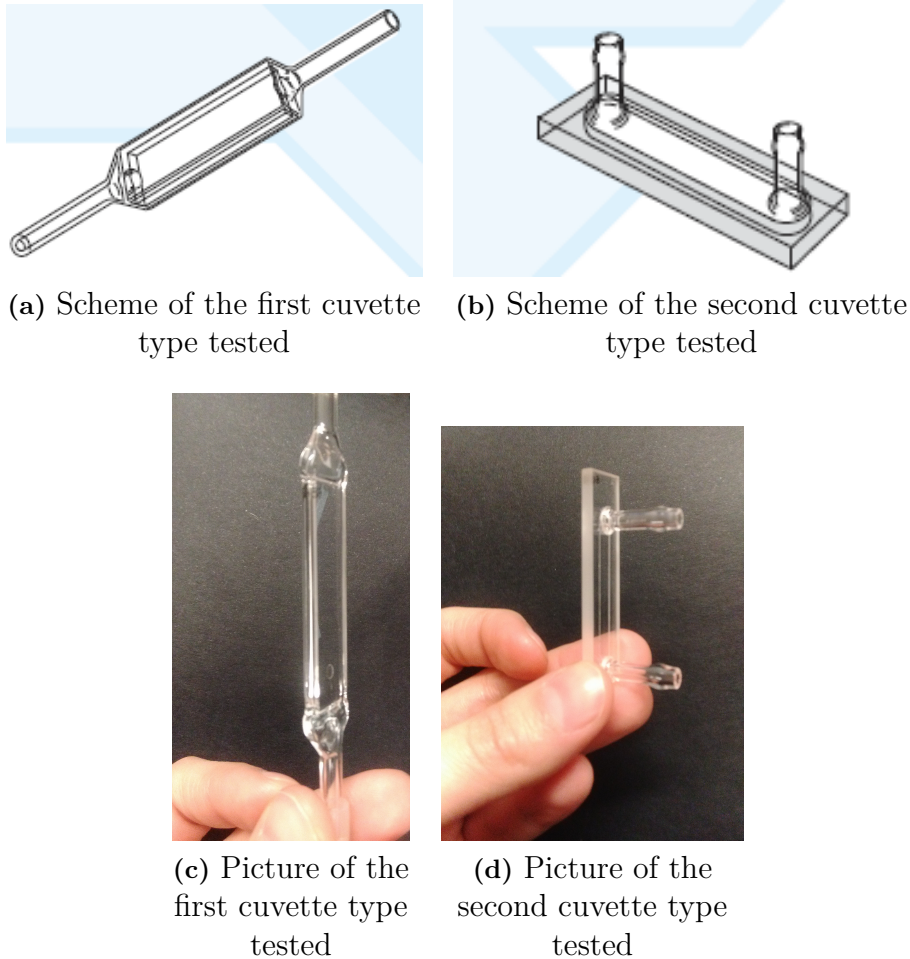
Material used	Function
Fluorescent particles	Particles used to evaluate the cuvette performance
Tubes	Where the fluid with the particles flow into the optical detection module
Peristaltic pump	Pumps the solution through the optical module
Quartz flow cell	Test subject
Laser source (532 nm)	To excite the fluorescent particles
Mirror	To direct the laser beam into lateral side of flow cell
Microscope objective	To amplify the particles flowing in the cuvette
CMOS camera	To record the amplified images of the flow of the particles within the cuvette

**Figure 5.7:** Picture exemplifying the set up used to perform the flow cell tests with one of the tested cuvettes mounted.

Tests showed that even though the path lengths were equal for both cuvettes, the cuvette depicted in the figure 5.8c had some disadvantages over the one depicted in figure 5.8d. The cuvette in figure 5.8c had a higher tendency for bubble formation than the cuvette in figure 5.8d due to its large window area. These bubbles can influence the flow direction since the flow is laminar, thus directly disturbing the data collection. Another disadvantage of this cuvette is that the design of the inlet/outlet for tubes made it a challenge to integrate it in the optical module. However, this cuvette presented one important advantage: whilst the cuvette in figure 5.8d did not have polished walls, the cuvette in figure 5.8c did. The influence of this



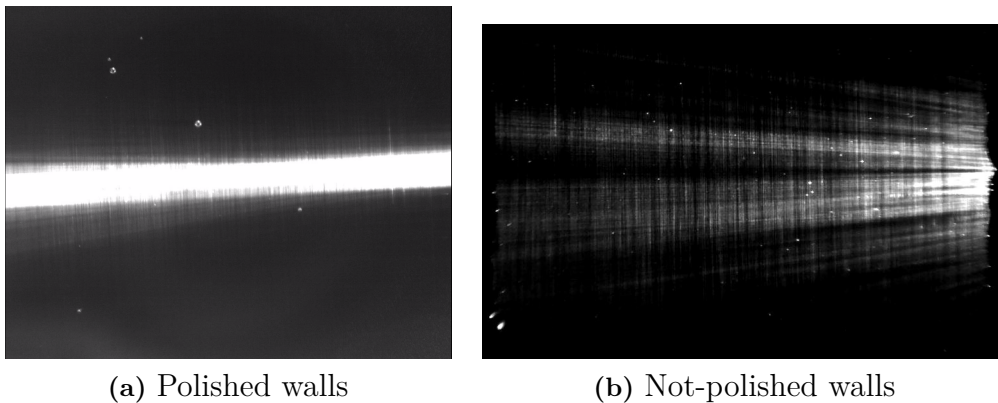
characteristic can be seen in figure 5.9.



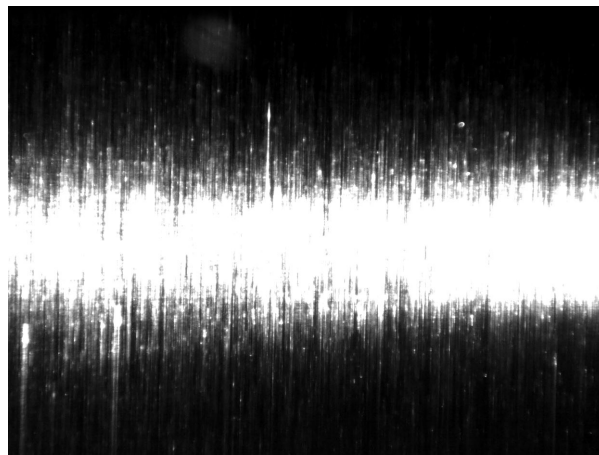
**Figure 5.8:** Schemes and pictures of the two cuvette types which were studied.

As shown in figure 5.9, when using the cuvette in figure 5.8d, the laser beam is scattered in different directions. It then suffers constructive and destructive interferences, which is shown in figure 5.9b. When using the cuvette in figure 5.8c, the laser beam barely suffers diffraction, resulting in a very homogeneous intensity of the laser throughout the whole path length. This difference in cuvette performance is very critical. This is because continuous laser intensity is of utmost importance for the acquisition of data, since the counting of the particles is made by considering the particles' path through sequential frames.

It was therefore of great interest to polish the sidewalls of the cuvette in figure 5.8d and keep all the benefits it presented when compared to the other cuvette: minimal or no bubble formation, easy to integrate in the optical module, and good transmission of the beam light. The performance of this cuvette after polishing is shown in figure 5.10.



**Figure 5.9:** Data obtained from the cuvette tests. On the left is the result from the cuvette pictured in figure 5.8c and on the right is the result from the cuvette pictured in figure 5.8d



**Figure 5.10:** Result of the cuvette depicted in figure 5.8d after it was submitted to polishing.

The result from the polishing, illustrated in figure 5.10, was determined to be sufficient for this thesis work. The obtained laser beam in the result is more focused, with a large reduction of the previously observed scattering. Therefore, it was deemed that the polishing of the cuvette brought forth improvements in the data collection, once the cuvette's interference in the obtained results was reduced.

In addition, it was observed that the shorter path length in the new cuvette of 0.1 mm, half of the previously used for the cuvette in the first release of the sensor, provided sharper images. The depth of focus of the previously used objective in the sensor system covered the main part of the path length, and thus it was not necessary to change this magnification. These sharper images contribute to a higher signal-to-noise ratio, improving the sensor system's overall performance.

# 6

## Integration

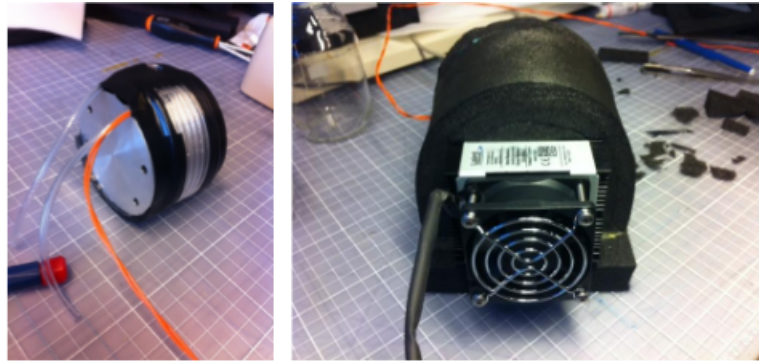
In this chapter, a short description of the changes made by Acreo in the Sensor system is made. Also, the results of tests using real water samples are shown. These tests evaluated the performance of the bacteria staining and the mixing done at the same conditions in which the sensor will be used. The assembling of the new version of the sensor is then described, where its functioning is briefly discussed. Lastly, final tests using the assembled sensor were executed, summarizing its performance as a whole.

### 6.1 New Elements developed by Acreo

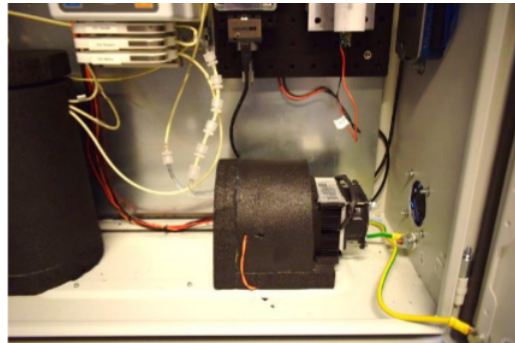
As previously discussed, the incubation during the labelling reaction has better performance at elevated temperatures. Therefore, Acreo developed a small incubator built into the sensor system. This incubator is composed of a tube that coils around a heating element and a thermometer to control the incubating temperature. The incubation occurs while the sample is in the tube and the tube currently has a maximum volume of 10 mL. Therefore, it is possible to incubate 10 mL of sample water at a time. Figure 6.1 shows a picture of the incubator with and without its casing and also its positioning within the sensor system.

In addition, to extend the antibodies life, the labelling agents should be stored in a cooled compartment, with temperatures between 4 to 8°C. Thus, Acreo implemented a Peltier operated mini fridge where the reagents are stored. This mini fridge also contains an external cooling system. Figure 6.2 has pictures of this cooling system implemented in the sensor.

In the previous version of the sensor three pumps were used in the hardware. To optimize the sensor space, Acreo substituted these three pumps into only one containing three channels. Each of these channels has its own purposes: one is to handle the reagents, the other is to pump the sample water and the third is to handle the cleaning agents. Therefore, the pump can also be used in the automation of the cleaning process. This new pump also has a smoother flow, due to the higher amount of rollers it contains and can be controlled by computer software. Figure 6.3 shows a picture of the pump implemented in the sensor.



(a) Incubator with and without its casing

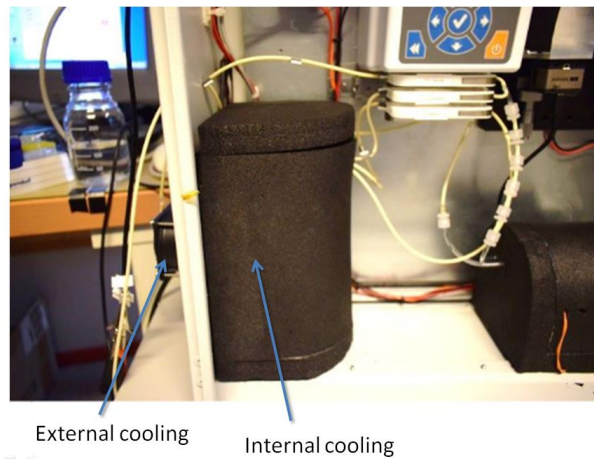


(b) Position of the incubator in the sensor system

**Figure 6.1:** Pictures of the incubator developed by Acreo for the sensor system.

Lastly, the approach used to build the optical module presented some challenges, specially regarding the use of a mirror to direct the focused beam to the cuvette. Since the beam was very small, it only covered up a partial depth of the cuvette's path length. Therefore it was crucial that no angular modification would occur to the mirror. This was hard to achieve since the mirror was not fully fixed due to its construction. Furthermore, the data collection would also be affected since the whole sample would not be subjected to equal laser intensity. This resulted in very bright signals for only a part of the analysed water, the portion struck by the laser beam.

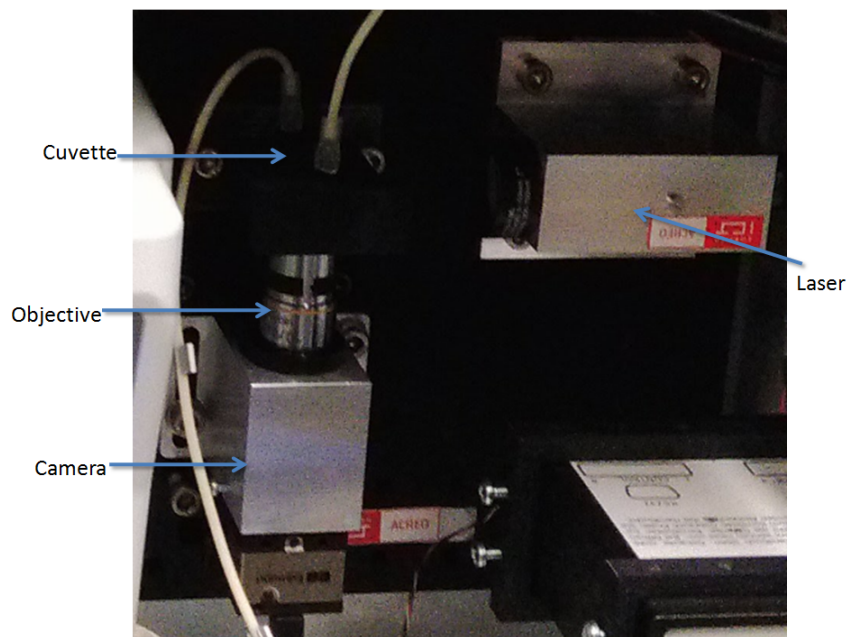
To deal with these weak points of the optical module construction, Acreo substituted the system of the focused laser beam by a laser that takes up the whole path length of the cuvette. Since no focusing of the laser was now necessary, it could be placed facing the side of the cuvette directly. The new optical module set up is presented in figure 6.4. Some advantages of this new arrangement is that now the whole sample is illuminated with same intensity, there is no more use for a mirror so the optical module is smaller containing less components, and all the items in this module are very stable and fixed. This contributes to a more reproducible data collection, with better performance as well.



**Figure 6.2:** Picture of the Peltier cooler where the reagents are stored.



**Figure 6.3:** Picture of the 3-channelled peristaltic pump used in the sensor system.



**Figure 6.4:** Picture of the top view of the new optical module of the sensor system.

## 6.2 Preparation for the final performance test

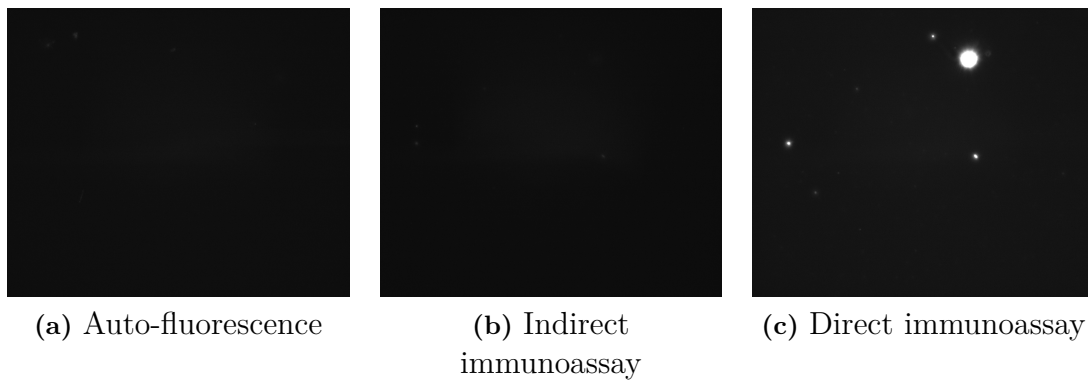
All experiments presented so far were conducted in a laboratory environment. For the evaluation of the bacteria staining, a lab strain of the *E. coli* bacteria harvested from an agar plates was used, with the fluorescent signals analysed through a fluorescent microscope. Aiming to validate if the same staining performance was obtainable with the sensor system, tests using sewage samples collected from WVZ were conducted.

There are many micro-organisms in sewage besides *E. coli*. Hence, if the specificity of the labelling performed is not good, cross-reactivity can occur, causing false positives. With this in mind, both indirect and direct immunoassay were performed simultaneously for these tests. This allows for the verification of the organisms being labelled by these two different immunoassay methods. If both methods are labelling the same organisms, there is a high likelihood that the quantum dots is not binding to an artifact in sewage. It is important to note however that the sensor system only performs direct immunoassay.

First an overview of the tests will be given, then their results are presented and discussed. The steps followed for both immunoassay methods were similar to the tests in 3.3. In the case studied here, the buffer used was composed of 30 mM NaCl in PB, 5 mM EDTA, 0.5% BSA and 0.1% Triton X-100. For the direct immunoassay 1:10000 IgY-functionalized quantum dots were used. For the indirect immunoassay 1:500 of the primary and of the secondary antibody were used.

The buffer solution was mixed with the sewage water, and with the primary antibody. The solution was left for 15 minutes at 40°C for incubation, followed by the washing procedure. Then the secondary antibody and the IgY-functionalized quantum dots antibodies were added and again left 15 minutes at 40°C for incubation, however no washing was done after this step and the solution could already be analysed. This solution was analysed with fluorescent microscope. Information on the steps followed in this test is presented in Appendix M.

The second washing procedure was not followed for these tests. This is because the intention was to simulate the process followed in the sensor system. The washing procedure can be neglected since the signals from the non immobilized quantum dots and organic fluorophores are not detected by the optical module. It is also important to note that the sewage might contain fluorescent particles itself, interfering with the data collection. This is because the fluorescence for any other organism could be counted as *E. coli*. To monitor this, pictures were first taken of the pure sewage, and compared with sewage after the immunoassay was executed. The results of the tests are illustrated in the pictures in figure 6.5.



**Figure 6.5:** Results for the bacteria staining in sewage for both direct and indirect immunoassay. Also presented is the auto-fluorescence of the sewage to verify its interference.

Pure sewage did not present a strong fluorescent signal, as seen in figure 6.5a. This is favourable for the applications in this work, since it will not present false signals. When comparing the results obtained with the two immunoassay methods, figures 6.5b and 6.5c, it is easy to see that their performance was very different. This was already expected, because it was previously seen in the tests in section 4.3 that the direct immunoassay has stronger signals than the indirect immunoassay when Triton X-100 is present in the buffer. Furthermore, it is possible, although somewhat difficult, to see that the detected fluorescent signals from the two different immunoassay methods were for the similar organism. This indicates that the quantum dots does not have non-specific binding. Therefore, successful labelling was obtained with the proposed buffer media when using sewage water, which is the necessary scenario for the sensor system to properly work.

Another test was performed to inspect if the mixing structure would obtain successful performance in the mixing of the substances to be used in the sensor system. Until here, tests were made with water and fluorescent particles only. Even though they are comparable fluids to the ones that will be mixed with the system, it is very important to have data upon the real fluids to be mixed.

To simulate the situation encounter in the sensor system, tests were conducted using the same elements to be used with the sensor system. Therefore, the tests mixed the following two fluids:

1. Buffer with IgY functionalized antibody with quantum dots. The buffer was composed of 150 mM NaCl in phosphate buffer, 5 mM EDTA, 0.5% BSA and 0.1% Triton X-100.
2. Harvested E.coli from agar plate suspended in tap water.

Since the sensor will analyse tap water with eventual sewage contamination, the chosen fluid of tap water with added E.coli to be used in the test is comparable with the fluid to be mixed in the real scenario.

The final flow rate was 1 mL/min, where the flow rate of fluid 1 was 10 times slower than that of fluid 2, simulating the flow rate to be used in the sensor system. It is important to comment that with this set-up the final concentration of the quantum dots was of 1:1000, and the molarity of NaCl in PB was of 30 mM. The steps followed were: fluid 1 was pumped with syringe pump, and fluid 2 was pumped with a peristaltic pump. Both fluids were joined through a T connector, and directed to the mixing structure, the same in figure 5.6. The final mixed fluid was collected and left for 15 minutes at 40°C for incubation.

To check the mixing performance between the bacteria and the reagents, the bacteria staining of the final fluid was evaluated through a fluorescent microscope. This is because the staining performance is directly related to the mixing degree. In this manner, good staining would be a reflection of a successful mixing between the staining agents and the *E. coli*. Figure 6.6 presents the obtained results.

As shown in figure 6.6, the labelling of the *E. coli* was strong, indicating that the mixing system works properly, and can be implemented in the sensor to be used. One interesting aspect of this result to point out is the clear presence of free quantum dots in the background of the picture in figure 6.6. But, as mentioned before, these do not interfere in the data processing.



**Figure 6.6:** Result obtained from the direct immunoassay representing the mixture effectiveness. The picture was taken from a fluorescent microscope.

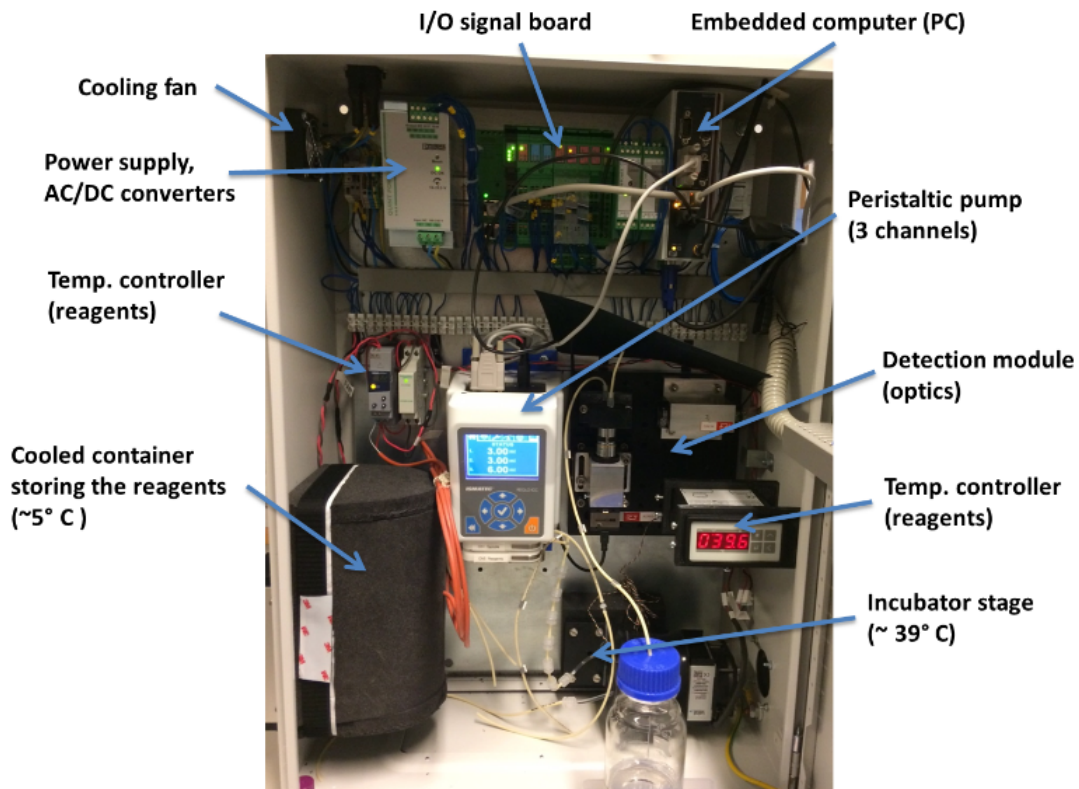
### 6.3 Final Sensor System and its Performance

In this section a description of the assembled sensor is given, followed by final tests and an evaluation of the sensor's performance. Lastly, a discussion of important parameters of the sensor is presented.

The sensor is assembled in a cabinet, along with all the needed systems for an automatic functioning of the sensor. A brief description of the outline of the components of the sensor is: a computer which plays the role of a transducer of the fluorescent



signals, one cooling station to store the reagents, a pump with three channels for the reagents, the sample water and the cleaning agents, an incubator where the labelling reaction takes place, and the optical module where the fluorescent signals are detected. In figure 6.7 is a picture of the inside of the sensor showing its components.



**Figure 6.7:** Picture of the inside of the assembled sensor.

The final value of the concentration of the bacteria can be shown in any regular monitor, through the connection of the monitor to the sensor. In addition, it is possible to see the final concatenated image and a graph exhibiting the number of particles and the threshold used. This threshold is set by eliminating signals coming mainly from the background, such as from free quantum dots.

The sensor was built to be operated in water facilities where periodic measurements should be performed with specific programmed intervals. However, it is possible to manually start a measurement if desired. The sensor system is set to warn if the measured bacteria concentration is equal or higher than 0.1 bacteria/ $\mu\text{L}$ .

A final step of this thesis work was to evaluate the final performance of the assembled sensor. For this case, measurements were started manually. The procedure followed by the sensor is: One channel of the pump injects the reagents into the system, and the sample is pumped with the second channel of the pump. Both fluids are mixed with the mixing structure composed of 7 barriers, and led to the incubator, which is set at 40°C. There, they are left for 15 minutes, and then sent to

the optical module. At this point, the laser turns on and the data collection starts. The buffer used was composed of 0.5% BSA, 5 mM EDTA, 0.01% Triton X-100 and 30 mM NaCl in PB, and the reagents concentration was of 1:10000. Different flow rates during data collection were tested in order to check if this parameter affects the results. The flow rates tested were: 2 mL/min, 0.5 mL/min and 0.2 mL/min. The concentration values for the tests under each flow rate are in table 6.1 below. It is important to add that the sample in these tests were made with sewage (collected from WVZ) diluted 10 times in tap water. This sample simulates a case of sewage contamination in water.

**Table 6.1:** E. coli concentration values for diluted sewage obtained at different flow rates

Flow rates (mL/min)	Concentration values (bacteria/ $\mu$ L)
0.2	0.156
0.5	0.047
1	0.01

It was seen that for same sample, the measured bacteria concentration varied depending on the flow rate. Comparing the values, it was clear that for flow rates equal or higher than 0.5 mL/min, the sensor could not detect every bacteria, which can be seen as a low measured bacteria concentration. For flow rate of 0.2 mL/min, the measured concentration was closer to the expected, since the reference value is of around 0.1 bacteria/ $\mu$ L. Therefore, it was seen that the flow rate indeed interferes with the data and that the results obtained suggest that higher flow rate results in lower measured E. coli concentration. Lastly, the flow rate of 0.2 mL/min returned expected concentration values and is the one to be used in further procedures.

Another parameter tested was the concentration of the labelling agents. Different concentration of antibody-conjugated quantum dots were used in the system in order to check if this parameter affects the data collection. These tests were conducted similarly to the one described above, using flow rate of 0.2 mL/min, and the tested reagents concentrations were: 1:5000 and 1:10000. The measured bacteria concentration for these tests are in table 6.2 below. It is important to add that the sample in these tests were made with tap water, which enabled the verification of the influence of different reagent concentrations.

**Table 6.2:** E. coli concentration values for tap water obtained at different reagent concentrations.

Reagents concentration	Concentration values (bacteria/ $\mu$ L)
1:5000	0.971
1:10000	0.02

The measured bacteria concentration varied depending on the used reagent concentrations. For the test conducted with QD concentration of 1:5000, the value measured was higher than expected, once it was thought to obtain a concentration of around 0.01 bacteria/ $\mu\text{L}$ . However, when the test was performed with concentration of 1:10000 for the QD, the measured bacteria concentration was in the same magnitude than the expected. Therefore it was seen that if high amount of free quantum dots are present in the background of the sample, they influenced the data collection, resulting in higher measured bacteria values than the real bacteria in the sample. In addition, the measured bacteria concentration when using 1:10000 QD was similar to the expected and can be used for future tests for verification of bacteria in water.

Final tests were made in order to compare the sensor system performance from different samples. One test was with pure tap water and the other with sewage. These tests were conducted similarly to the aforementioned steps, and by using final flow rate of 0.2 mL/min and 1:10000 reagent concentration. The measured values of the bacteria concentration with the sensor system are in table 6.3

**Table 6.3:** Measured concentration values of *E. coli* for samples from different sources.

Sample	Concentration values (bacteria/mL)
tap water	0.01
sewage	0.9

Satisfactory results were obtained with these parameters, where the sensor could discern tap water from sewage. In addition the values obtained were similar to the expected once it was thought to obtain a concentration around 0.05 bacteria/ $\mu\text{L}$  in tap water. Interesting to note is that the measured *E. coli* concentration in sewage was approximately 10 times higher than of the one obtained in a previous test with the 10 times diluted sewage, with same parameters of QD concentration and flow rate, which was 0.156 as shown in table 6.1. This suggests that the sensor system is consistent with the measured bacteria concentration in the sample water.

Some difficulties were presented regarding the data analyses with this sensor system. The analyses are made from the light emitted from each bacteria, however this information is not the same for every bacteria. This is because the immunoassay used for labelling does not stain each organism equally. This challenge can affect the data since some bacteria signal might not be detected. Besides that, one other interesting cause for losses in bacteria signal was also noticed. It is very common in nature for several bacteria to cluster together, which was also seen in the sewage. These clusters, formed by several bacteria, will have a very strong fluorescent signal and be counted as only one bacteria by the sensor.

Furthermore, it is important to highlight that there is a limitation of the concentration of bacteria in the sample to be analysed. If the bacteria concentration is too

high, the software can not discriminate all the signals obtained, and the final measured concentration is not the true concentration. However, this would correspond to a case where a lot of sewage is present in the water. In such cases, it would be clear that contamination is taking place and that the sampled water should not be distributed to the community.

Besides these limitations, this system has some favourable aspects for the purpose studied, when comparing its performance with the Viable Count (VC), which is the standard method for bacteria counting nowadays. The VC is a method that takes much more time and demands much more manual work than by using the sensor system. In addition, the VC can not count dead bacteria, only live bacteria. This is, in general, considered a positive aspect of the VC method. However, if the intention is to use the bacteria as indication that sewage has contaminated the water, it is important to be able to detect all the bacteria, even the dead ones. The sensor system developed is capable of doing this automatically, demanding nearly no hands-on work.

# 7

## Final remarks

This chapter presents the conclusions of this thesis work along with suggestions for future work in the studied area.

### 7.1 Conclusion

It was established that the proper buffer to perform the direct immunoassay in this study case is composed of 30 mM NaCl in PB, 5 mM EDTA, 0.5% BSA, 0.1% Triton X-100. It was seen that the binding reaction between the antibody and antigen should take place in a saline environment, and that the molarity of 30 mM NaCl in PB sufficed this need. In addition, the signals of the direct immunoassay were very strong with the combination of the chelator EDTA with Triton X-100 in the buffer. This indicates that sterical hindrance were reduced by the addition of these components. Furthermore, the specificity of the obtained immunoassay was high, where cross-reactivity was avoided indicating that the use of the combination of detergent and protein to block unoccupied sites in the quantum dots served its purpose well.

It was also realized that the concentration value of 1:10000 IgY-antibody functionalized quantum dot properly labelled the *E. coli*. This was, as seen, in conjunction with an incubation process that lasted 15 minutes and was performed at a temperature of 40°C. In addition, this quantum dot concentration presented a satisfying behaviour for the analysis of the obtained fluorescent signals, allowing for the background noise from the free quantum dots to be neglected by the software during data processing. It is interesting to highlight that the proposed buffer and diluted reagents produce a cost of roughly 1 euro for testing a sample water of 4 mL.

The obtained results shows that this buffer can be used to enhance and optimize immunoassay, and can be used as a starting point for performing other immunoassay. However empirical tests are recommended to be executed for each unique system to confirm the proper functioning of the immunoassay.

Another result from this thesis work is the development of a structure to mix the aforementioned buffer and quantum dots with water, being this the structure in figure 5.6. This structure obtained a proper and suitable mixing for the sensor system to work. However, for its implementation in the system, more barriers were used to have a higher certainty of its performance, given that the system will test different

sample fluids that might have different characteristics. In addition, the data acquisition has better performance now through the use of a quartz flow cell, as opposed to the previously used PMMA flow cell. This is because the new flow cell shows lower autofluorescence, has less bubble formation which benefits the flow direction, has smaller path length which results in sharper images of the bacteria, and has a high chemical resistance allowing for periodical washing procedures to take place.

It was seen that the sensor system properly works for its purpose. Final tests with the assembled sensor showed that this sensor can discriminate between potable water and contaminated water, delivering the expected values of bacteria concentration in the sample. The assembled sensor system is already in a in situ test period at the time of the publication of this thesis. This test period is being carried out in water utilities in Jerusalem and Zürich to later be implemented for its use as an early warning system.

## 7.2 Future work

It was noticed that the sensor system sometimes underestimated bacteria concentrations. This was considered to be a result of how the data is processed in the system. It is speculated that due to clusters of bacteria in the sample, and due to different signal strength from each bacteria in the sample, the sensor miscounted the amount of bacteria. Therefore it is of interest to improve this weakness of the sensor in the future. This could be done by developing a better discrimination between signals from cluster of bacteria and from single bacterium. Consequently, the data processing should be changed. Now the software only analyses fluorescent signals detected in the video, not being able to differentiate one bacterium from a cluster. But, if it could also distinguish sizes of the particles, it would be able to do this needed discernment.

Furthermore, the specificity upon the labelling was determined by performing tests with two simultaneous immunoassay methods, allowing for the comparison of the labelled organisms from both methods. This was considered to be sufficient to state that the *E. coli* were being labelled in this work. However, this was not investigated to a high precision. Therefore, it would be of interest to perform an in depth investigation upon the specificity of the immunoassay detection method. This would give a high assurance that all the stained organisms are indeed *E. coli*, and that all present *E. coli* have been labelled. One approach to do this investigation with high degree of reliability is through a method called Fluorescence in situ hybridisation, known as FISH. This method uses DNA strands that bind into the organism's chromosomes to fluorescently stain these organisms. Therefore, it can label bacteria with a very high specificity. Performing this method in combination with the immunoassay, with different excitation spectrum for both fluorescent signals, would allow for the comparison between the stained organisms. Hence it would be possible to know if the immunoassay is behaving as desired.

Lastly, it is important to point out that this sensor system was developed in a way that it allows for it to be used for different purposes. In this work, the sensor system was solely used to perform the detection of *E. coli*. However, with appropriate changes to the reagents used, the sensor system would be able to detect other organisms. Therefore, possible future utilizations of the developed technology in this thesis work and by Acreo are not limited to water quality verification, but present a broad spectrum of possible applications.





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# A

## SiteClick™ Antibody Labeling Kits

### 1. Antibody Concentration and/or Buffer Exchange (Optional)

- 1.1 Add 450  $\mu\text{L}$  of  $\text{dH}_2\text{O}$  to the small antibody concentrator (Component B) and cap the device.
- 1.2 Centrifuge for 6 minutes at  $5000 \times g$ , ensuring that the cap strap and one membrane panel of the concentrator faces the centre of the rotor.
- 1.3 Discard the flow through.
- 1.4 Add a sufficient volume of antibody solution to contain 100–125  $\mu\text{g}$  of antibody to the small antibody concentrator. For example, if the antibody concentration is 1  $\text{mg}/\mu\text{L}$ , add 125  $\mu\text{L}$ .
- 1.5 Dilute the added antibody to 500  $\mu\text{L}$  using antibody preparation buffer (Component A).
- 1.6 Centrifuge for 6 minutes at  $5000 \times g$ , ensuring that the cap strap and one membrane panel of the concentrator faces the centre of the rotor.
- 1.7 Discard the flow through.
- 1.8 Add 450  $\mu\text{L}$  of antibody preparation buffer (Component A) to the small antibody concentrator (Component B) and centrifuge for 6 minutes at  $5000 \times g$ , ensuring that the cap strap and one membrane panel of the concentrator faces the centre of the rotor. Note: If antibody volume in concentrator is greater than 50  $\mu\text{L}$  following Step 1.8, centrifuge for an additional 3 minutes at  $5000 \times g$  or until the appropriate volume is achieved.
- 1.9 Invert the small antibody concentrator (Component B) into the collection tube (Component C).
- 1.10 Centrifuge for 3 minutes at  $1000 \times g$  to collect the concentrated antibody. Following collection, you should have approximately 50  $\mu\text{L}$  of concentrated antibody in the collection tube.

### 2. Modification of Antibody Carbohydrate Domain

- 2.1 Add 10  $\mu\text{L}$  of  $\beta$ -galactosidase (Component D) to the antibody collected in Step 1.10.
- 2.2 Wrap the tube cap with Parafilm® laboratory film or similar and incubate

for 4 hours at 37°C.

### 3. Azide Attachment

- 3.1 Prepare the azide modification solution by adding the following components to the tube containing UDP-GalNAz (Component E): 75  $\mu\text{L}$  of  $\text{dH}_2\text{O}$ ; 10  $\mu\text{L}$  of 20 $\times$  Tris buffer, pH 7.0 (Component F); 25  $\mu\text{L}$  of buffer additive (Component G); 80  $\mu\text{L}$  of GalT enzyme (Component H).
- 3.2 Vortex the reaction components and then add the modified antibody from Step 2.2 to the tube.
- 3.3 Briefly centrifuge the tube, wrap the tube cap with Parafilm® laboratory film or similar, and incubate overnight at 30°C.

### 4. Purification and Concentration of Azide-Modified Antibody

- 4.1 Prepare 10 mL of 1 $\times$  Tris, pH 7.0 by adding 500  $\mu\text{L}$  of 20 $\times$  Tris, pH 7.0 (Component F) to 9.5 mL of  $\text{dH}_2\text{O}$  in a 15-mL conical tube. Vortex briefly to mix. Note: TBS may also be used for the purification and collection of the modified antibody (Steps 4.2 – 4.12). 20 $\times$  Tris, pH 7.0 is provided for convenience.
- 4.2 Remove the conical collection tube from the large antibody concentrator (Component I).
- 4.3 1 mL of 1 $\times$  Tris, pH 7.0 to the large antibody concentrator (Component I) and centrifuge for 10 minutes at 1200  $\times$  g, ensuring that one membrane panel of the concentrator faces the centre of the rotor.
- 4.4 Discard flow through.
- 4.5 Add 1.75 mL of 1 $\times$  Tris, pH 7.0 and 250  $\mu\text{L}$  of the azide modified antibody from Step 3.3 to the large antibody concentrator (Component I).
- 4.6 Centrifuge for 6 minutes at 1200  $\times$  g, ensuring one membrane panel of the concentrator faces the centre of the rotor.
- 4.7 Discard flow through.
- 4.8 Add 1.8 mL of 1 $\times$  Tris, pH 7.0 to the large antibody concentrator (Component I) and centrifuge for 10 minutes at 1200  $\times$  g, ensuring that one membrane panel of the concentrator faces the centre of the rotor.
- 4.9 Discard flow through and repeat Step 4.8 once more.
- 4.10 Add 1.8 mL of 1 $\times$  Tris, pH 7.0 to the large antibody concentrator (Component I) and centrifuge for 10 minutes at 1400  $\times$  g. Discard flow through. The final volume in the concentrator should be approximately 80–120  $\mu\text{L}$ . Note: If antibody volume in concentrator is greater than 100  $\mu\text{L}$ , centrifuge for an additional 5 minutes at 1400  $\times$  g or until the appropriate volume is achieved.
- 4.11 Invert the antibody concentrator into the conical collection tube.
- 4.12 Centrifuge for 3 minutes at 1000  $\times$  g to collect the concentrated antibody.

4.13 Transfer the antibody from the conical collection tube to a 1.5 mL centrifuge tube. If the final collected volume is less than 100  $\mu\text{L}$ , dilute antibody to 100  $\mu\text{L}$  with 20 $\times$  Tris, pH 7.0. Note: At this stage, the antibody can be stored at 2–8°C for attachment of the DIBO-modified label at a later time.

## 5. Conjugation with DIBO-modified Label

- 5.1 Add 50  $\mu\text{L}$  of Qdot® DIBO (Component J) to the azide-modified antibody in the 1.5-mL centrifuge tube.
- 5.2 Vortex the reaction mixture, briefly centrifuge, and incubate overnight at 25°C.
- 5.3 The antibody conjugate can now be stored at 2–8°C, protected from light (see Antibody Conjugate Storage) or optionally purified of excess antibody (Step 6).

## 6. Purification and Concentration of Antibody Conjugate (Optional)

- 6.1 Add 500  $\mu\text{L}$  of dH<sub>2</sub>O to the purification concentrator (Component K) and centrifuge for 5 minutes at 1500  $\times$  g. Discard flow through.
- 6.2 Add 150  $\mu\text{L}$  of the labelled antibody conjugate from Step 5.2 to the purification concentrator (Component K).
- 6.3 Add buffer (TBS or PBS) to the purification concentrator to bring the volume to 500  $\mu\text{L}$  and centrifuge for 10 minutes at 1500  $\times$  g. Discard flow through.
- 6.4 Add 500  $\mu\text{L}$  of buffer (TBS or PBS) to the purification concentrator and centrifuge for 10 minutes at 1500  $\times$  g.
- 6.5 Discard flow through and repeat Step 6.4 once more.
- 6.6 Collect the purified labelled antibody conjugate from the top of the concentrator and transfer to a new 1.5 mL centrifuge tube. The antibody conjugate can now be stored at 2–8°C, protected from light (see Antibody Conjugate Storage).

## 7. Storing and Using Antibody Conjugates

Store the antibody conjugate labelled with the Qdot® nanocrystal at 2–8°C, in the dark, until needed. DO NOT FREEZE. Sodium azide or thimerosal can be added at this stage to a final concentration of 0.02% (w/v) for long term storage, if preferred.





# B

## Alexa Fluor<sup>®</sup> 488 Labeling kit

### 1. Labeling the Protein

- 1.1 Prepare a 1 M solution of sodium bicarbonate by adding 1 mL of deionized water (dH<sub>2</sub>O) to the provided vial of sodium bicarbonate (Component B). Vortex or pipet up and down until fully dissolved. The bicarbonate solution, which will have a pH 8–9, can be stored at 2–8°C for up to two weeks.
- 1.2 If the antibody to be labelled has a concentration of  $\geq 1$  mg/mL and is in an appropriate buffer (see Preparing the Protein, above), dilute it to 1 mg/mL and then add 1/10th volume of 1 M sodium bicarbonate buffer (prepared in step 1.1). If the protein is a powder lyophilized from an appropriate buffer, prepare a 1 mg/mL solution of the antibody by adding an appropriate amount of 0.1 M sodium bicarbonate buffer to the protein. Prepare 0.1 M sodium bicarbonate buffer by diluting the 1 M solution 10-fold with dH<sub>2</sub>O. Note: Bicarbonate, pH 8–9, is added to raise the pH of the reaction mixture, since succinimidyl esters and TFP esters react efficiently at alkaline pH.
- 1.3 Transfer 100  $\mu$ L of the protein solution (from step 1.2) to the vial of reactive dye. Cap the vial and gently invert it a few times to fully dissolve the dye. Violent agitation of the protein solution can result in protein denaturation. Note: To visually confirm that the dye has fully dissolved, it may help to peel the label off the vial of reactive dye.
- 1.4 Incubate the solution for 1 hour at room temperature. Every 10–15 minutes, gently invert the vial several times in order to mix the two reactants and increase the labelling efficiency. Note: During the incubation period, proceed to steps 2.1–2.4, below, to prepare a spin column for the purification of the labelled protein. This will take  $\sim$ 15 minutes.

### 2. Purifying the Labelled Protein

The purification step removes the unbound dye from the dye-conjugated protein. In applications that utilize repeated wash steps after labelling with the dye-conjugated antibody, purification may not be necessary.

- 2.1 Place a spin column in a 13  $\times$  100-mm glass tube. Note: The enclosed

spin column should have two frits inserted at the bottom. If the two frits are not present, one or both of them may be in the plastic bag. Insert the second or both frits into the column and push them down to the bottom of the column with a glass or plastic stir rod. If any resin from the column get past the frits and ends up in the collection tube with the conjugate, it will do no harm and removing it is optional.

- 2.2 Stir the purification resin (Component C), then add 1.0 mL of the suspension into the column and allow it to settle by gravity.
- 2.3 Continue to add more of the suspension until the resin bed volume is  $\sim 1.5$  mL.
- 2.4 Allow the column buffer to drain from the column by gravity. Initially, some pressure may be required to cause the first few drops of buffer to elute. Place the spin column in one of the provided collection tubes and centrifuge the column for 3 minutes at  $1100 \times g$  using a swinging bucket rotor. Note: A fixed angle rotor will suffice if a swinging bucket rotor is not available.
- 2.5 Load the 100  $\mu\text{L}$  reaction volume (from step 1.4) drop wise onto the centre of the spin column. Allow the solution to absorb into the resin bed.
- 2.6 Place the spin column into the empty collection tube and centrifuge for 5 minutes at  $1100 \times g$ .
- 2.7 After centrifugation, the collection tube will contain labelled protein in approximately 100  $\mu\text{L}$  of PBS, pH 7.2, with 2 mM sodium azide; free dye will remain in the column bed. Discard the spin column.

# C

## General Assay optimization steps followed

### C.1 Direct Immunoassay

#### 1. Washing bacteria strain

- 1.1 Add 1 mL PBS in a 1.5-mL centrifuge tube.
- 1.2 Suspend the bacteria strain collected from agar plate in this PBS.
- 1.3 Centrifuge the tube for 4 minutes at  $6000 \times g$ .
- 1.4 Discard the flow through.

#### 2. Addition of primary antibody

- 2.1 Add 1 mL buffer in the bacteria pellet.
- 2.2 Vortex or pipet up and down until the bacteria pellet is fully dissolved.
- 2.3 Add 1:1000 diluted antibody functionalized quantum dots (with initial concentration of 1 mg/mL).
- 2.4 Rapidly vortex the tube for mixing.
- 2.5 Incubate the solution with the reagents for 30 minutes at room temperature.

#### 3. Washing procedure

This step is necessary to discard the unbound fluorescently labelled antibodies, reducing background signals.

- 3.1 Centrifuge the solution in the centrifuge tube for 4 minutes at  $6000 \times g$ .
- 3.2 Discard the flow through.

#### 4. Finalization of the immunoassay

This step is necessary to dissolve the labelled bacteria pellet to analyse the immunoassay performance in the resultant solution.

- 4.1 Add 1 mL buffer in the resultant pellet.
- 4.2 Vortex or pipet up and down until the pellet is fully dissolved.

**Table C.1:** General materials and its functions used for direct immunoassay tests

Material used	Function
Bacteria strain	E. coli strain to be labelled
Chicken $\alpha$ -E. coli	Immobilized antibody in quantum dot
Buffer	Tested media in which the reaction takes place
MilliQ <sup>®</sup> water	To adjust molarities when necessary
PBS	To wash the bacteria
Fluorescent microscope equipped with camera	Used to take pictures for analyses of the binding performance

## C.2 Indirect Immunoassay

### 1. Washing bacteria strain

- 1.1 Add 1 mL in a 1.5-mL centrifuge tube.
- 1.2 Suspend the bacteria strain collected from agar plate in this PBS.
- 1.3 Centrifuge the tube for 4 minutes at  $6000 \times g$ .
- 1.4 Discard the flow through.

### 2. Addition of primary antibody

- 2.1 Add 1 mL buffer in the bacteria pellet.
- 2.2 Vortex or pipet up and down until the bacteria pellet is fully dissolved.
- 2.3 Add 1:500 of not functionalized Chicken  $\alpha$ -E. coli.
- 2.4 Rapidly vortex the tube for mixing.
- 2.5 Incubate the solution with the antibodies for 30 minutes at room temperature.

### 3. Washing procedure

This step is necessary to discard the unbound antibodies.

- 3.1 Centrifuge the solution in the tube for 4 minutes at  $6000 \times g$ .
- 3.2 Discard the flow through.

## 4. Addition of secondary antibody

- 4.1 Add 1 mL buffer in the pellet.
- 4.2 Vortex or pipet up and down until the pellet is fully dissolved.
- 4.3 Add 1:500 of functionalized Rabbit  $\alpha$ -Chicken.
- 4.4 Rapidly vortex the tube for mixing.
- 4.5 Incubate the solution with the reagents for 30 minutes at room temperature.

## 5. Washing procedure

This step is necessary to discard the unbound fluorescently antibodies to reduce background noise.

- 5.1 Centrifuge the solution in the centrifuge tube for 4 minutes at  $6000 \times g$ .
- 5.2 Discard the flow through.

## 6. Finalization of the immunoassay

This step is necessary to dissolve the labelled bacteria pellet to analyse the immunoassay performance in the resultant solution.

- 6.1 Add 1 mL buffer in the resultant pellet.
- 6.2 Vortex or pipet up and down until the pellet is fully dissolved.

**Table C.2:** General materials and its functions used for indirect immunoassay

Material used	Function
Bacteria strain	E. coli strain to be labelled
Chicken $\alpha$ -E. coli	Primary antibody
Rabbit $\alpha$ -chicken	Secondary antibody conjugated with organic fluorophore
Buffer	Tested media in which the reaction takes place
MilliQ <sup>®</sup> water	To adjust molarities when necessary
PBS	To wash the bacteria
Fluorescent microscope equipped with camera	Used to take pictures for analyses of the binding performance

### C. General Assay optimization steps followed

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# D

## Indirect immunoassay for bacteria strain selection

### 1. Washing bacteria strain

- 1.1 Add 1 mL PBS in three separate 1.5-mL centrifuge tube.
- 1.2 Suspend K-12 bacteria strain collected from agar plate in one of the tubes with PBS.
- 1.3 Suspend XL1-Blue bacteria strain collected from agar plate in the second tube with PBS.
- 1.4 Suspend XL10-Gold bacteria strain collected from agar plate in the third tube with PBS.
- 1.5 Centrifuge all three tubes for 4 minutes at  $6000 \times g$ .
- 1.6 Discard the flow through.

### 2. Addition of primary antibody

- 2.1 Add 1 mL PBS 150 mM NaCl/PBS in each of the three tubes now containing the bacteria pellet.
- 2.2 Vortex or pipet up and down until the bacteria pellet of each tube is fully dissolved.
- 2.3 Add 1:500 of not functionalized Chicken  $\alpha$ -E. coli in each tube.
- 2.4 Rapidly vortex the three centrifuge tubes for mixing.
- 2.5 Incubate the three solutions with the antibodies for 30 minutes at room temperature.

### 3. Washing procedure

This step is necessary to discard the unbound antibodies.

- 3.1 Centrifuge the three tubes containing the solutions for 4 minutes at  $6000 \times g$ .
- 3.2 Discard the flow through.

4. Addition of secondary antibody

- 4.1 Add 1 mL PBS 150 mM NaCl/PBS in each of the three tubes now containing the pellet.
- 4.2 Vortex or pipet up and down until the pellet of each tube is fully dissolved.
- 4.3 Add 1:500 of functionalized Rabbit  $\alpha$ -Chicken.
- 4.4 Rapidly vortex for mixing.
- 4.5 Incubate the solution with the reagents for 30 minutes at room temperature.

5. Washing procedure

This step is necessary to discard the unbound fluorescently antibodies to reduce background noise.

- 5.1 Centrifuge the three tube containing the solutions for 4 minutes at 6000  $\times$  g.
- 5.2 Discard the flow through.

6. Finalization of the immunoassay

This step is necessary to dissolve the labelled bacteria pellet to analyse the immunoassay performance in the resultant solution.

- 6.1 Add 1 mL PBS 150 mM NaCl/PBS in each of the three tubes now containing the pellet.
- 6.2 Vortex or pipet up and down until the pellet in each tube is fully dissolved.

**Table D.1:** Material and quantities used for the E. coli strain tests for indirect immunoassay

Material used	Function	Quantities used in the tests
Chicken $\alpha$ -E. coli	primary antibody	0.2%
Rabbit $\alpha$ -chicken	secondary antibody	0.2%
K-12, XL10-Gold, XL1-Blue	different E. coli strains	unknown quantities taken from agar plate
PBS	Standard saline buffer	150 mM NaCl/PBS
Fixed-angle centrifuge	Used to centrifuge in washing procedures	1
Fluorescent microscope equipped with camera	Used to take pictures for analyse of the binding performance	1



# E

## Direct immunoassay for buffer salt molarity tests

### 1. Washing bacteria strain

- 1.1 Add 1 mL of 150 mM NaCl/PB in six different 1.5-mL centrifuge tube.
- 1.2 Suspend the bacteria strain K-12 collected from agar plate in each of these centrifuge tube.
- 1.3 Centrifuge all the tubes for 4 minutes at  $6000 \times g$ .
- 1.4 Discard the flow through.

### 2. Addition of antibody functionalized quantum dot

- 2.1 Add 1 mL of MilliQ<sup>®</sup> to the bacteria pellet in the first tube.
- 2.2 Add 1 mL of 7.5 mM NaCl/PBS to the bacteria pellet in the second tube.
- 2.3 Add 1 mL of 15 mM NaCl/PBS to the bacteria pellet in the third tube.
- 2.4 Add 1 mL of 30 mM NaCl/PBS to the bacteria pellet in the fourth tube.
- 2.5 Add 1 mL of 75 mM NaCl/PBS to the bacteria pellet in the fifth tube.
- 2.6 Add 1 mL of 150 mM NaCl/PBS to the bacteria pellet in the sixth tube.
- 2.7 Vortex or pipet up and down until the bacteria pellet in all the tubes are fully dissolved.
- 2.8 Add 1:10000 antibody functionalized quantum dots in each tube.
- 2.9 Rapidly vortex the tubes for mixing.
- 2.10 Incubate the solutions with the reagents for 30 minutes at room temperature.

### 3. Washing procedure

This step is necessary to discard the unbound fluorescently labelled antibodies, reducing background signals.

- 3.1 Centrifuge the solutions in the centrifuge tubes for 4 minutes at  $6000 \times g$ .
- 3.2 Discard the flow through.

4. Finalization of the immunoassay

This step is necessary to dissolve the labelled bacteria pellet to analyse the immunoassay performance in the resultant solution.

- 4.1 Add 1 mL of 150 mM NaCl in PB in the pellet in all the six tubes.
- 4.2 Vortex or pipet up and down until the pellet in all the tubes are fully dissolved.

**Table E.1:** Material and quantities used for the buffer salt molarity tests

Material used	Function	Quantities used in the tests
Chicken $\alpha$ -E. coli	Immobilized antibody in quantum dot	1:10000 for all tests
K-12	E. coli strain	unknown quantity taken from agar plate
PBS (different molarities)	Standard saline buffer	0; 7.5; 15; 30; 75; 150 mM NaCl in PB
MilliQ <sup>®</sup> water	To adjust the PBS molarity	volumes to dissolve the NaCl for required molarities
Fixed-angle centrifuge	Used to centrifuge in washing procedures	1
Fluorescent microscope equipped with camera	Used to take pictures for analyse of the binding performance	1

# F

## Direct immunoassay for blocking agents tests

### 1. Washing bacteria strain

- 1.1 Add 1 mL of 150 mM NaCl in PB in four different 1.5-mL centrifuge tube.
- 1.2 Suspend the bacteria strain K-12 collected from agar plate in each of these centrifuge tube.
- 1.3 Centrifuge all the tubes for 4 minutes at  $6000 \times g$ .
- 1.4 Discard the flow through.

### 2. Addition of antibody functionalized quantum dot

- 2.1 Add 1 mL of BBG solution to the bacteria pellet in the first tube.
- 2.2 Add 1 mL of SuperBlock™ to the bacteria pellet in the second tube.
- 2.3 Add 1 mL of solution containing 5 mM of EDTA and 30 mM NaCl in PB, to the bacteria pellet in the third tube.
- 2.4 Add 1 mL of solution containing 0.5% BSA and 30 mM NaCl in PB, to the bacteria pellet in the fourth tube.
- 2.5 Vortex or pipet up and down until the bacteria pellet in all the tubes are fully dissolved.
- 2.6 Add 1:10000 antibody functionalized quantum dots in each tube.
- 2.7 Rapidly vortex the tubes for mixing.
- 2.8 Incubate the solutions with the reagents for 30 minutes at room temperature.

### 3. Washing procedure

This step is necessary to discard the unbound fluorescently labelled antibodies, reducing background signals.

- 3.1 Centrifuge the solutions in the centrifuge tubes for 4 minutes at  $6000 \times g$ .
- 3.2 Discard the flow through.

4. Finalization of the immunoassay

This step is necessary to dissolve the labelled bacteria pellet to analyse the immunoassay performance in the resultant solution.

- 4.1 Add 1 mL of 150 mM NaCl in PB in the pellet in all the four tubes.
- 4.2 Vortex or pipet up and down until the pellet in all the tubes are fully dissolved.

**Table F.1:** Material and quantities used for tests using protein as blocker agents

Material used	Function	Quantities used in the tests
Chicken $\alpha$ -E. coli	Immobilized antibody in quantum dot	1:10000 for all tests
K-12	E. coli strain	unknown quantity taken from agar plate
PBS	Standard saline buffer	30 mM NaCl in PB
BSA	Standard protein used as blocking agent	0.5%
BBG	commercial blocking agent	1 mL
SuperBlock™	commercial blocking agent	1 mL
EDTA	chelator used to increase hydrophobicity of the bacteria membrane	5 mM
Fixed-angle centrifuge	Used to centrifuge during washing procedures	1
Fluorescent microscope equipped with camera	Used to take pictures to analyse of the binding performance	1

# G

## Direct immunoassay for ionic and non-ionic detergents tests

### 1. Washing bacteria strain

- 1.1 Add 1 mL of 150 mM NaCl/PBS in three different 1.5-mL centrifuge tube.
- 1.2 Suspend the bacteria strain K-12 collected from agar plate in each of these centrifuge tube.
- 1.3 Centrifuge all the tubes for 4 minutes at  $6000 \times g$ .
- 1.4 Discard the flow through.

### 2. Addition of antibody functionalized quantum dot

- 2.1 Add 0.5% of BSA diluted in 30 mM NaCl in PB in the bacteria pellet in all the three tubes.
- 2.2 Add 0.05% of Tween-20 in the first tube.
- 2.3 Add 0.1% of Triton X-100 in the second tube.
- 2.4 Add 0.1% of SDS in the third tube.
- 2.5 Vortex or pipet up and down until the bacteria pellet in all the tubes are fully dissolved.
- 2.6 Add 1:10000 antibody functionalized quantum dots in each tube.
- 2.7 Rapidly vortex the tubes for mixing.
- 2.8 Incubate the solutions with the reagents for 30 minutes at room temperature.

### 3. Washing procedure

This step is necessary to discard the unbound fluorescently labelled antibodies, reducing background signals.

- 3.1 Centrifuge the solutions in the centrifuge tubes for 4 minutes at  $6000 \times g$ .
- 3.2 Discard the flow through.

## 4. Finalization of the immunoassay

This step is necessary to dissolve the labelled bacteria pellet to analyse the immunoassay performance in the resultant solution.

4.1 Add 1 mL of 150 mM NaCl in PB in the pellet in all the three tubes.

4.2 Vortex or pipet up and down until the pellet in all the tubes are fully dissolved.

**Table G.1:** Material and quantities used for tests using ionic and non-ionic detergents

Material used	Function	Quantities used in the tests
Chicken $\alpha$ -E. coli	Immobilized antibody in quantum dot	1:10000 for all tests
K-12	E. coli strain	unknown quantity taken from agar plate
PB	Standard phosphate buffer	30 mM NaCl in PB
BSA	Standard protein used as blocking agent	0.5%
Tween-20	non-ionic detergent	0.05%
Triton X-100	non-ionic detergent	0.1%
SDS	ionic detergent	0.1%
Fixed-angle centrifuge	Used to centrifuge during washing procedures	1
Fluorescent microscope equipped with camera	Used to take pictures to analyse of the binding performance	1

# H

## Direct and Indirect immunoassay performed for non-ionic detergents tests

### 1. Washing bacteria strain

- 1.1 Add 1 mL of 150 mM NaCl/PBS in two different 1.5-mL centrifuge tube.
- 1.2 Suspend the bacteria strain K-12 collected from agar plate in each of these centrifuge tube.
- 1.3 Centrifuge all the tubes for 4 minutes at  $6000 \times g$ .
- 1.4 Discard the flow through.

### 2. Addition of antibody functionalized quantum dot and of primary antibody

- 2.1 Add 0.5% of BSA and 5 mM EDTA diluted in 30 mM PBS in the bacteria pellet in the two tubes.
- 2.2 Add 0.05% of Tween-20 in the first tube.
- 2.3 Add 0.1% of Triton X-100 in the second tube.
- 2.4 Vortex or pipet up and down until the bacteria pellet in all the tubes are fully dissolved.
- 2.5 Add 1:10000 antibody functionalized quantum dots for the direct immunoassay in the two tubes.
- 2.6 Add 1:500 of primary antibody for the indirect immunoassay in the two tubes.
- 2.7 Rapidly vortex the tubes for mixing.
- 2.8 Incubate the solutions with the reagents for 30 minutes at room temperature.

### 3. Washing procedure

This step is necessary to discard the unbound antibodies.

- 3.1 Centrifuge the solutions in the centrifuge tubes for 4 minutes at  $6000 \times g$ .

3.2 Discard the flow through.

4. Addition of secondary antibody

4.1 Add 0.5% of BSA and 5 mM EDTA diluted in 30 mM PBS in the bacteria pellet in the two tubes.

4.2 Add 0.05% of Tween-20 in the first tube.

4.3 Add 0.1% of Triton X-100 in the second tube.

4.4 Vortex or pipet up and down until the bacteria pellet in all the tubes are fully dissolved.

4.5 Add 1:500 of secondary antibody, functionalized with organic fluorophore for the indirect immunoassay in the two tubes.

4.6 Rapidly vortex the tubes for mixing.

4.7 Incubate the solutions with the reagents for 30 minutes at room temperature.

5. Washing procedure

This step is necessary to discard the unbound fluorescently labelled antibodies, reducing background signals.

5.1 Centrifuge the solutions in the centrifuge tubes for 4 minutes at  $6000 \times g$ .

5.2 Discard the flow through.

6. Finalization of the immunoassay

This step is necessary to dissolve the labelled bacteria pellet to analyse the immunoassay performance in the resultant solution.

6.1 Add 1 mL of 150 mM NaCl in PB in the pellet in all the two tubes.

6.2 Vortex or pipet up and down until the pellet in all the tubes are fully dissolved.



**Table H.1:** Material and quantities used for tests using non-ionic detergents

Material used	Function	Quantities used in the tests
Chicken $\alpha$ -E. coli	Immobilized antibody in quantum dot	1:10000 for all tests
Chicken $\alpha$ -E. coli	primary antibody (not conjugated)	1:500 for all tests
Rabbit $\alpha$ -Chicken	secondary antibody (conjugated antibody with organic fluorophore)	1:500 for all tests
K-12	E. coli strain	unknown quantity taken from agar plate
PBS	Standard saline buffer	30 mM NaCl in PB
BSA	Standard protein used as blocking agent	0.5%
EDTA	Standard chelator	5 mM
Tween-20	non-ionic detergent	0.05%
Triton X-100	non-ionic detergent	0.1%
SDS	ionic detergent	0.1%
Fixed-angle centrifuge	Used to centrifuge during washing procedures	1
Fluorescent microscope equipped with camera	Used to take pictures to analyse of the binding performance	1



# I

## Direct and Indirect immunoassay for Triton X-100 tests

### 1. Washing bacteria strain

- 1.1 Add 1 mL of 150 mM NaCl/PBS in four different 1.5-mL centrifuge tube.
- 1.2 Suspend the bacteria strain K-12 collected from agar plate in each of these centrifuge tube.
- 1.3 Centrifuge all the tubes for 4 minutes at  $6000 \times g$ .
- 1.4 Discard the flow through.

### 2. Addition of antibody functionalized quantum dot and of primary antibody

- 2.1 Add 0.5% of BSA and 5 mM EDTA diluted in 30 mM PBS in the bacteria pellet in the two tubes.
- 2.2 Add 0.02% of Triton X-100 in the first tube.
- 2.3 Add 0.04% of Triton X-100 in the second tube.
- 2.4 Add 0.06% of Triton X-100 in the third tube.
- 2.5 Add 0.1% of Triton X-100 in the fourth tube.
- 2.6 Vortex or pipet up and down until the bacteria pellet in all the tubes are fully dissolved.
- 2.7 Add 1:10000 antibody functionalized quantum dots for the direct immunoassay in the four tubes.
- 2.8 Add 1:500 of primary antibody for the indirect immunoassay in the four tubes.
- 2.9 Rapidly vortex the tubes for mixing.
- 2.10 Incubate the solutions with the reagents for 30 minutes at room temperature.

### 3. Washing procedure

This step is necessary to discard the unbound antibodies.

- 3.1 Centrifuge the solutions in the centrifuge tubes for 4 minutes at  $6000 \times g$ .

3.2 Discard the flow through.

4. Addition of secondary antibody

4.1 Add 0.5% of BSA and 5 mM EDTA diluted in 30 mM PBS in the bacteria pellet in the four tubes.

4.2 Add 0.02% of Triton X-100 in the first tube.

4.3 Add 0.04% of Triton X-100 in the second tube.

4.4 Add 0.06% of Triton X-100 in the third tube.

4.5 Add 0.1% of Triton X-100 in the fourth tube.

4.6 Vortex or pipet up and down until the bacteria pellet in all the tubes are fully dissolved.

4.7 Add 1:500 of secondary antibody, functionalized with organic fluorophore for the indirect immunoassay in the four tubes.

4.8 Rapidly vortex the tubes for mixing.

4.9 Incubate the solutions with the reagents for 30 minutes at room temperature.

5. Washing procedure

This step is necessary to discard the unbound fluorescently labelled antibodies, reducing background signals.

5.1 Centrifuge the solutions in the centrifuge tubes for 4 minutes at 6000 × g.

5.2 Discard the flow through.

6. Finalization of the immunoassay

This step is necessary to dissolve the labelled bacteria pellet to analyse the immunoassay performance in the resultant solution.

6.1 Add 1 mL of 150 mM NaCl/PBS in the pellet in all the four tubes.

6.2 Vortex or pipet up and down until the pellet in all the tubes are fully dissolved.

**Table I.1:** Material and quantities used for tests using different Triton X-100 concentrations

Material used	Function	Quantities used in the tests
Chicken $\alpha$ -E. coli	Immobilized antibody in quantum dot	1:10000 for all tests
Chicken $\alpha$ -E. coli	primary antibody (not conjugated)	1:500 for all tests
Rabbit $\alpha$ -Chicken	secondary antibody (conjugated antibody with organic fluorophore)	1:500 for all tests
K-12	E. coli strain	unknown quantity taken from agar plate
PBS	Standard saline buffer	30 mM NaCl/PBS
BSA	Standard protein used as blocking agent	0.5%
EDTA	Standard chelator	5 mM
Triton X-100	non-ionic detergent	0.1%, 0.06%, 0.04%, 0.02%
Fixed-angle centrifuge	Used to centrifuge during washing procedures	1
Fluorescent microscope equipped with camera	Used to take pictures to analyse of the binding performance	1



# J

## Direct and indirect immunoassay tests for different incubation temperatures

1. Washing bacteria strain
  - 1.1 Add 1 mL of 150 mM NaCl/PBS in four different 1.5-mL centrifuge tube.
  - 1.2 Suspend the bacteria strain K-12 collected from agar plate in each of these centrifuge tube.
  - 1.3 Centrifuge all the tubes for 4 minutes at  $6000 \times g$ .
  - 1.4 Discard the flow through.
  
2. Addition of antibody functionalized quantum dot and of primary antibody
  - 2.1 Add 0.5% of BSA, 5 mM EDTA and 0.1% Triton X-100 diluted in 30 mM PBS in the bacteria pellet in the four tubes.
  - 2.2 Vortex or pipet up and down until the bacteria pellet in all the tubes are fully dissolved.
  - 2.3 Add 1:10000 antibody functionalized quantum dots for the direct immunoassay in all the tubes.
  - 2.4 Add 1:500 of primary antibody for the indirect immunoassay in all the tubes.
  - 2.5 Rapidly vortex the tubes for mixing.
  - 2.6 Incubate the solutions with the reagents for 15 minutes, each tube in different temperatures: one tube at room temperature, the second at  $37^{\circ}\text{C}$ ; the third at  $40^{\circ}$  and the fourth at  $45^{\circ}\text{C}$ .
  
3. Washing procedure

This step is necessary to discard the unbound antibodies.

  - 3.1 Centrifuge the solutions in the centrifuge tubes for 4 minutes at  $6000 \times g$ .
  - 3.2 Discard the flow through.

4. Addition of secondary antibody

- 4.1 Add 0.5% of BSA, 5 mM EDTA and 0.02% Triton X-100 diluted in 30 mM PBS in the bacteria pellet.
- 4.2 Vortex or pipet up and down until the bacteria pellet in all the tubes are fully dissolved.
- 4.3 Add 1:500 of secondary antibody, functionalized with organic fluorophore for the indirect immunoassay in all the tubes.
- 4.4 Rapidly vortex the tubes for mixing.
- 4.5 Incubate the solutions with the reagents for 15 minutes, each tube in different temperatures: one tube at room temperature, the second at 37°C; the third at 40°and the fourth at 45°C.

5. Washing procedure

This step is necessary to discard the unbound fluorescently labelled antibodies, reducing background signals.

- 5.1 Centrifuge the solutions in the centrifuge tubes for 4 minutes at 6000 × g.
- 5.2 Discard the flow through.

6. Finalization of the immunoassay

This step is necessary to dissolve the labelled bacteria pellet to analyse the immunoassay performance in the resultant solution.

- 6.1 Add 1 mL of 150 mM NaCl in PB in the pellet in all the tubes.
- 6.2 Vortex or pipet up and down until the pellet in all the tubes are fully dissolved.



**Table J.1:** Material and quantities used for tests varying the incubation temperature

Material used	Function	Quantities used in the tests
Chicken $\alpha$ -E. coli	Immobilized antibody in quantum dot	1:10000 for all tests
Chicken $\alpha$ -E. coli	primary antibody (not conjugated)	1:500 for all tests
Rabbit $\alpha$ -Chicken	secondary antibody (conjugated antibody with organic fluorophore)	1:500 for all tests
K-12	E. coli strain	unknown quantity taken from agar plate
PB	Standard phosphate buffer	30 mM NaCl/PB
BSA	Standard protein used as blocking agent	0.5%
EDTA	Standard chelator	5 mM
Triton X-100	non-ionic detergent	0.1%
Fixed-angle centrifuge	Used to centrifuge during washing procedures	1
Fluorescent microscope equipped with camera	Used to take pictures to analyse of the binding performance	1



# K

## Direct immunoassay for different incubation times

1. Washing bacteria strain
  - 1.1 Add 1 mL of 150 mM NaCl/PBS in five different 1.5-mL centrifuge tube.
  - 1.2 Suspend the bacteria strain K-12 collected from agar plate in each of these centrifuge tube.
  - 1.3 Centrifuge all the tubes for 4 minutes at  $6000 \times g$ .
  - 1.4 Discard the flow through.
  
2. Addition of antibody functionalized quantum dot and of primary antibody
  - 2.1 Add 0.5% of BSA, 5 mM EDTA and 0.1% Triton X-100 diluted in 30 mM NaCl/PB in the bacteria pellet in all tubes.
  - 2.2 Vortex or pipet up and down until the bacteria pellet in all the tubes are fully dissolved.
  - 2.3 Add 1:10000 antibody functionalized quantum dots in all the tubes.
  - 2.4 Rapidly vortex the tubes for mixing.
  - 2.5 Incubate the solutions with the reagents at  $40^\circ$  for different times: the first tube for 5 minutes, the second for 10 minutes, the third for 15 minutes, the fourth for 20 minutes and the fifth for 25 minutes.

**Table K.1:** Material and quantities used for tests varying the incubation temperature

Material used	Function	Quantities used in the tests
Chicken $\alpha$ -E. coli	Immobilized antibody in quantum dot	1:10000 for all tests
K-12	E. coli strain	unknown quantity taken from agar plate
PBS	Standard phosphate buffer	30 mM NaCl/PB
BSA	Standard protein used as blocking agent	0.5%
EDTA	Standard chelator	5 mM
Triton X-100	non-ionic detergent	0.1%
Fluorescent microscope equipped with camera	Used to take pictures to analyse of the binding performance	1

# L

## Mixing test with T connector

1. High concentration of fluorescent particles diluted in tap water were pumped with a syringe pump at flow rate of 0.1 mL/min
2. Tap water was pumped with a peristaltic pump at flow rate of 0.9 mL/min
3. The two fluids were joined by a T connector, which is connected to another tube where both joined fluids are flowing.
4. The tube with the joined fluids are connected to a plastic flow cell which is in a fluorescent microscope.
5. The final mixing in the flow cell is analysed through the microscope by using an adequate light source emission. The images of the final mixing are taken with a camera coupled to the microscope.

**Table L.1:** Material and its functions used to verify the mixing by using a T connector

Material used	Function
Fluorescent particles diluted in tap water	Fluid 1 to be mixed
Tap water	Fluid 2 to be mixed
Syringe pump	To pump fluid 1
Peristaltic pump	To pump fluid 2
PMMA flow cell	Used to verify the final mixture
Fluorescent microscope equipped with camera	Used to take pictures to analyse of the mixing performance



# M

## Direct immunoassay for sewage tests

### 1. Preparation of buffer with primary antibody

This buffer is to be diluted in sewage water by 1:2 dilution. Therefore the concentrations in this buffer is initially higher, which the correct concentration values is achieved after this dilution occurs.

- 1.1 Add 1% of BSA, 10 mM EDTA and 0.2% Triton X-100 in 60 mM PBS.
- 1.2 Add 1:2500 of primary antibody for indirect immunoassay.

### 2. Preparation of buffer with secondary antibody and quantum dots

This buffer is not diluted in sewage water, instead it is used to dilute the bacteria pellet after washing procedures. Therefore, the concentrations is already at their normal values in his buffer.

- 2.1 Add 0.5% of BSA, 5 mM EDTA and 0.1% Triton X-100 in 30 mM PBS.
- 2.2 Add 1:500 of secondary antibody for indirect immunoassay. Add 1:10000 of antibody conjugated quantum dot for direct immunoassay as well.

### 3. Dilution of the buffer containing the primary antibody in the sewage water and incubation.

- 3.1 Take 500  $\mu$ L of the buffer containing the primary antibody and dilute in 500  $\mu$  of sewage water in a 1.5 mL centrifuge tube.
- 3.2 Rapidly vortex the tubes for mixing.
- 3.3 Incubate the solutions with the reagents at 40° for 15 minutes.

### 4. Washing procedure

This step is necessary to discard the unbound antibodies.

- 4.1 Centrifuge the solutions in the centrifuge tubes for 4 minutes at  $6000 \times g$ .
- 4.2 Discard the flow through.
  
- 5. Addition of the buffer containing the secondary antibody and the quantum dots.
  - 5.1 Add 1 mL of the buffer containing the secondary antibody and the quantum dots into the bacteria pellet.
  - 5.2 Vortex and pipet up and down the solution until the pellet is diluted.
  - 5.3 Incubate the solution with the reagents at  $40^\circ$  for 15 minutes.

**Table M.1:** Material and its functions used for tests with sewage

Material used	Function
Chicken $\alpha$ -E. coli	Immobilized antibody in quantum dot
Sewage water	To detect the E. coli present in it
PBS	Standard saline buffer
BSA	Standard protein used as blocking agent
EDTA	Standard chelator
Triton X-100	non-ionic detergent
Fluorescent microscope equipped with camera	Used to take pictures to analyse of the binding performance