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Automation of a solid-phase proximity ligation assay for biodefense applications

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Title (English)

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Abstract

The extent of devastation caused by a biological warfare attack is highly correlated to the time from release to detection. As a step towards lowering the detection time the international project TWOBIAS was launched. Here, the main goal is to develop an automated, specific and sensitive combined detection and identification instrument capable of identifying a biological threat within an hour. The identification unit is comprised of a sample preparation module, an amplification module and a detection module and utilizes a proximity ligation assay in combination with circle-to-circle amplification in order to detect a biological threat. This thesis describes the automation of the sample preparation steps of the assay and the integration with the downstream units. The functionality of the sample preparation module was verified by subjecting it to biological samples in a laboratory and at a real-life location. The results showed that the sample preparation module was capable of preparing a sample collected in a complex environment with the same results as a sample prepared in a laboratory.

Keywords

Biological warfare, proximity ligation assay, real-life location trial, automated pathogen detection, air samples, liquid handling robot

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Automation of a solid-phase proximity ligation assay for biodefense applications

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Populärvetenskaplig sammanfattning

Användning av biologiska vapen har länge varit förbjudet men trots detta har sådana vapen använts vid flera tillfällen. Hur stor effekt en biologisk attack har är starkt korrelerat till hur lång tid som går från utsläpp till upptäckt. Den här rapporten beskriver utvecklingen av en automatiserad provberedningsmodul som kan användas för att detektera luftburna biologiska hot inom en timme från utsläpp. Systemet i fråga använder proteinanalysmetoden närhetsligering (proximitetsligering) för att upptäcka och identifiera en viss bakterie. Det är en känslig och specifik metod som kan känna igen specifika proteiner på ytan av t.ex. en bakteriespor eller en viruspartikel. Metoden bygger på att tillsatta antikroppar binder till dessa ytproteiner. När två antikroppar bundit tillräckligt nära varandra på en spor bildas en DNA cirkel. Denna cirkel kan kopieras och förstärkas till ett stort linjärt DNA nystan vilket gör det möjligt att upptäcka bakterien.

En design som uppfyllde de uppsatta målen togs fram och implementerades i provberedningsmodulen. Efter implementering undersöktes modulens förmåga att bereda ett prov, dels i en labbmiljö men även på en offentlig plats. Genom att jämföra resultat från modulen med manuella resultat visades att modulen generar ett likvärdigt resultat. Modulen integrerades med de övriga delarna av identifieringsinstrumentet och utsattes för spridning av *Bacillus atrophaeus* sporer i en tunnelbanestation i Prag. Instrumentet visade att det även i en komplex miljö klarade av att detektera de utsläppta sporerna.

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Abbreviations

ASMD	Amplified single-molecule detection
BDU	Biological detection unit
BG	*Bacillus atrophaeus*
BIU	Biological identification unit
BSA	Bovine serum albumin
C2CA	Circle-to-circle amplification
CAD	Computer-aided design
CNC	Computer numerical control
EDTA	Ethylenediaminetetraacetic acid
FP7	EU's Seventh Framework Program
FOI	Swedish defence research agency
PBS	Phosphate buffered saline
PLA	Proximity ligation assay
RCA	Rolling circle amplification
RCP	Rolling circle products
RSP9000	Robotic sample processor, the robotic arm used
SASS2300	Air sampler
SPM	Sample processing module
TWOBIAS	Two Stage Rapid Biological Surveillance and Alarm System for Airborne Threats

1 Introduction

1.1 The project

1.1.1 Background

This master degree project was part of an EU-financed project, involving ten partners and running over three years.

1.1.1.1 Q-linea AB

The project was performed at Q-linea AB (hereinafter Q-linea), a privately held company situated in Uppsala Science Park. The company develops systems used for single molecule detection and analysis, in order to identify and detect microorganisms. At the moment, Q-linea is focusing on two areas; the defence sector, with the aim to detect biological threats; and the healthcare sector, focusing on infection diagnostics and investigating antibiotic susceptibility. The degree project was part of the former category.

1.1.1.2 TWOBIAS

1.1.1.2.1 Background

During the past 20 years great advances have been made in genetic engineering allowing scientists to design organisms to better suite their needs. As our knowledge of biological systems increase, especially regarding disease-causing microorganisms, it is important to address the impact genetically modified pathogens can have if used as warfare agents. In 1925 the Geneva Protocol was established prohibiting the use of biological weapons. But despite this international treaty the use of such weapons has been recorded at several occasions during the 20th century resulting in many casualties [1].

In order to reduce the potential devastation of biological warfare, early detection, identification and warning is of utmost importance. Minimizing the time from outbreak to response will minimize spreading of the agent, lower the time needed to trigger countermeasures and save lives. For a system to be effective, in addition to giving a fast result, it must be able to detect threat agents with high sensitivity and very low false alarm rates in order to avoid unnecessary and expensive actions. At the moment, commercially available systems fail on the latter requirement. The problem for these combined detection and warning systems is to distinguish a potential threat from the naturally occurring biological background. A major challenge for a novel instrument is to attain a sensitivity low enough to detect particles small enough to be respirable, and still have very low false alarm rates.

1.1.1.2.2 Objective of TWOBIAS

The international project TWOBIAS is part of the EU´s Seventh Framework Program (FP7). The aim of the project was to develop a modular demonstrator capable of detecting and

identifying airborne biological threats, a so-called Two Stage Rapid Biological Surveillance and Alarm System for Airborne Threats (TWOBIAS).

The system will consist of two alarm stages, StageONE and StageTWO, used to detect-to-warn and to detect-to-treat, respectively. StageONE is composed of a biological detection unit (BDU) with the capability of continuously measuring the naturally occurring biological background. If a biological incident should occur, the BDU will detect the biological changes, in the form of increased levels of a potential threat agent in the local environment, and provide an early warning. By doing so, countermeasures, such as evacuation or isolation, can be put in at an early stage.

Upon a positive alarm from the BDU, StageTWO is initialized in order to identify the detected bioaerosol [2]. The biological identification unit (BIU) utilizes a microfluidic platform in order to identify agents with high sensitivity. A positive alarm from the BIU ensures that correct treatment is given to exposed and non-exposed individuals. Also, it confirms the StageONE alarm and subsequently verifies a biological hazard.

The system is intended to be placed at high-risk sites, such as airports and train stations, where a bioterrorist attack would result in many casualties.

Up to this moment, real-life location testing of a combined BDU/BIU system is lacking. In order to investigate if the system is operational in such an environment a real-life location trial was set up. The TWOBIAS system will be placed in the Prague metro where a biological threat agent simulant – *Bacillus atrophaeus* – will be aerosolised. Upon dissemination, the BDU will alarm and consequently trigger the BIU to start its identification procedure. Approximately one hour post dissemination, the BIU can confirm the presence of BG spores.

1.2 Project objective

The main goal of this degree project was to automate the molecular technique used in the BIU, namely a proximity ligation assay (PLA). If sufficient time was available, the project would also include integration of the instrument with the upstream and downstream parts of the BIU.

1.2.1 Limitations

The project was limited to automation of the sample preparation steps of the protocol as the downstream parts already were automated. Also, only the solid-phase PLA protocol was to be automated. Prior to the project it was decided that the sample preparation module would be built on a RSP9000 robotic sample processor and a SASS2300 air sampler.

2 Background

2.1 The molecular technique applied

2.1.1 Amplified single-molecule detection (ASMD)

In order for the TWOBIAS system to be reliable, a specific and highly sensitive detection technique must be employed. In addition, the technique must be possible to multiplex as well as capable of processing complex environmental samples. Commonly, PCR is used in identification instruments [3]. However, it can be sensitive to the latter criterion.

As described by Jarvius *et al.* [4] amplified single-molecule detection proves to be an efficient method for biomolecule enumeration, illustrated in **fig. 1**. The method aims to enlarge an individual molecule to an enumerable object. For nucleic acid or protein detection pad-lock probes and proximity ligation assay can be used, respectively. As bacterial spores will be used as the simulant in the TWOBIAS project, a proximity ligation assay was applied.

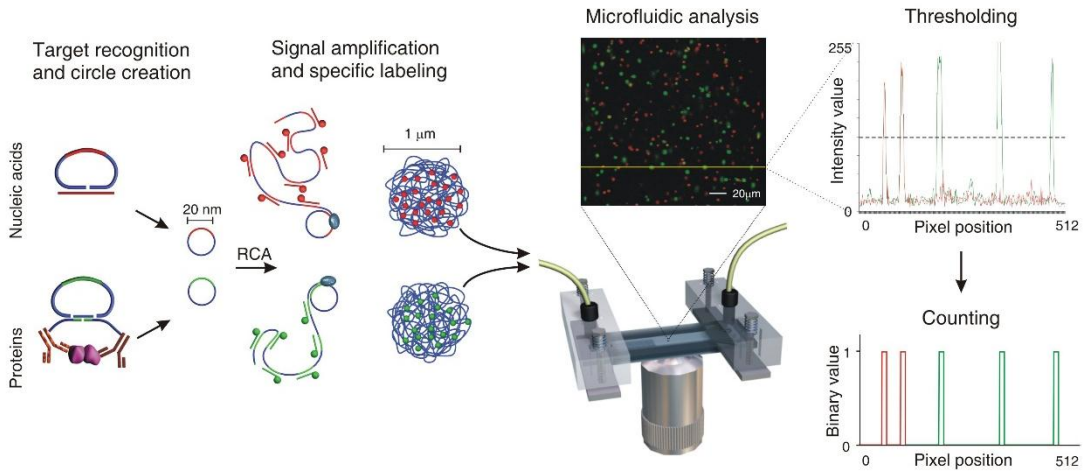

Figure 1. The mechanism of ASMD. Padlock probes or a proximity ligation assay enables enumeration of nucleic acids or proteins, respectively. Binding of the target results in the formation of a circular DNA molecule. Several cycles of rolling circle replication are employed to amplify the DNA circle and consequently form an enumerable object. The rolling circle products (RCP) are labelled with fluorescent tags and detected using a dedicated detection instrument. The output data is analysed with regards to the intensity of the fluorescently labelled RCPs. An intensity value above a set threshold is considered to correspond to an RCP and the number of RCPs in the image can thus be determined. *Figure used with permission from Q-linea.*

2.1.1.1 Solid-phase proximity ligation assay (PLA)

Proximity ligation assay (PLA) is a sensitive and specific protein detection assay. It utilizes the proximal binding of probes to a target protein and can be used to create a circular DNA molecule that can be amplified and detected using ASMD [5].

The BIU will employ a solid-phase PLA protocol using streptavidin-coated magnetic beads as the stationary phase. The PLA system is depicted in **fig. 2**. Here, protein specific biotinylated antibodies – capture antibodies – bind to the magnetic bead by a strong biotin-streptavidin

bond. By binding the capture antibody, the target molecule is immobilized. Furthermore, two additional antibodies – PLA probes – with attached oligonucleotides are added to the protein solution. As the PLA probes bind to the target protein the oligonucleotides are brought in proximity of each other resulting in the formation of a DNA template [6, 7]. Two additional oligonucleotides, a backbone and a splint, can hybridize to the template in such a way that a single-stranded DNA circle is formed. After enzymatic ligation, the circle can be amplified using rolling circle amplification (RCA).

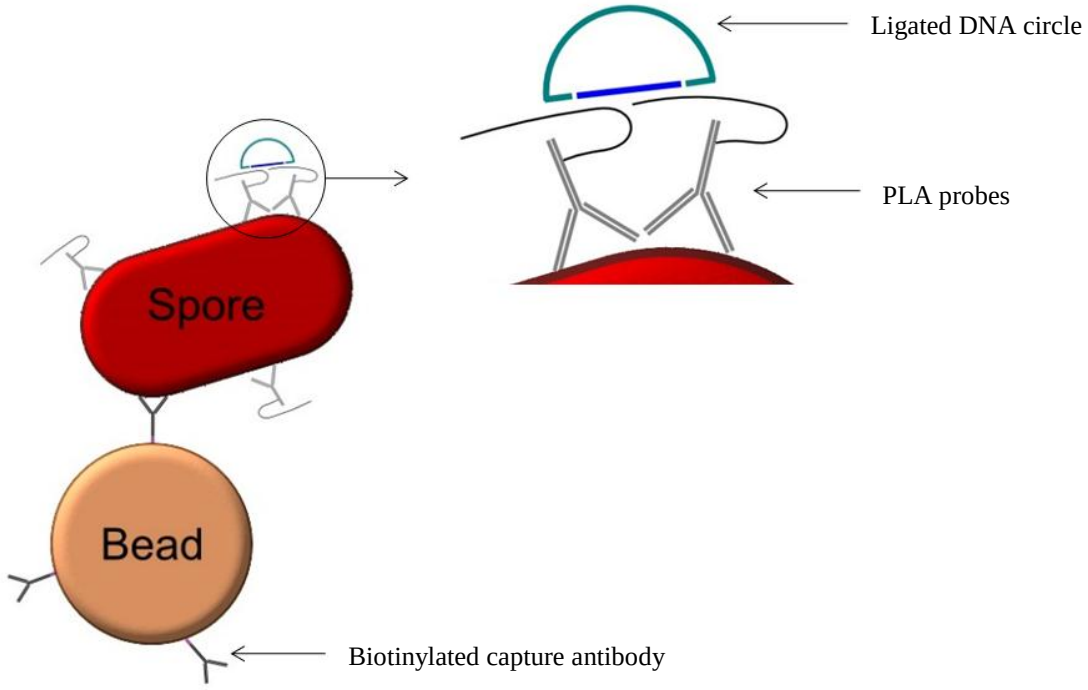

Figure 2. The PLA system. Biotinylated capture antibodies bind the streptavidin coated magnetic bead and the target molecule. In the close-up, two PLA probes have bound to the target in proximity of each other. The splint (blue) and the backbone (green) hybridize to the two oligonucleotides on the PLA probes allowing the formation of a DNA circle upon ligation.

2.1.1.2 Rolling circle amplification (RCA)

RCA, analogous to the replication mechanism utilized by several viruses, is the process where a single stranded circular DNA or RNA molecule is amplified [8].

The method is initiated by the hybridization of a primer oligonucleotide to the backbone of the PLA product. A polymerase, commonly phi29, copies the circle numerous times. The resulting replication product is a long linear DNA strand composed of multiple tandem complementary copies of the template circle. As the template is circular it is theoretically possible to achieve an endless amplification. However, the properties of the polymerase are limiting.

2.1.1.3 Circle-to-circle amplification (C2CA)

C2CA is applied within the ASMD scheme to further enlarge the PLA product and achieve an easier detection of the target protein. **Fig. 3** illustrates the C2CA mechanism.

The replication products are enzymatically digested into monomers complementary to the original circle. Furthermore, the monomers are circularized by ligation allowing them to undergo a second RCA cycle upon the addition of a complementary primer, as described above [4].

As the C2CA cycles have been repeated a desired number of times the amplification reactions are halted prior to monomerization. The long DNA strands collapse into large DNA coils (rolling circle products or RCPs), that can be labelled with fluorescent tags used for detection.

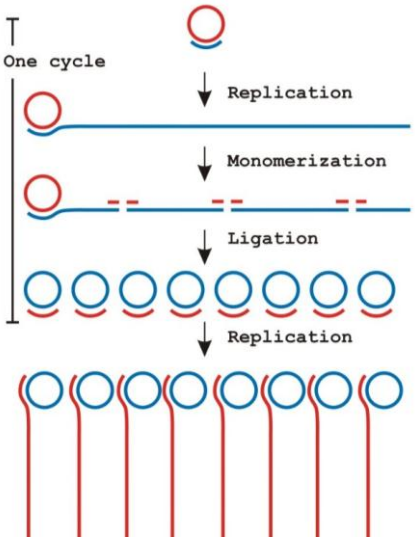

Figure 3. The mechanism of C2CA. The circular DNA molecule is amplified using RCA. The replication products are monomerized and ligated. The scheme is then repeated a desired number of times. Here, the red and blue colors indicate the different polarity of the DNA molecule. Jarvius *et al.* 2006. *Figure used with permission from Q-*

2.1.2 Detection

Detection of the labelled replication products is carried out using a dedicated high-speed fluorescent detection instrument (Q-linea AB, Uppsala, Sweden). The labelled replication products are passed through a flow cell under illumination by lasers of multiple wavelengths. In response to illumination, the labels emit a fluorescent light of a specific detectable wavelength allowing enumeration of the replication products [3].

2.2 The biological target

Bacillus atrophaeus (BG), previously *globigii*, is a spore-forming, non-pathogenic bacterium. Due to its similar appearance to *Bacillus anthracis* it is widely used and has a long history of being used as a biowarfare simulant [10]. In the experiments described in this report, BG spores were used as the simulant.

2.3 Biological identification unit (BIU)

The identification unit of the TWOBIAS system, the BIU, is composed of three sub-units; a sample preparation module, an amplification module and a detection module in said order, as depicted in **fig. 4**. The different components are connected through tubing, allowing the sample to be transferred internally without human involvement. The focus in this report is mainly on the sample preparation module.

Programming of the amplification and the detection module was not part of the degree project, however integration with the sample preparation module became part of it. Prior to the project it was decided that the SASS2300 air sampler and the RSP9000 robotic arm were to be used in the preparation module, consequently this could not be changed during the project. The tubing configuration and the nature of the integration was investigated as part of the project.

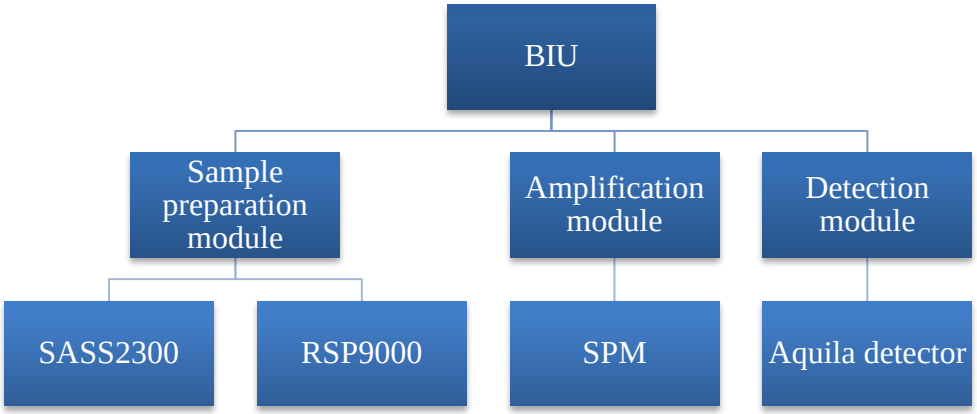

Figure 4. Schematic illustration of the BIU. The BIU is composed of a sample preparation module (the SASS2300 air sampler and the RSP9000 robotic arm), an amplification module (the SPM) and a detection module (the Aquila detector).

The BIU utilizes a proximity ligation assay (PLA) in combination with circle-to-circle amplification (C2CA) in order to identify a biological threat agent. Briefly, the air sampler will collect samples and transfer them to the preparation module. Here, all preparation steps of the PLA protocol are performed. As the sample is fully prepped it is transferred to the amplification module for the subsequent processing. The sample is further transferred to the detection module for detection and analysis.

2.3.1 The sample preparation module

The sample preparation module (**Fig. 5**) is composed of several components. However, the SASS2300 and the RSP9000 are the two main components. Prior the project the valve and pump was fitted to the RSP9000.

Figure 5. The sample preparation module. The sample preparation module, here equipped with a SASS2300 air sampler (on top) and a RSP9000 robotic

2.3.1.1 RSP9000

The RSP9000 (Cavro Scientific Instruments, San Jose, USA) is a robotic arm (**Fig. 6A**), that when integrated with pumps, valves and tubes can function as a complete liquid handling system. It is also fitted with a tip-head connected to two tips. It is controlled from an external host and any integrated valves and pumps are also controlled through it. The arm is operational along three axes, X/Y/Z, each with its own motor. It can be designed to move to specific positions specified by the external host.

2.3.1.2 Rheodyne TitanEX valve

A six-port liquid distribution valve (Rheodyne, California, USA) is mounted on the RSP9000 (**Fig. 6B**). Through a number of tubes it is connected to the robotic arm, the pump and the liquids used.

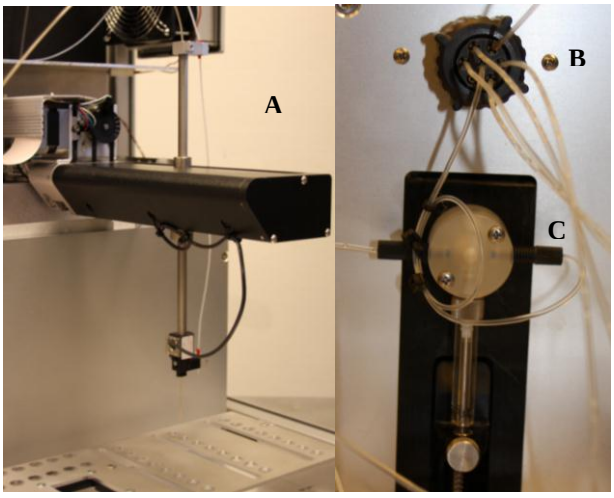

Figure 6. Close-up of the parts of the sample preparation module. A) An RSP9000 robotic arm. B) The six-port liquid distribution valve and C) the two-port valve and syringe with connected tubing.

2.3.1.3 Cavro pump

The Cavro XCalibur Modular Digital Pump (Tecan Systems, San Jose, USA) used is equipped with a two-port valve, one inlet and one outlet, and a 250 µl syringe. The two-port valve is connected to the six-port valve via a sample loop (**Fig. 6C**).

2.3.1.4 SASS2300 air sampler

The SASS2300 (Research International, Monroe, USA) is a wetted-wall cyclone air sampler capable of collecting particulates from air (**Fig. 7**). In the beginning of a sampling period a defined volume of water, typically 5 ml, is pumped from the internal water reservoir into the cyclone. This water is never exchanged during one sampling cycle. The cyclone is fitted with a liquid level sensor that enables the sampler to compensate for possible evaporation. All particulates collected during sampling are mixed with the water in the cyclone. The liquefied sample is transferred to the sample preparation module for further processing.

Figure 7. The SASS2300 air sampler. The air sampler is connected to the RSP9000 through tubing.

2.3.2 Amplification module – Sample processing module (SPM)

All ligation and amplification reactions occur in the SPM (Q-linea, Uppsala, Sweden). The system is designed so that samples can be processed continuously with given intervals. It utilizes a carousel platform, with room for 60 reaction cartridges, which moves one position every minute. The sample preparation module is connected to the SPM through tubing, which enables a prepped sample to be transferred to a reaction cartridge in the carousel. Different steps of the processing occur at different positions and 45 min after sample injection the fully processed sample is transferred to the detection module.

2.3.3 Detection module – Aquila detector

The Aquila detector (Q-linea, Uppsala, Sweden) will be used for detection. The technique used is briefly described in section 2.1.2.

3 Materials and methods

3.1 Protocol

The solid-phase PLA protocol was designed and verified by employees at Q-linea. It was slightly adapted to fit the capacity of the robot regarding pipetting volumes.

3.1.1 Antibodies and target

The BG spores were provided by FOI. At Q-linea they were diluted in PLA buffer to a suitable concentration. Biotinylated capture antibodies were coupled to Dynabeads MyOne™ Streptavidin T1 beads as described by Göransson *et al*. [3] and diluted in PLA buffer and incubated for a minimum of 5 min.

3.1.2 Sample preparation protocol

Initially, the antibody-coupled magnetic beads were mixed with PLA buffer (0.1 % BSA (Sigma-Aldrich), 1 mM D-biotin (Invitrogen), 0.1 g/l salmon sperm DNA (Invitrogen), 5 mM EDTA, 0.1 uM goat IgG, 1 x PBS, 0.05 % Tween-20) and incubated for five minutes. The supernatant was removed from the beads and both PLA probes and the sample were added. PLA buffer was added to reach a concentration of 0.5 nM and 5000 spores/5 µl for each PLA probe and sample, respectively. The solution was gently mixed and left to incubate in room temperature for a minimum of five minutes. After incubation a magnet was placed against the tube allowing the sample-coupled beads to pellet and the supernatant to be removed. Three washing cycles consisting of addition and removal of PLA wash-buffer (1 x PBS, 0.05 % Tween-20) were performed. The washing was followed by the addition of 50 µl of pre-ligation buffer (0.2 g/l BSA, 1 x Amp buffer) and the pellet was resuspended by vortex.

3.1.3 Sample processing protocol

The sample processing was initiated by the addition of 10 µl of ligation mixture (100 nM backbone, 100 nM splint, 33 nM replication oligonucleotide, 0.2 g/l BSA, 1 x Amp buffer, 50 mU/µl ampligase) after which the solution was incubated at 50°C for five minutes. To remove unreacted PLA probes the solution was washed twice using Mg wash-buffer (33 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 % Tween-20) employing the same procedure as during the PLA wash, after which the remaining supernatant was removed.

The circularized amplifiable products were further subjected to C2CA. Firstly, 50 µl of amplification mixture (0.2 g/l BSA, 125 µM dNTPs, 100 mU/µl phi29 polymerase, 1 x phi29 polymerase buffer (Thermo-Scientific; 33 mM Tris-acetate (pH 7.9 at 37 °C), 10 mM Mg-acetate, 66 mM K-acetate, 1 % (v/v) Tween-20, 1 mM DTT)) was added followed by incubation and inactivation for eleven minutes at 37°C and two minutes at 75 °C, respectively. The amplified products were monomerized by the addition of 20 µl of digestion mixture (0.2 g/l BSA, 1 x phi29 polymerase buffer, 200 mU/µl Alu1, 90 nM replication oligonucleotide) and incubated and inactivated for one minute at 37 °C and one minute at 75 °C, respectively. The beads were separated from the monomers using a magnet after which the supernatant was transferred to a fresh tube. The monomers were circularized, amplified and labelled by the addition of 0.2 g/l BSA, 14 mU/µl T4 DNA ligase, 1 x phi29 polymerase buffer, 5 nM detection probe I, 5 nM detection probe II and 60 mU/µl phi29 polymerase. Detection and enumeration was carried out in an instrument utilizing the same technique as the Aquila detector.

4 Design

4.1 Requirements

A requirement specification was constructed early on to present all requirements that the sample preparation module must fulfil. The tabulated requirements in tables 1-5 were decided by the author. Requirements that were pre-defined and therefore could not be changed during the project are not included. Such requirements were that the RSP9000, SASS2300, SPM and

Aquila detector was to be used. The specification is divided into different categories either regarding to a specific function or where the requirements belong to a certain part of the module.

The overall aim of the sample preparation module was to automatically prepare a sample, collected by an air sampler at a real-life location, for solid-phase PLA. More precisely, the sample should be collected by the air sampler and transferred to the sample preparation module. Antibodies and magnetic beads should be added to the sample. Furthermore, the sample should undergo several washing steps after which a pre-ligation buffer will be added. The fully prepped sample will then be transferred to the SPM.

4.1.1 Functionality

Table 1 presents some basic requirements for the unit to function properly.

Table 1. Requirements regarding the functionality of the sample preparation module.

ID	Requirement
1	The unit must keep reagents cooled
2	Capable to process a sample at least every eight minutes
3	Process one sample within 16 minutes
4	Avoid contamination of the reagents
5	Avoid contamination of the sample
6	Avoid cross contamination between samples

4.1.2 The protocol

The sample preparation module must fulfil the requirements in **table 2** in order to follow the sample preparation protocol presented in section 3.1.2.

Table 2. Requirements regarding the molecular protocol.

ID	Requirement
7	Add antibody coupled magnetic beads to sample tubes
8	Add and mix 5 µl of PLA probe 1 with beads
9	Add and mix 5 µl of PLA probe 2 with beads
10	Dilute the sample
11	Add and mix 5 µl of sample with the PLA probe and bead solution
12	Incubate the sample for a minimum of five minutes
13	Separate the solid-phase from the liquid phase
14	Perform a minimum of three washes using PLA wash-buffer
15	Add and mix 50 µl of pre-ligation buffer with the sample solution

4.1.3 The air sampler

There are a number of requirements, presented in **table 3**, that the air sampler must fulfil in order to reach the overall objective of the BIU.

Table 3. Requirements regarding the air sampler.

ID	Requirement
16	Collect air samples simultaneously as the processing
17	Deliver a collected sample every eight minutes
18	Purge the sample chamber post sample delivery

4.1.4 Processing- and buffer deck

All reagents and buffers needed to meet the requirements presented in **table 4** are placed in tubes in a processing- or buffer deck, respectively. They must be designed in such a way that all samples, buffers and reagents can fit. The volumes are calculated by multiplying the amount needed during one sample of each solution with the maximum capacity, namely 16 samples and adding a few extra samples used as a spare volume.

Table 4. Requirements regarding the processing- and buffer deck.

ID	Requirement
19	24 samples received from the air sampler
20	16 samples to be processed
21	16 samples to be diluted
22	115 µl of PLA probe 1
23	115 µl of PLA probe 2
24	1200 µl of pre-ligation buffer
25	20 ml of PLA wash-buffer
26	100 ml of system buffer
27	25 ml of wash solution

4.1.5 Integration

Table 5 presents the requirements to be fulfilled regarding integration.

Table 5. Requirements regarding the integration.

ID	Requirement
28	Automated transfer from the air sampler to the RSP
29	Automated transfer from the RSP to the SPM

4.2 Design

This section aims to describe the design developed to fulfil the requirements.

4.2.1 Syringe and valve

A distribution valve is mounted on the sample preparation module, as illustrated in **fig. 8**. The valve is equipped with six ports used for liquid distribution and one extra port which via a sample loop connects the valve with a 250 µl syringe. A second valve, though equipped with two ports – one inlet and one outlet – is connected to the syringe. The outlet port is connected to the sample loop and consequently to the distribution valve and the tube connected to the inlet port is immersed in system buffer. Furthermore, port 1 on the distribution valve is connected to the robotic arm. The tubes connected to port 2, 5 and 6 are immersed in PLA wash solution, a waste tube or wash solution, respectively. Port 3 is connected to the SPM.

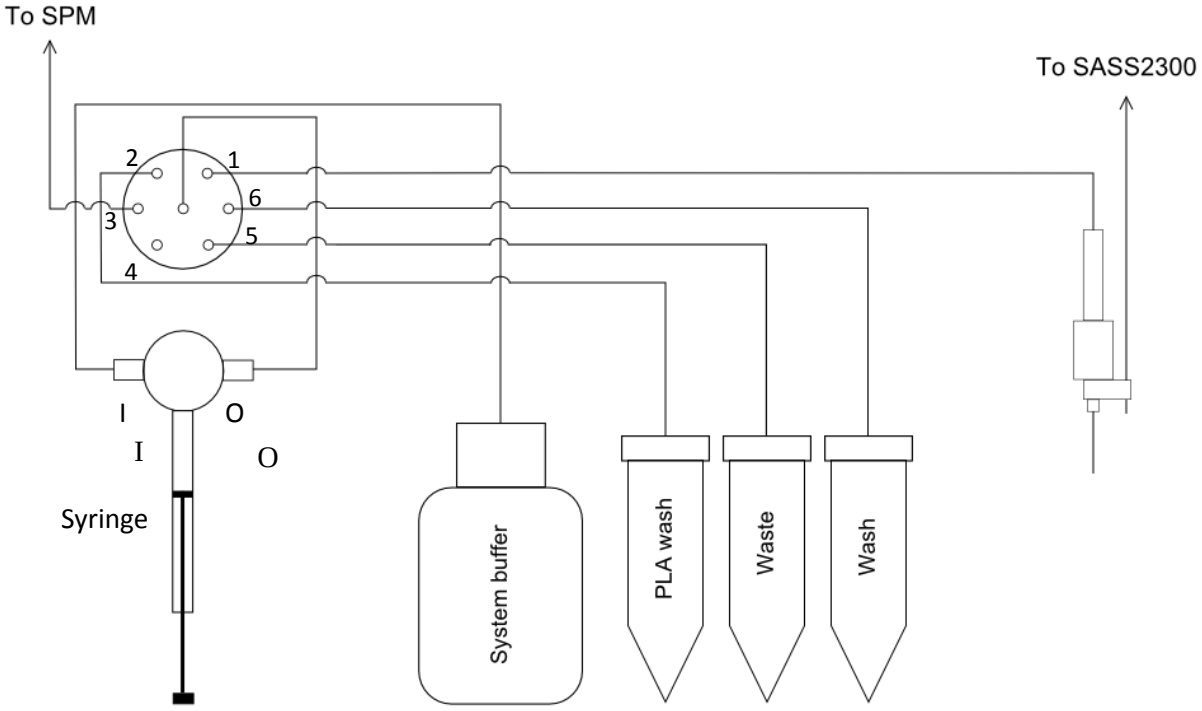

Figure 8. Schematic illustration of the fluidics in the sample preparation module. The six-port distribution valve is connected to the outlet port (O) of the two-port valve and the syringe via a sample loop. Port 1 of the six-port valve is connected to the tip-head and port 3 to the SPM. The remaining ports are either connected to specific buffers or not used.

4.2.2 Tip-head layout

The tip-head is mounted on the robotic arm. As illustrated in **fig. 9**, it is fitted with two tips; the collection tip and the processing tip that in turn are connected to the air sampler or to the distribution valve, respectively. The collection tip is connected to the air sampler through a switch. A second tube is connected to the switch that in turn is placed in a waste container. This allows the air sampler to pump liquid either to the RSP for further processing or to discard it through the waste tube. By default the switch is in the waste position.

The collection tip is dedicated to transferring the collected sample from the air sampler while the processing tip is dedicated to perform all sample preparation operations. The collection tip is used only once during each sample and must not interfere with the downstream operations performed by the processing tip. Hence, it is much shorter than the processing tip, which also reduces the risk of contamination.

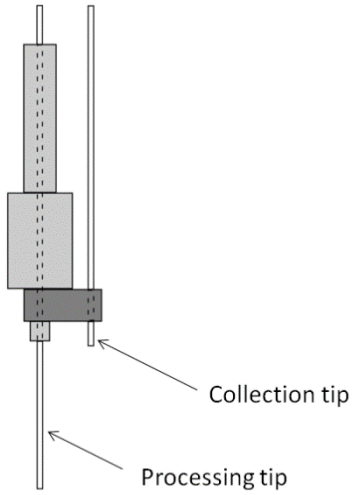

Figure 9. Illustration of the tip head mounted on the robotic arm. The processing tip and the collection tip is connected to the distribution valve and to the air sampler, respectively. The collection tip transfers collected samples from the air sampler to the RSP. All other operations are carried out by the longer processing tip.

4.2.3 Processing- and buffer deck

The robotic arm can only move within a limited area of approximately 150 x 550 mm. The processing deck had to be designed with this in mind while still fulfilling the requirements. The design was created using the 3D CAD software SolidWorks and the decks were cut out using a Datron M8 CNC milling machine.

The buffer deck is composed of four holes with diameters of 35 mm each with room for a 50 ml falcon tube.

The processing deck consists of several holes with different functions, as illustrated in **fig. 10**. In the bottom left side 16 holes, with diameters of 6 mm, are placed in two columns. Each column can fit one strip of eight 200 µl PCR tubes. The PCR tubes are henceforth referred to as the Processing tubes or Processing positions. A square-shaped hole is placed in the bottom centre of the plate. A cooling block with room for ten Eppendorf tubes can be placed in this position (Reagent positions/tubes), ensuring that the reagents used stay cooled. The right half of the deck is occupied by 24, eleven mm diameter, holes distributed over three columns. Eppendorf tubes can be placed in these positions (Collection tubes/positions) and used for storage of collected samples transferred from the air sampler. Due to the design of the tip head, a groove is positioned to the left of each column. As the collected sample is transferred from the air sampler, the longer processing tip can be lowered into the groove allowing the shorter collection tip to be immersed into a collection tube. In the upper left side of the plate, 16 eleven mm holes are placed in two rows. An Eppendorf tube can be placed in each position (Dilution tube/position) allowing the sample to be diluted prior to further processing.

A wash station is placed in the upper left corner of the processing deck. It is positioned in such a way that the processing tip will not interfere while cleaning the collection tip. The cleaning station is composed of two cylinders, one narrow located inside a wider. In the bottom of the narrow cylinder is a small hole allowing liquid to travel into the wider one. The wider cylinder is, through a hole in the bottom and tubing, connected to a waste container. Furthermore, it is connected to a vacuum pump in order to ensure sufficient suction.

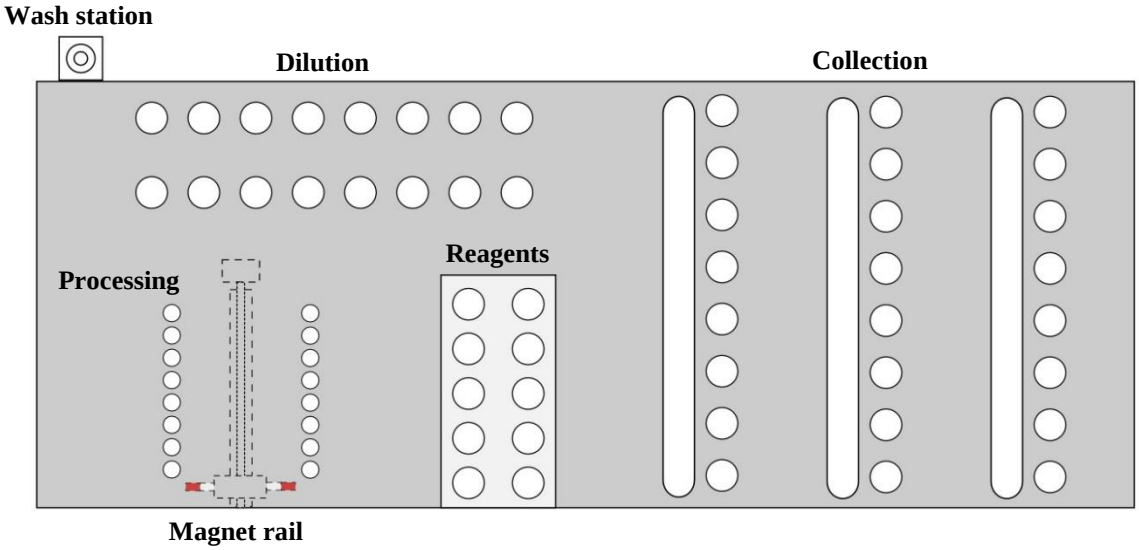

Figure 10. Illustration of the processing deck. The dilution, collection and reagent positions can fit 16, 24 and 10 Eppendorf tubes, respectively. The processing positions can fit 16 tubes á 250 µl. The magnet rail is fitted underneath the deck. It is equipped with two magnets on a cart that can slide back and forth on the rail. The wash station is designed so that the tip is cleaned on both the inside and the outside.

A larger bottom plate supports the entire sample preparation unit. The buffer- and processing decks are mounted on stilts connected to the bottom plate, thus leaving sufficient height for Eppendorf tubes. Additionally, a rail is mounted underneath the sample holder in between the two strips of processing tubes (**Fig. 11**). A cart connected to a motor is placed on the rail allowing it to slide back and forth. The cart has two magnets attached, one on each side facing a strip. Consequently, as the cart is moved into position the left side and right side magnets are placed against a processing tube on the left and right strip, respectively.

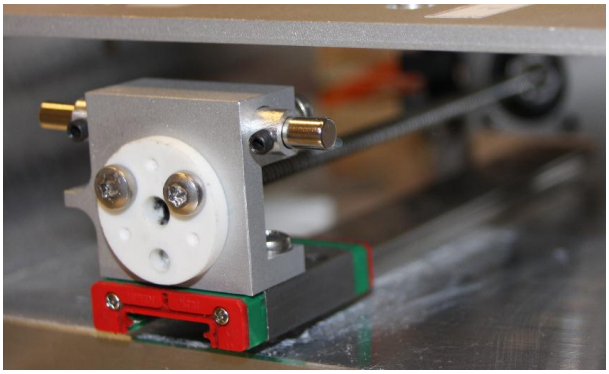

Figure 11. The magnet cart with two magnets attached. The cart is fitted on a rail underneath the processing positions of the processing deck. The magnet on each side of the cart can be positioned against a processing tube.

4.2.4 Magnet cart movement

The magnet cart movement must fulfil some additional requirements to fulfil the overall goal. As will be stated in section 4.2.5, two samples overlap during the processing procedure meaning that one sample is incubating when the second is to be washed. The wash is initiated by the cart moving to the active processing tube where the magnetic beads consequently form a pellet against the tube wall. As the wash is finished the cart moves away, allowing the pellet to be resuspended. These movements must not disrupt the incubating sample. Additionally, as the distance between the processing tubes is rather small each magnet affects three tubes. Hence, there must be a gap of at least on tube between the incubating sample and the one to be washed.

Each sample has a corresponding processing tube. The order of which processing tube to be used is different from that of the collection tubes. The generic order would be to start at tube number 1 and end at number 16. However, this sequence would interfere with the incubating sample. Therefore, the sequence depicted in **fig. 12** was created. Also, the magnet position corresponding to each processing position is depicted. As illustrated in **table 6** each sample number has one corresponding processing position and three magnet positions. Prior to the wash the magnet cart moves to the Wash position and stays put for the duration of the wash. As the wash is finished it moves to the Transfer position allowing the resuspended sample to be transferred to the downstream unit. After the transfer and prior to the collection of the next sample, the cart moves to the End position.

Table 6. The three positions the magnet move between for each sample.

Sample No.	Processing pos.	Wash pos.	Transfer pos.	End pos.
1	2	2	0	0
2	4	4	2	2
3	6	6	4	6
4	8	8	6	6
5	12	4	6	4
6	10	2	4	4
7	14	6	4	6
8	16	8	6	6
9	3	3	5	3
10	1	1	3	3
11	5	5	3	5
12	7	7	5	5
13	11	3	5	3
14	9	1	3	3
15	13	5	3	3
16	15	7	0	0

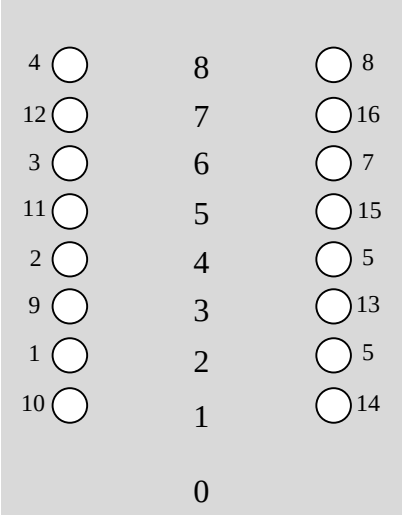

Figure 12. The order in which the processing tubes are used. The numbers next to the tubes indicate which sample is processed in that specific tube. The numbers in the middle represent the magnet positions.

4.2.5 Event description

The sample preparation procedure is divided into six events where each event corresponds to one or several parts of the preparation protocol. The events are depicted in **table 7**. A complete run of one sample occupies approximately 16 minutes. However, the sample preparation unit is run in cycles of eight minutes during which all events are carried out. Each sample is to be incubated post event 2 for a minimum of five minutes leaving the RSP vacant. Consequently, the preparation of the subsequent sample can start as the former sample is incubating.

Table 7. The six events performed during one eight-minute cycle.

Event No.	Event
0	Collection of air samples
1	Delivery of an air sample to the RSP
2	Addition of PLA probes and dilution of sample
3	Incubation
4	PLA wash and addition of pre-ligation buffer
5	Transfer of the prepped sample to the SPM

Each cycle starts with event 1 – transfer of a collected air sample to a collection position in the RSP. Event 0 – collection of an air sample – is carried out simultaneously as events 1-5 in order to maximize the sampling time. Since the processing of two samples is overlapping during one cycle, events 1-3 are carried out for sample X while events 4-5 are carried out for sample X-1, as further described in 4.2.6. Furthermore, event 0 is carried out for sample X+1.

The events are comprised of several operations, which will be further explained in this section. The operations involving transfer of a liquid from one tube to another during a cycle are depicted in **fig. 13**. It also illustrates in which order the operations are carried out.

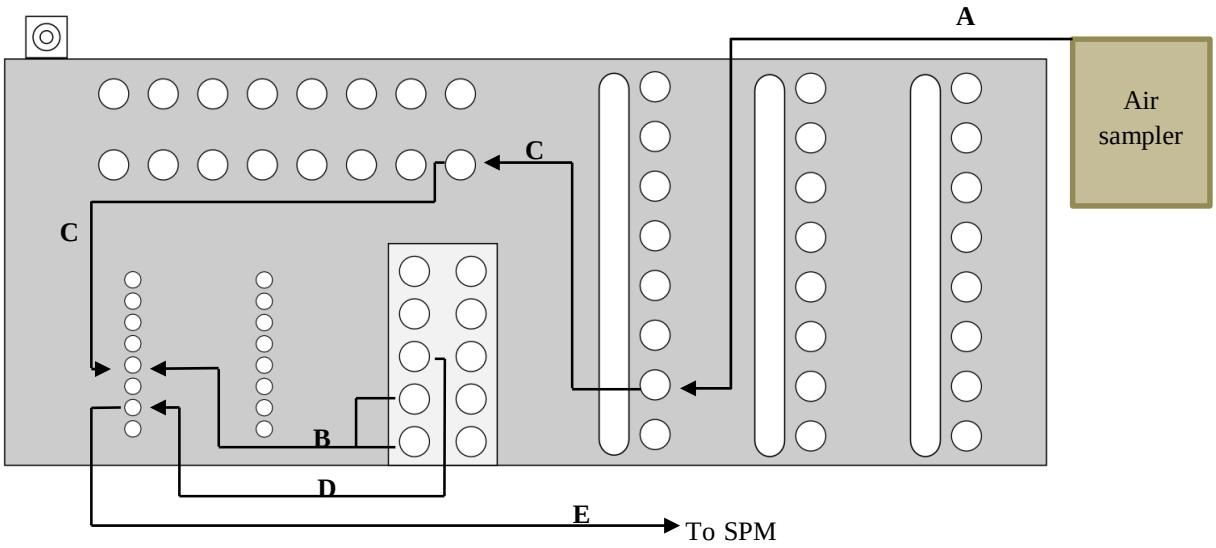

Figure 13. Illustration of the operations carried out during an eight-minute cycle. A) Delivery of sample X from the air sampler to a collection tube. B) Addition of PLA probes to the processing tube corresponding to sample X. C) Dilution of sample X in a dilution tube followed by the transfer to its processing tube. D) Addition of pre-ligation buffer to the processing tube corresponding to sample X-1. E) Transfer of sample X-1 to the SPM.

4.2.5.1 Collect air samples

During the on-going processing the air sampler will continuously collect air samples. However, prior to the first sample the air sampler will not have undergone a complete sampling cycle. Instead, as the system is prepared for a run a 20 s sampling period is performed to fill the cyclone. Except from this first sample, the air sampler will work in eight-minute cycles.

A sampling cycle starts as the fan is turned on filling the cyclone with 5 ml water from the reservoir.

4.2.5.2 Delivery of a sample to the RSP

Each processing cycle starts with the delivery of a collected air sample. The delivery starts by turning off the fan and aborting the sampling. The collection tip is positioned at the wash station allowing it to be primed by the air sampler. Upon complete priming the RSP moves to the corresponding collection tube. Here, the air sampler delivers 1 ml of the collected sample. These are the only operations carried out by the collection tip during each cycle. For the remainder of the cycle the processing tip is used. After sample delivery the RSP moves the collection tip back to the wash station. The air sampler pump is turned off and the switch on the tube connecting the air sample with the RSP is in waste position to diminish dripping and contamination. The sample chamber is completely emptied through the waste tube after which the fan is turned back on marking the beginning of a new sampling period.

The collected samples will either be just collected or collected and processed. Upon a given command entered by the user, processing of the upcoming sample will start. If not aborted by

the user the system will continue to process samples until the maximum capacity (16 processed samples) is reached. When the last sample has been processed the system returns to only collecting.

4.2.5.3 Addition of PLA probes and dilution of sample

If a sample is to be processed the first step is to add PLA probes to the corresponding processing tube. The RSP moves to reagent position 1 and aspirates 5 µl of air. All aspirations are preceded by aspiration of 5 µl of air. As the system is completely primed the aspirated liquid would become diluted without the air gap in between. The processing tip is immersed into the reagent tube, aspirates 5 µl of PLA probe 1, moves to a processing tube and dispenses the liquid.

After addition of the first probe, the arm moves to the wash station and immerses the processing tip. The tip is cleaned by pumping, in said order, 250 µl of system buffer, 125 µl of 20 % NaOCl and 250 µl of system buffer through the processing tube. The same procedure is used for all washes throughout the sample preparation.

Addition of the second probe is identical to the first with the exception that the arm moves to reagent tube 2.

After the second wash the arm moves to a collection tube and aspirates 20 µl of sample. It is dispensed in the corresponding dilution tube, resulting in a 5-fold dilution. The solution is mixed by a repeated aspiration and dispensation procedure. After proper mixing, the processing tip aspirates 5 µl and transfers it to the corresponding processing tube. The mixture containing the diluted sample, PLA probes and magnetic beads is mixed using the previous technique. After mixing the tip is washed and the sample mixture is left for incubation.

4.2.5.4 PLA wash and addition of pre-ligation buffer

During incubation of the sample mixture, processing of the preceding sample is resumed. The incubated sample is washed using PLA wash-buffer to remove unreacted PLA probes and various debris in the supernatant.

The magnet cart must be positioned against the processing tube to enable the magnetic beads to form a pellet against the tube wall throughout the wash. The wash is initiated by the movement of the magnet cart to a wash position followed by a 20 second pelleting pause. The arm moves to the processing tube, aspirates the supernatant and moves back to the wash station where the tip is washed and primed with PLA wash-buffer. It moves back to the processing tube, dispenses 100 µl of PLA wash-buffer and after a 3 second pause the supernatant is once again aspirated. The washing procedure is then repeated an additional two times.

As the supernatant has been removed after the last wash, the magnet cart moves to the transfer position and the tip is washed and moved to reagent tube 3. It aspirates 50 µl of pre-ligation

buffer and dispenses it in the processing tube. The pellet is partly resuspended in the buffer by pumping the liquid up and down.

4.2.5.5 Transfer of the prepped sample to the SPM

Some additional mixing, to completely resuspend the pellet, is performed before the transfer commences. Large air gaps are used before and after the sample to keep it well separated from the system buffer in order to minimize dilution.

The transfer is divided into three sub events; Pre-transfer, Transfer and Post-transfer. The transfer procedure is initiated with the pre-transfer, which transfers the sample to the end of the transfer tube. The sub-event starts with the processing tip aspirating the first air gap. Thereafter, the tip is immersed into the sample and aspirating both the sample and the second air gap. These first aspirations leave the sample and the air gaps in the processing tube just prior to the distribution valve. The next aspiration transfers the sample and air gaps into the sample loop. The following step is synced with the SPM. The liquid in front of the sample must be discarded before the sample can be transferred to a reaction cartridge. Therefore, after an okay from the SPM, the liquid in front of the sample is discarded and consequently the sample is transferred from the sample loop to the end of the transfer tube. Upon this action the pre-transfer is finished. The sub-event transfer is commenced when the SPM is ready. As a reaction cartridge is placed in the correct position the sample is dispensed leaving the air gap in the transfer tube. The last sub-event – the post-transfer – is carried out as the reaction cartridge has changed position. Washing of the transfer and processing tube with a series of flushing steps, using 20 % NaOCl and water, completes the transfer.

As the washing is finished the magnet cart moves to the End position ensuring it does not affect the upcoming sample. With this operation the cycle is finished and a new eight-minute cycle begins.

4.2.6 Time table

One of the objectives of TWOBIAS is to develop an instrument with the ability to sample and identify airborne biological threats within a short period of time. To meet this goal the requirements state that the sample preparation unit must be able to process a sample every eight minutes and a total of 16 samples within 2.5 hours. By always collecting one and processing two samples simultaneously the overall handling time of the sample preparation unit is reduced.

The sample preparation unit is run in cycles of eight minutes. Each cycle comprises all six preparation events. However, events 0, 1-3 and 4-5 (**Table 7**) are carried out for the following sample, the current sample and the former sample, respectively.

Fig. 14 illustrates the collection of four samples and processing of three, where the third sample can be likened with the 16^{th}, meaning that no more processing will occur after sample 3 but only collection. The pattern of the sample 2 and 3 is repeated for the remaining 13 samples. In between the events short pauses are integrated, ensuring that the instrument has

sufficient time to complete each event. The first cycle starts at 0 min with a 5 s pause, followed by the delivery of a collected air sample. However, as this is the first sample to be delivered the air sampler has not performed a complete sampling period. Instead, a short sampling period of 20 s is performed prior to cycle start. The air sampler is turned off for 26 s in order to deliver a sample and empty the cyclone. The second sampling cycle starts as the cyclone is emptied and is performed simultaneously as the processing of sample 1. Following the delivery of sample 1 is a short pause and the addition of PLA probes and dilution of the collected sample. Sample 1 is incubated for approximately eight minutes. While sample 1 is incubating the RSP is unused until the second cycle starts. Also here, the cycle starts with delivery of the collected sample. As sample 2 has been diluted, PLA probes has been added to the processing tube and incubation has started, sample 1 is once again active. The PLA wash is performed and the pre-ligation buffer is added before the sample can be transferred.

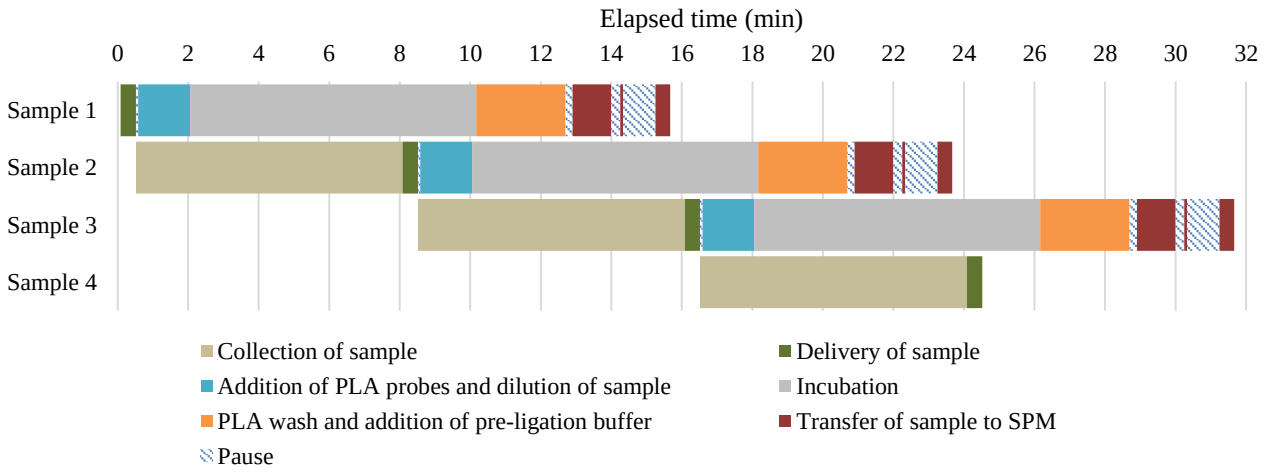

Figure 14. Time table. Illustration of how the collection and processing procedures are overlapping. Here, four samples (1-4) are collected and three of these (1-3) are processed. Every eight-minute cycle starts with the delivery of a sample (green), which is directly followed by the collection of the subsequent sample (beige). Addition of PLA probes and dilution of the sample (blue) is carried out for the newly delivered sample followed by incubation (grey). During incubation the subsequent sample is delivered (beige). After incubation PLA wash is performed (orange) followed by transfer to the SPM (red).

To ensure that the sample is correctly transferred to a reaction cartridge the transfer process is synced with the SPM. The SPM utilizes a carousel platform, with room for 60 reaction cartridges, which changes position every minute. The RSP and the SPM run on different clocks to enable the transfer to be as time efficient as possible. Due to the nature of the transfer it requires three carousel positions, one for each of the sub events. The pre-transfer is only dependent of the SPM during the end of the event when the sample is transferred to the end of the transfer tube. Therefore, the pre-transfer is scheduled to finish just before the SPM performs a position change. Also here a safety margin in the form of a few seconds pause is used before and after the position change. When the reaction cartridge is in the transfer position the sample is dispensed. Since the transfer occurs just after a carousel movement, it is followed by a rather long pause. Just after the upcoming position change the last transfer event, post-transfer, starts.

When the post-transfer is finished the processing of sample 1 is finished but Sample 2 is still incubating, which allows the third cycle to start. The pattern is repeated for the remaining samples. However, like sample 1 sample 16 will be processed partially without overlapping another sample. As only collection and sample delivery will occur post sample 16 the last part of the incubation will occur with a vacant RSP.

5 Verification

The main goal of the project was to automate the sample preparation steps of an existing PLA protocol. The module was to be integrated with three other modules in order to function as a biological threat agent detection and identification instrument.

For the system to function properly several requirements had to be fulfilled. This section aims to describe if the requirements were fulfilled and how it was verified.

Fig. 15 portrays the workflow of the verification process. Firstly, the functionality of the RSP was verified by controlling its capability to transfer liquids and the consistency regarding volumes. Secondly, the RSP was used to prepare samples which were further processed in the laboratory. Furthermore, the entire BIU system was used to perform the PLA protocol and finally it was tested at a real-life location.

Figure 15. Verification and validation process workflow. The verification and validation was performed in the four steps depicted.

5.1 Functionality verification

In order to verify the functionality of the sample preparation module several experiments were performed. Depending on which requirements were to be verified, different experimental set-ups were applied.

5.1.1 Liquid transfer

To ensure liquids are transferred in a correct manner, several experiments using food colour were set up. In three respective experiments coloured Milli-Q water was manually dispensed in the collection tubes, dilution tubes and processing tubes. In the first experiment the transfer of the simulated sample from a collection tube to a dilution tube was verified by running the

corresponding program and visually assuring the liquid was properly transferred. The procedure was repeated in the second experiment but instead verifying the transfer from a dilution tube to a processing tube.

The transfer of a sample from the sample preparation module to the downstream unit was investigated and verified in the third experiment. Due to the limited moving range of the RSP9000 the sample must be transferred via the distribution valve. If the sample is aspirated into completely primed processing tubing, the dilution degree will be rather high. Also, as the sample is dispensed into a reaction cartridge it must be well secluded from the surrounding liquid. Therefore, large air gaps were aspirated on both sides of the sample. Again, coloured water was used to simulate the sample. While running the transfer events the sample path was followed from the processing tube to the reaction cartridge. As the sample halted at the end of the transfer tube the size of the air gap in front of it was visually controlled to be of sufficient size. Also, as the sample had been dispensed in the reaction cartridge, the size of the second air gap was controlled.

The experiment was repeated but instead using magnetic beads in PLA buffer as the simulated sample. The test showed that the transfer procedure had to be somewhat adapted to fit the more viscose PLA buffer. The sample became more elongated, when compared to water, which caused it to stop too close to the end of the transfer tube during pre-transfer. As a consequence, the air gap left in the tube was too small. Therefore, the distance the sample is transferred in the transfer tube was slightly decreased.

5.1.2 Volumes

For the sample preparation module to be comparable with laboratory work, all aspirations and dispensations must be performed with a high consistence regarding volumes especially small ones. Several dispensations of varying volumes were performed after which the volume was measured using a pipette. It was discovered that the lower limit was about 5 µl when using the 250 µl syringe and the current tubing configuration.

The consistency regarding the volume transferred to the SPM was also investigated. The processing tubes were filled with 50 µl of water and the three transfer events were carried out several times after which the volumes were measured. The transferred volumes varied between 45 and 47 µl meaning about 6-10 % of the sample is lost, either it is left in the processing tube or lost in the RSP tubing. When repeating the experiment using magnetic beads in PLA buffer the same result was achieved.

5.1.3 Solid phase handling

The capability of the RSP to handle a solid phase, especially during the PLA wash, was investigated. Two processing tubes were prepared, each with 5 µl of magnetic beads dissolved in 45 µl of water. One of the tubes was placed in a processing position after which the magnetic cart was positioned against the tube, making the beads form a pellet on the wall. The

PLA wash operations were performed while visually assuring that the pellet never left the tube wall. Upon the wash, 45 µl of water was manually added and the beads were dissolved. When dissolving the beads the solution takes on a brown colour making it possible to compare the two tubes. It was decided that the similarity in colour between them was satisfying confirming that no great loss of beads occurred during the PLA wash.

5.1.4 Mixing

The mixing ability of the RSP was investigated by dispensing a highly concentrated volume of coloured water or magnetic beads in a tube with un-coloured water and visually controlling the mixing. The test illustrated that the mixing procedure employed resulted in a homogenous solution.

5.2 Experimental verification and validation

This section aims to describe how it was verified that the sample preparation module can handle biological samples.

5.2.1 Experimental set-up of the sample preparation module

Prior to all experiments, preparations were performed both in the laboratory and on the instruments.

All reagents used in the BIU were prepared in the laboratory as described in section 3.1.

The entire RSP system was flushed with water to minimize the risk of contamination. In the dilution positions 16 tubes were placed each containing 80 µl of PLA buffer. The 16 processing tubes were each filled with 70 µl of PLA buffer and 5 µl of magnetic beads and placed in the processing positions. The cooling block was normally stored in +4 °C and just before start placed in the reagent position. The two PLA probe solutions were distributed in two tubes placed in the first two positions of the cooling block. Additionally, the pre-ligation buffer was dispensed in a tube placed in the third position of the cooling block and 24 empty tubes were placed in the collection positions. Two tubes containing PLA wash-buffer and wash solution were placed in the buffer plate whereas a 250 ml flask containing the system buffer – Milli-Q water – was placed next to the instrument. Also, an empty waste tube was placed in the buffer plate. As the PLA wash-buffer tubing, waste tubing, wash solution tubing and system buffer tubing was immersed in the corresponding tube, the system was primed.

The water reservoir in the air sampler was filled with approximately 800 ml of MilliQ water and the tubing connecting it with the RSP was connected. The last operation was to run the air sampler for 5 min in order to flush the cyclone with water and thereby minimising the risk of contaminations.

After each test the RSP was flushed with water, washed with 20 % NaOCl and again flushed repeatedly with water. The air sampler was only emptied, no extra washing was performed.

5.2.2 Biological verification of the sample preparation module

Several experiments were performed to verify that the sample preparation module is capable of preparing a sample for downstream processing. To verify the response from the RSP on a biological sample, the sample preparation protocol was performed in the RSP while the sample processing protocol was performed in the laboratory. Also, both protocols were performed in the laboratory and the results were compared with those from the sample preparation module.

The system was set up as previously described but with a few adaptations. The collection tubes were pre-filled with PLA buffer or BG spores diluted in PLA buffer used as negative and positive samples, respectively. To ensure no liquid was transferred from the air sampler to the collection tubes, the tubing connecting it with the RSP was placed in a waste container. This way the air sampler could run allowing the integration with the RSP to be tested without interfering with the verification of the RSP. Furthermore, the dilution rate was increased from a 5-fold to a 20-fold.

In the experiment presented in **fig. 16**, the RSP gave lower background values and lower RCP counts on positive samples when compared to laboratory samples. Both the RSP and lab samples resulted in a distinct difference between signal and background. By dividing the mean signal with the mean background, a signal-to-noise (S/N) value is attained. This exemplifies how much higher the signal is to the background. The S/N values for the RSP and the laboratory samples were 110 and 109, respectively.

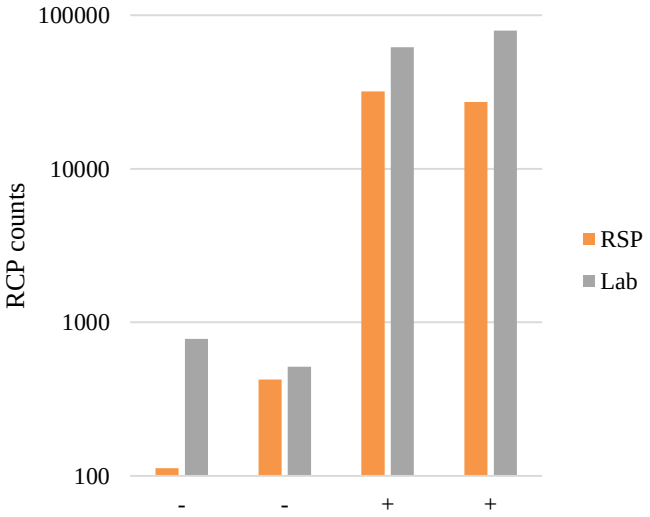

Figure 16. The sample preparation module prepares biological samples with good results. Comparison between samples prepared in the sample preparation module (orange) and manually prepared samples (grey). Two positive (+) and two negative samples (-), containing only PLA buffer, were run resulting in a similar amount of counted RCPs.

Next, an experiment was set up to investigate the carryover between samples prepared in the sample preparation module. Also here was the set-up of the RSP as previously described and the sample processing protocol and detection was performed offline. Five samples were run alternating negative and positive. As depicted in **fig. 17** there was once again a distinct difference between signal and background. The first positive sample was followed by a low count in the negative sample. The pattern was repeated for the second positive sample. Also, the second and third negative samples are just slightly higher than the first.

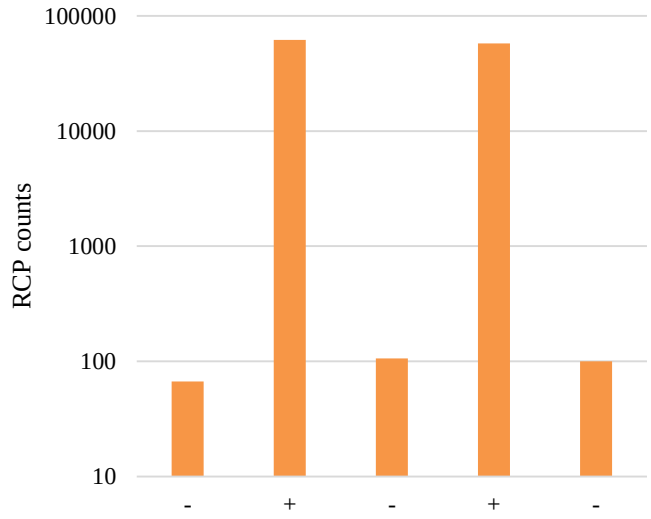

Figure 17. Investigation of carryover. Five samples, three negative (-) and two positive (-), were prepared using the sample preparation module in order to investigate the amount of carryover between samples. The similarity of the three negative samples indicate that no carryover was occurring.

5.2.3 Biological verification of the BIU

At a similar trial as the one described in section 5.2.4, performed a year before the current trial, air samples were collected both with and without spore disseminations. These samples were run in the instrument during the verification as a preparation for the upcoming trial.

The entire BIU was tested with the exception of the air sampler where the same procedure as for the preceding experiment was employed. The instrument was set up as previously described. However, no experiments were performed in the laboratory but the results can be compared with those in **fig. 16**. **Fig. 18*A*** displays the results from running two negative and two positive samples in the BIU. Again, there was a distinct difference between signal and background.

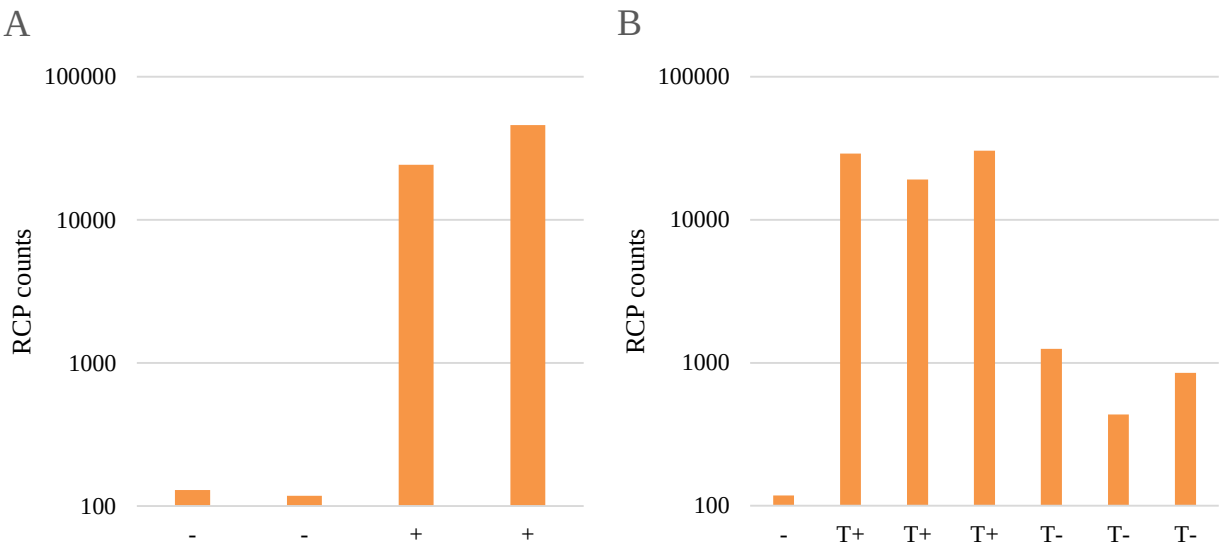

Figure 18. Biological verification of the BIU. The entire BIU was subjected to biological samples. A) Two negative (-) and two positive (+) samples were run through the identification instrument. B) Samples collected at a previous real-life location trial were analysed. The T+ samples were collected during a dissemination while T- were not. The first sample (-) was a negative control containing only PLA buffer.

In **fig. 18*B*** the first sample (-) was a negative control containing only PLA buffer. Samples 2-4 (T+) were collected during a dissemination at the previous trial and would therefore supposedly contain spores whereas samples 5-7 (T-) were collected without dissemination and therefore expected to be negative. The negative trial samples resulted in higher counts than the negative control while the positive trial samples were in the same range as the positive controls in **fig. 18*A***. Due to the higher RCP counts in the T- samples the difference between signal and background was not as distinct as in previous experiments.

5.2.4 Validation of the BIU

As part of the TWOBIAS project a trial was performed at a real-life location. The trial took place 6-11th of October 2013 in a metro station centrally located in Prague. The aim of the trial was to demonstrate that it is possible for a system to automatically detect and identify a dissemination of BG spores in the natural environment existing at a public site. The results from three tests, each performed during the closed hours of the metro, are reported here.

The BDU was comprised of several detectors, these will not be described in this report but rather focus will be on the BIU.

5.2.4.1 Site description

The trial site, consisting of four levels, is a meeting point of two metro lines used by many passengers every day. On the fourth and bottom level, where all disseminations occurred, was one platform with trains running in two directions. An escalator led up to the third level, which was a passage between the two platforms. The second level was reached with an escalator leading up to the second platform, also with trains running in two directions. A last set of escalators led up to the first level and the metro entrance.

The BIU together with some instruments of the BDU were placed on the third level just at the top of the escalators. The systems were controlled from the control bridge situated on the first level. The disseminated spores were transferred to the third level by the airflow created either by running the trains or by the ventilation system.

5.2.4.2 Test description

The execution of each of the three tests differed slightly but the preparation of the BIU remained the same. The BIU was set up as described in section 5.2.1 with the exception that the air sampler was re-connected to the system. Also, in response to the results in **fig. 18*B*** the dilution rate was re-set to a 5-fold in order to get even higher counts and therefore a more distinct difference between signal and background. **Table 8** portrays the nature of the disseminations regarding duration and whether the trains were running. The results from the tests are presented in **fig. 19** where the width of the grey bars illustrate the length of the specific dissemination.

Table 8. Tabulation of the dissemination profile.

Test	Dissemination ID	Duration of dissemination (mm:ss)	Trains
A	1	02:00	No
B	2	10:00	Yes
	3	10:00	Yes
C	4	00:56	No
	5	01:58	No
	6	01:20	No

During the first test, no trains were running and one dissemination was performed. The BIU was started approximately 45 min prior to the dissemination in order to get a sufficient number of background samples. Sample 1 was a manually added negative control used for comparison with the natural background in the metro. **Fig. 19*A*** shows that the BIU detected the agent in the samples collected during and after the dissemination. There was not enough

time to run any more samples in order to verify if the counts would go down to the same level as before the dissemination.

Two disseminations were performed during the second test (**Fig. 19*B***). No trains were running while collecting the first three samples but the remaining samples were collected while the trains were running. The BIU detected spores after both disseminations and the RCP counts decreased in between, however not down to the same level as the background. Also, the background level was significantly higher than when compared with the first test.

During the third test (**Fig. 19*C***) three disseminations with approximately 30 min in between were performed, all without any trains. The first dissemination resulted in a high peak followed by a rather rapid decrease. As a response to the second dissemination the RCP counts were once again increased followed by a not as rapid and deep decrease. The third dissemination, however, showed indecisive results starting with a small decrease followed by a high count.

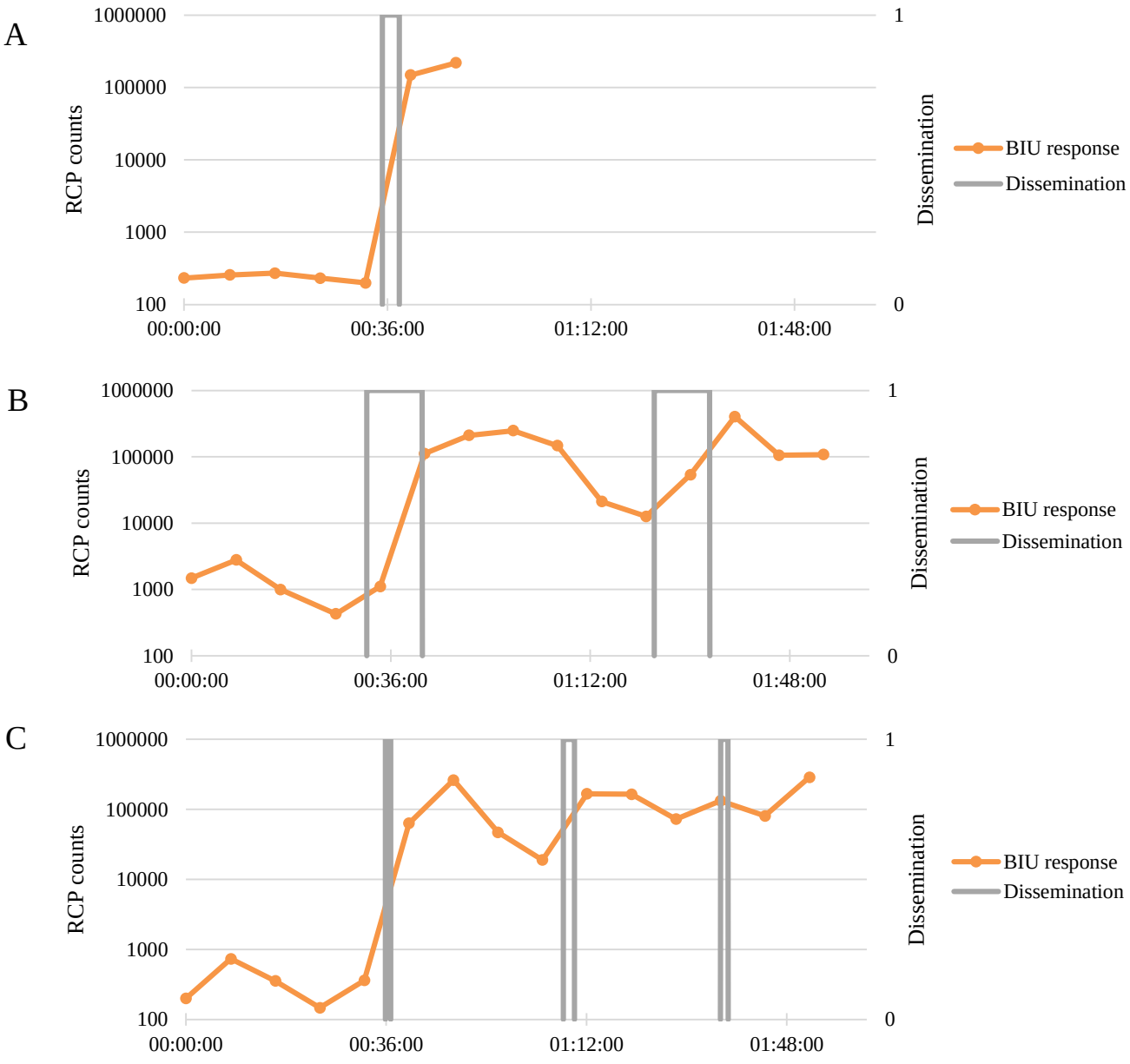

Figure 19. Validation of the BIU at a Prague metro station. Here, the results from three tests are presented. The BIU response is displayed in orange and the BG spore disseminations are displayed in grey, where the width of the bars represent the length of the dissemination. A) One dissemination was performed which generated a distinct BIU response. B) Two longer disseminations were performed. The peak after each dissemination indicate that the BIU was capable of detecting spores from both disseminations. C) During the last test, three short disseminations were performed. The BIU detected spores from the first two, but during the third the amount of spores present was probably to high making it difficult to differentiate between the disseminations.

6 Discussion

This report has described the design of an automated sample preparation unit. The overall aim of the project was to automate the preparation steps of Q-linea's proximity ligation assay protocol. The unit was supposed to be able to collect air samples and transfer these to the RSP9000. PLA probes should be added to a mix containing antibody coupled magnetic beads and the collected sample should be diluted and added to the same mix. The solution was then to be incubated for a minimum of five minutes. After incubation the supernatant was to be removed and three cycles of PLA wash should be performed. An enzyme free solution should be added to the washed sample after which it is transferred to the downstream unit – the SPM. All steps was to be performed without contaminating the reagents or the sample and also without cross contamination between samples. A sample was to be processed in 16 minutes starting a new processing cycle every eight minutes.

A requirement specification was constructed early on in order to visualize all requirements needed to be fulfilled. **Table 9** illustrates if the requirements have been fulfilled using the design reported in this thesis. If a requirement was fulfilled the table refers to the section where this has been verified.

Table 9. Tabulation illustrating whether the requirements were fulfilled or not.

ID	Fulfilled	Verified in section
1	Yes	4.2.3
2	Yes	4.2.6
3	Yes	4.2.6
4	Yes	5.2
5	Yes	5.2
6	Yes	5.2.2
7	Yes	5.2.1
8	Yes	4.2.5.3
9	Yes	4.2.5.3
10	Yes	4.2.5.3
11	Yes	4.2.5.3
12	Yes	4.2.6
13	Yes	5.1.3
14	Yes	4.2.5.4
15	Yes	4.2.5.4
16	Yes	4.2.6
17	Yes	4.2.6
18	Yes	4.2.5.2
19	Yes	4.2.3
20	Yes	4.2.3
21	Yes	4.2.3
22	Yes	4.2.3
23	Yes	4.2.3
24	Yes	4.2.3
25	Yes	4.2.3
26	Yes	4.2.3
27	Yes	4.2.3
28	Yes	4.2.5.2
29	Yes	5.1.1

The results presented in this report verifies that the main goal of the unit has been fulfilled, namely to function as an automated preparation module integrated with downstream units in a complex environment. **Fig. 16** verifies that the sample preparation module, in a lab environment, can prepare a sample for downstream processing with the same quality as a manual preparation. In **fig. 17** it is evident that there is no carry over between samples in the RSP. When running positive and negative samples in the entire BIU, again the results are good.

Up to this point the samples had been added manually in the collection tubes while the samples collected in the air sampler were directed to a waste container. Hence, the capability

of the air sampler to collect a sample that can be used for further processing had not been tested. This was instead verified during the Prague trial. The slow decrease after the dissemination in **fig. 19*B*** indicates carry over from the positive sample. As this never was a problem prior to the trial, it is most probably caused by the air sampler. The best solution to this problem would be to use two air samplers in combination. When the first has delivered a sample it can undergo a thorough washing cycle while the second is sampling. This way, the wash is not time dependent and no sampling time will be lost.

As displayed in **fig. 19*B*** and ***C*** the RCP count is not reduced to the background level after a dissemination and after the second dissemination in C the level is barely reduced. If the disseminations were further apart the level would probably go down to the background level. Interestingly, there was no difference in BIU response regardless of the length of the dissemination and whether the trains were running or not.

6.1 Conclusion

This thesis reports that it is possible for the described instrument to automatically prepare a sample, collected in a natural environment, for downstream processing and detection. By utilizing proximity ligation assay the entire system was able to detect disseminated BG spores in the Prague metro. The results indicate that the system functions satisfyingly in a complex environment and therefore can be used in biodefense applications.

6.2 Future outlook

6.2.1 Possible improvements

To further improve the functionality of the sample preparation module some improvements can be done.

- A second air sampler would probably reduce the carry over between samples.
- To better fit Q-linea's product line the sample preparation module should be integrated with the SPM by utilizing a similar platform.
- Change the pump to one with higher precision and lower volume limits. This would reduce the amount of reagents needed both in the samples and as a spare volume

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