MILESTONE REPORT TEMPLATE

PROJECT CODE:	2017-7049
MILESTONE NUMBER:	**10**
PREPARED BY:	Michael Breadmore
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1.0 MILESTONE DESCRIPTION

As per the milestone table (below) and the agreed variation (1/9/2020), this report will address milestones 10.

milestone	Achievement Criteria	Due Date
1	Delivery to and approval by AMPC of a project progress report, in the form of a milestone report	1/04/2018
2	Evaluate commercial swabs for uptake and release of bacteria from meat processing surfaces. Swabs will be ranked in terms of efficiency of uptake, and efficiency of release. *phD student research* Delivery to and approval by AMPC of a milestone report	1/10/2018
3	Construct prototype miniaturized electrophoresis instrument with fluorescence detector. Instrument will feature pumps, high voltage control, fluorescence detector, with a laptop/tablet for control and data acquisition. Delivery to and approval by AMPC of a milestone report and a project snapshot	1/10/2018
4	Delivery to and approval by AMPC of a project progress report, in the form of a milestone report	1/04/2019
5	Major Milestone Review	1/06/2019
6	Optimise extraction chemistry for cells to ensure maximum recovery of cells from swab in conditions which are suitable for rapid staining of cells *phD student research* Delivery to and approval by AMPC of a milestone report	1/10/2020
7	Modify current prototype extraction system to be compatible with selected swab and connect prototype electrophoresis instrument to the extraction system. Delivery to and approval by AMPC of a milestone report and a project snapshot report.	1/10/2020
8	Delivery to and approval by AMPC of a project progress report, in the form of a milestone report	1/04/2021
9	Major Milestone Review	1/06/2021
10	**Demonstrate detection of cells from surface using swab and instrument in a meat processing facility.** ***phD student research*** **Delivery to and approval by AMPC of a Final Report and a project snapshot report**	**1/12/2021**

2.0 ABSTRACT

In the first phase of the project, swabs and chemistry for the uptake and release of bacteria are to be developed. Four swabs were evaluated: CleanFoam®, FLOQSwabs™, Cotton swabs, and HydraFlock swabs. The uptake efficiency was 80%, 87%, 98%, and 90%, respectively. Eight different electrolyte systems (Tris-Borate Buffer, Phosphate Buffered Saline Phosphate Buffered Saline with Tween 80 (0.25%), Tris-Borate Buffer with Tween 80 (0.25%), MOPS Buffer(3-(N-morpholino)propanesulfonic acid), Tris-MOPS Buffer, Tris-HEPES (4-(hydroxyethyl)-1-piperazineethanesulfonic acid) Buffer, and Tris TAPS([tris(hydroxymethyl)methylamino] propanesulfonic acid)) were used to release captured cells from the swab. Considering the releasing efficiencies and the compatibility with the isotachophoretic staining, Tris TAPS was used for further experiments. The isotachophoresis staining method developed previously was modified and two nucleic acid stains(SYTO 9 and Propidium iodide) were used to detect total viable cell count(Phung, Nai et al. 2015). The limit of detection of the developed method is 131 viable cells/mL. The required microbiological safety limits for food contact surfaces (10 CFU/ cm^2) can be detected using the developed method both on steel and plastic surfaces as well as on meat, with excellent agreement with traditional plate counts. The electrophoresis methods requires approximately 30 min when implemented on a laboratory instrument.

3.0 PROJECT OBJECTIVES

The overall objective is to create an automated sample-to-answer system for the rapid quantitation of bacteria on meat and in meat processing facilities. This is divided into three milestones

- Develop a swab system and chemistry for the efficient uptake and release of bacteria from meat and processing surfaces that are compatible with isotachophoretic staining and concentration of cells
- Modify the existing prototype extraction system for new swab type and chemistry, construct a small electrophoresis instrument with a fluorescence detector, and to integrate the two
- Demonstrate quantitative detection of bacteria from surfaces using the new system

4.0 METHODOLOGY (OPTIONAL)

Determining Extraction (cells uptake) efficiency

A series of solutions with a range of cell concentrations were prepared using an overnight culture of GFP *E.coli*. A known volume (100 µL) of 10^{-3} dilution was spread evenly on a defined area (2 × 5 cm^2) of a clean microscopic glass slide. The slide was allowed to dry for one hour to facilitate the adherence of cells to the surface. Swabbing was done according to the following procedure.

The swab was held at a 30° angle between the handle of the swab and the surface. When swabbing in one direction, the swab tip was rotated slowly throughout the sampling area. The direction of the swabbing motion was changed by 90° and continued the swabbing. The remaining number of bacterial cells on the slide were counted using a fluorescent microscope. A control slide was used to determine the initial number of cells on the slide.

$$\text{Extraction efficiency} = \frac{\text{Extracted number of cells} \times 100\%}{\text{Inoculated number of cells}}$$

Determining the efficiency of cell release.

An overnight culture of GFP *E.coli* was directly inoculated (100 µL of the 10^{-3} dilution) onto the swab tip. The swab was placed into a releasing buffer (1.00 mL) for 5 s. The buffer solution was then filtered using a membrane filter (polycarbonate (PCTE) membrane filters (diameter-25 mm, pore size-0.2 µm) Images of 10 random microscopic fields of the filter were taken using a fluorescence microscope. As a control, 100 µL of the 10^{-3} dilution of GFP E.coli was directly filtered, and the cells on the filter paper counted. This is considered as the initial count.

$$\text{Release efficiency} = \frac{\text{number of cells released from the swab} \times 100\%}{\text{Inoculated number of cells}}$$

Eight different buffer solutions were selected: Phosphate buffered saline (PBS), Tris-Borate buffer (TB), Phosphate Buffered Saline with Tween 80 (0.25%), Tris-Borate Buffer with Tween 80 (0.25%), MOPS, Tris-MOPS, Tris HEPES, Tris TAPS. The impact of the releasing buffer on cell release from the swabs was evaluated as described above. Five replicates were carried for each experiment (n=5).

Simultaneously the releasing efficiency of swabs using TB and PBS buffers was evaluated using culturing techniques. An overnight *E.coli* culture was serially diluted, and 100 µL of three different cell concentrations (10^6, 10^5,10^4 cells/mL) was spiked onto swab tips separately. Then the cells were released into the releasing buffer (1.00 mL). 100 µL of this solution was plated onto LB agar plates. The plates were incubated at 37 °C for 24 hr. After 24 hr, colonies present on agar plates were counted, and the efficiency was calculated using the same equation mentioned above.

Isotachophoretic quantification of bacterial cells.

Electrolytes – For all ITP experiments, the leading electrolyte (LE) used was 50 mM Tris HCl (pH 8.0) with 0.05% HEC w/v. The terminating electrolyte (TE) was 10 mM Tris TAPS (pH 7.8). The spacer electrolyte (SE) used was 90 mM MES adjusted with Tris to pH 8.0. All solutions were prepared using 18 MΩ Milli- Q water.

Bacterial culture preparation – *E.coli* M23 strain was cultured in a solid LB agar plate at 37 °C. The broth culture was prepared when necessary (before the experiment) by inoculating a single colony into sterile LB broth and incubating overnight at 37 °C. The cells were harvested by transferring 10 mL of the overnight culture into a sterile 15 mL centrifuge tube and centrifuging at 3600 rpm for 5 min at room temperature. The supernatant was discarded carefully and the cell pellet was resuspended in sterile PBS. This cell suspension was stored at 4 °C until required. The cell concentration of the cell suspension was calculated using the plate count method.

Sample preparation – 100 µL of *E.coli* in PBS was centrifuged at 3600 rpm for 5 min at room temperature. The supernatant was discarded carefully, and the cell pellet was resuspended in 100 µL of 10 mM TE (Tris TAPS). Serial dilutions were prepared using this cell suspension using 5 mM TE with the final volume of each dilution of 1000 µL.

To determine cell counts (both dead and alive), the *E.coli* cells(100 µL) were added into a microtube with sterile TE(850 µL), and then the cells were stained by adding 50 µL of 20 mM SYTO 9. The tube was covered with aluminum foil. 200 µL of the cell sample is transferred into a sterile PCR vial for analysis. To quantify viable cells, the sample was prepared by adding SYTO 9 (50 µL, 20 mM), propidium iodide (60 µL, 100 µM), cell culture (100 µL) into sterile TE(790 µL).

Capillary Electrophoresis – All the experiments were performed using a Beckman Coulter P/ACE MPQ Capillary Electrophoresis System equipped with a 488-nm Argon laser module. Experiments were conducted at 25 ^{o}C using an unmodified fused silica capillary of 50 µm i.d. (Polymicro Technologies, AZ, USA) with a total length of 40 cm (effective length to the detector,30 cm). A different sample vial and cap with a spring that holds the PCR vials was used for sample injection allowing the capillary and electrodes to each the end of the tube with equal distribution of cells to be injected into the capillary data was collected and analyzed using the 32 Karat software version 8.

Capillary conditioning and isotachophoresis – Prior to analysis, a new capillary was preconditioned at 275,790 Pa (40 psi) in the following procedure: 1 M NaOH (30 min), milli-Q water (20 min), 1 M HCl (20 min), milli-Q water (10 min) followed by 1% w/v PVP at 45 psi for 45 min. Finally, the capillary was conditioned with 0.05% HEC 50 mM Tris HCl with 0.05% W/V HEC (pH 8.0) at -16 kV for 10 min.

Each analysis began by flushing with 1.6 % w/v HEC for 8 min at 75 psi, LE solution for 9 min at 10 psi, EKI injection of spacer electrolyte solution at -16 kV with counter pressure of 18 psi for 0.5 min, EKI injection of sample bacterial cells in TE solution at -16 kV with counter pressure of 18 psi for 2 min, followed by EKI injection of TE solution at -16 kV.

Using the developed method to quantify microbial load on red meat carcasses.

Bacterial culture preparation.

A small colony of *E.coli* M23 from an agar plate was inoculated in LB broth (40 mL) and incubated for 8 – 12 h. 10mL of the overnight culture was transferred into another sterile flask and centrifuged (3600 rpm, room temperature). The supernatant was discarded. The cell pellet was resuspended in PBS(10 mL). The initial cell concentration was determined by the plate count method.

Inoculating the bacteria onto the meat surface and swabbing.

A dilution series with different cell concentrations were prepared using the *E.coli* M23 broth culture. A grid was used to divide 10 × 10 cm^2 area into 1cm^2 small squares. 2 µL of cell culture was inoculated onto 50 squares according to the following pattern. 2 µL of cell culture from appropriate dilution was inoculated per square. 50 squares of the grid were inoculated covering a total area of 100 cm^2.

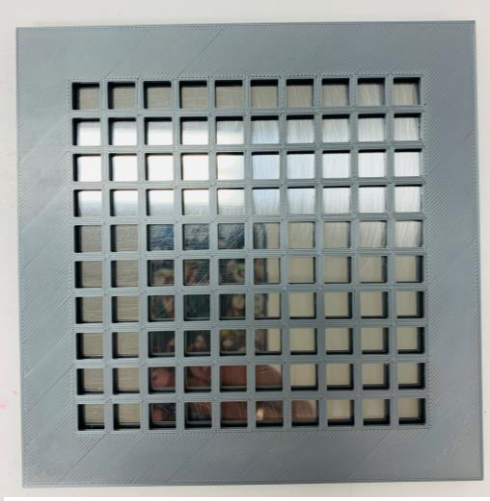

Figure 1: The grid used to divide the area into equal 1cm2 squares

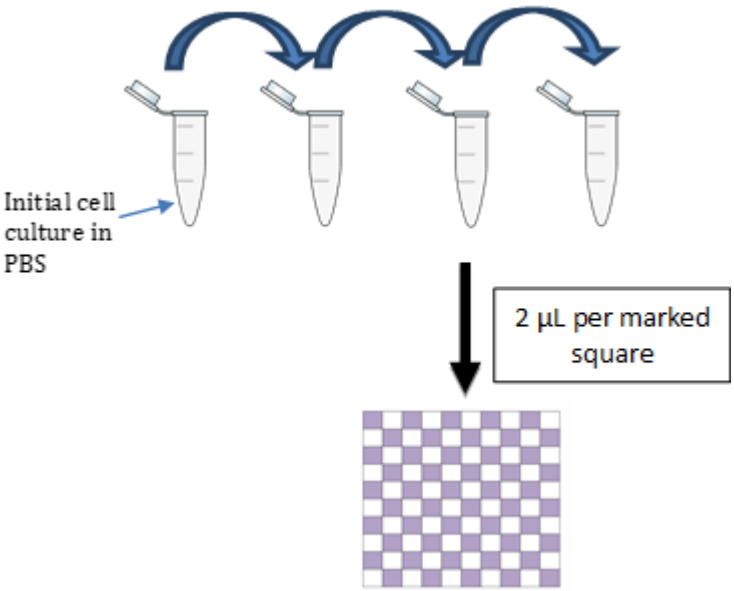

Figure 2: colored squares were inoculated with bacteria (E.coli M23)

A sterile Puritan Hydra Flock swab was used to swab an area of 100 cm^2 and the extracted cells were released into a 1.5 mL Eppendorf tube with extraction buffer(TRIS TAPS) (10 mM, 890 µL). The cells were then stained using SYTO9 (20 mM, 50 µL) and Propidium iodide (100 mM, 60 µL). The tube was gently vortexed to mix everything, and 200 µL from the extract was transferred into a small 200µL CE vial.

This same method was used to quantify bacteria from other food contact surfaces as well.

Capillary conditioning and isotachophoresis

Prior to analysis, a new capillary was preconditioned at 275,790 Pa (40 psi) in the following procedure: 1 M NaOH (30 min), milli-Q water (20 min), 1 M HCl (20 min), milli-Q water (10 min) followed by 1% w/v PVP at 45 psi for 45 min. Finally, the capillary was conditioned with 0.05% HEC 50 mM Tris HCl with 0.05% W/V HEC (pH 8.0) at -16 kV for 10 min.

Each analysis began by flushing with 1.6 % w/v HEC for 8 min at 75 psi, LE solution for 9 min at 10 psi, EKI injection of spacer electrolyte solution at -16 kV with counter pressure of 18 psi for 0.5 min, EKI injection of sample bacterial cells in TE solution at -16 kV with counter pressure of 18 psi for 2 min, followed by EKI injection of TE solution at -16 kV.

Home-made fluorescence detector for a customized instrument

Two different detector designs were evaluated for fluorescence detection. The first consisted of the interface from a Beckman MDQ instrument. The second was a sheath-flow interface based on the design of Dada et al (Dada, Huge et al. 2012). Both were evaluated for the separation of Fluorescein using a 488 nm argon-ion laser, an in-house controlled PMT, and an Agilent Capillary Electrophoresis unit.

5.0 STAGE SUMMARY (REVIEW OF PREVIOUS STAGE WHERE APPLICABLE)

Swab Evaluation (Milestone 2)

Four swabs were evaluated: CleanFoam®, FLOQSwabs™, Cotton swabs, and HydraFlock with more details found in Appendix 1. The uptake efficiency was 80%, 87%, 98%, and 90% respectively.

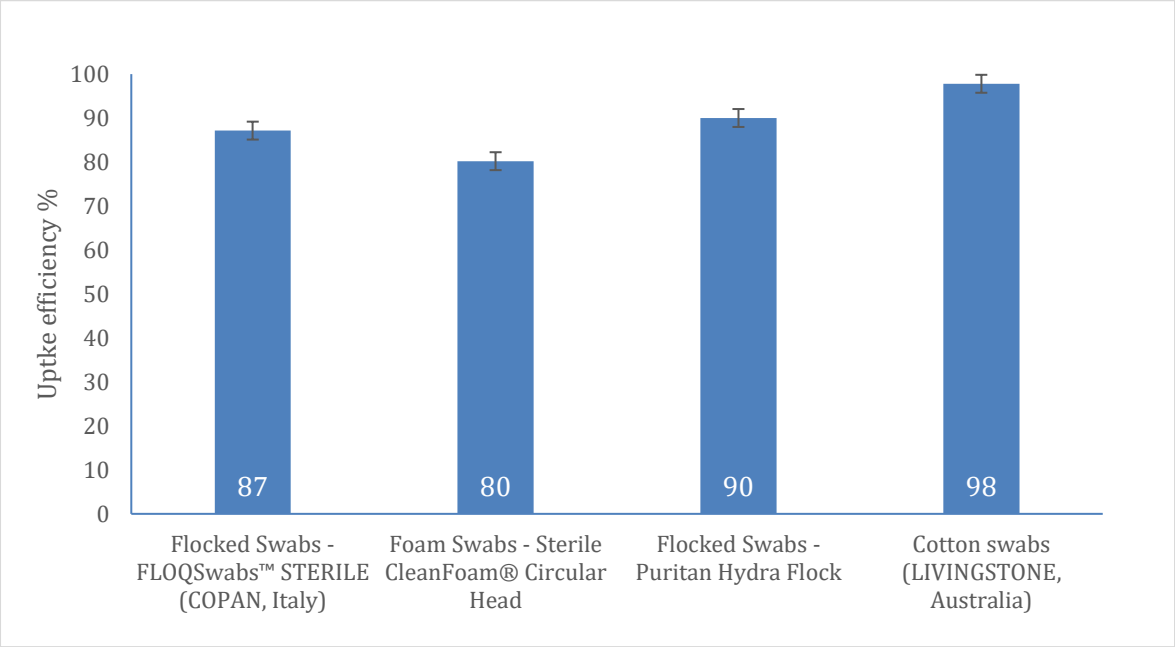

Figure 3: Uptake efficiency of different swab types. The experiment was repeated three times.

The efficiency of the release of the captured cells from the swab tip was evaluated by using two different methods (Epifluorescence microscopy and Microbiological culturing methods) (Table 1 and Figure 3).

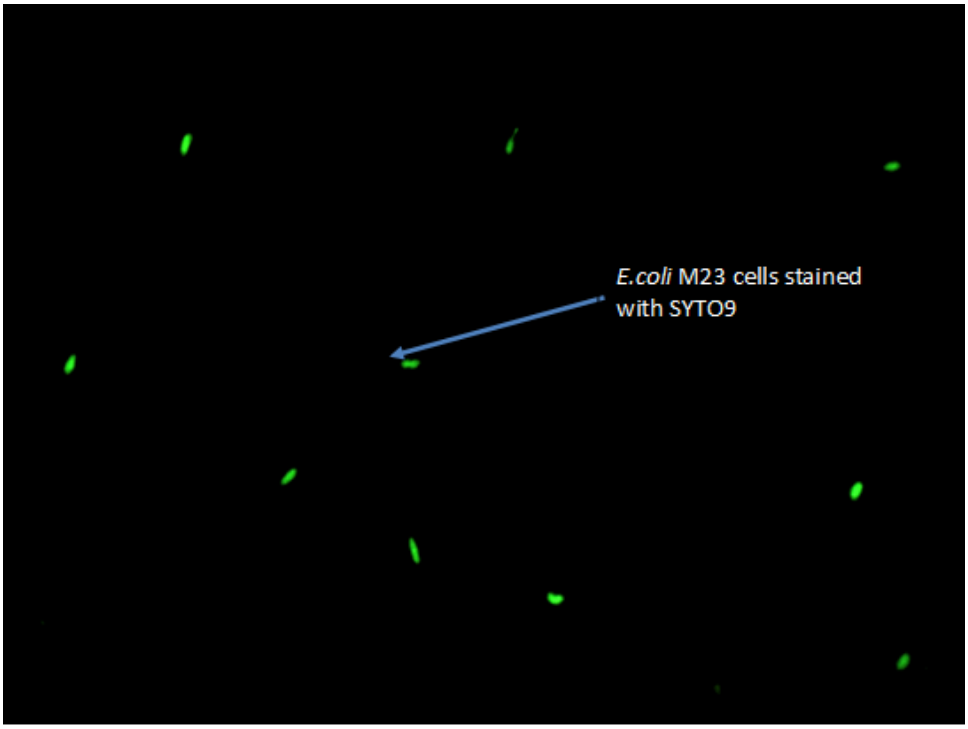

Figure 4: E.coli M23 cells and meat cells stained with SYTO9 (meat cells are not stained with SYTO9)

Table 1: Release efficiency (%) of different solutions using the four different swabs. Data was obtained using filters and fluorescent microscope imaging.

Buffer	Flocked Swabs - FLOQSwabs™ STERILE (COPAN, Italy)	Foam Swabs - Sterile CleanFoam® Circular Head	Flocked Swabs - Puritan Hydra Flock	Cotton swabs (LIVINGSTONE, Australia)	ETD-100 swab (GreyScan, Australia)
PBS	30.7 ± 15.4	28.5 ± 11.0	61.0 ± 17.1	19.1 ± 8.49	
TB	71.2 ± 14.1	42.5 ± 12.1	66.8 ± 9.53	46.0 ± 25.1	
PBS with Tween 80 (0.25%)	64.7 ± 15.5	43.2 ± 4.50	77.2 ± 12.3	64.7 ± 6.63	
TB with Tween 80 (0.25%)	79.9 ± 34.3	73.4 ± 34.7	69.3 ± 6.01	17.5 ± 17.8	
MOPS	81.8 ± 33.7	78.4 ± 29.2	77.1 ± 29.2	37.3 ± 10.6	
Tris MOPS	79.6 ± 4.17	91.7 ± 9.02	94.7 ± 3.07	32.8 ± 4.01	
Tris HEPES	75.6 ± 17.2	88.0 ± 11.8	80.7 ± 19.8	40.0 ± 2.26	
Tris TAPS	80.4 ± 12.7	61.9 ± 15.0	87.8 ± 18.3	46.2 ± 14.0	73.5 ± 29.4

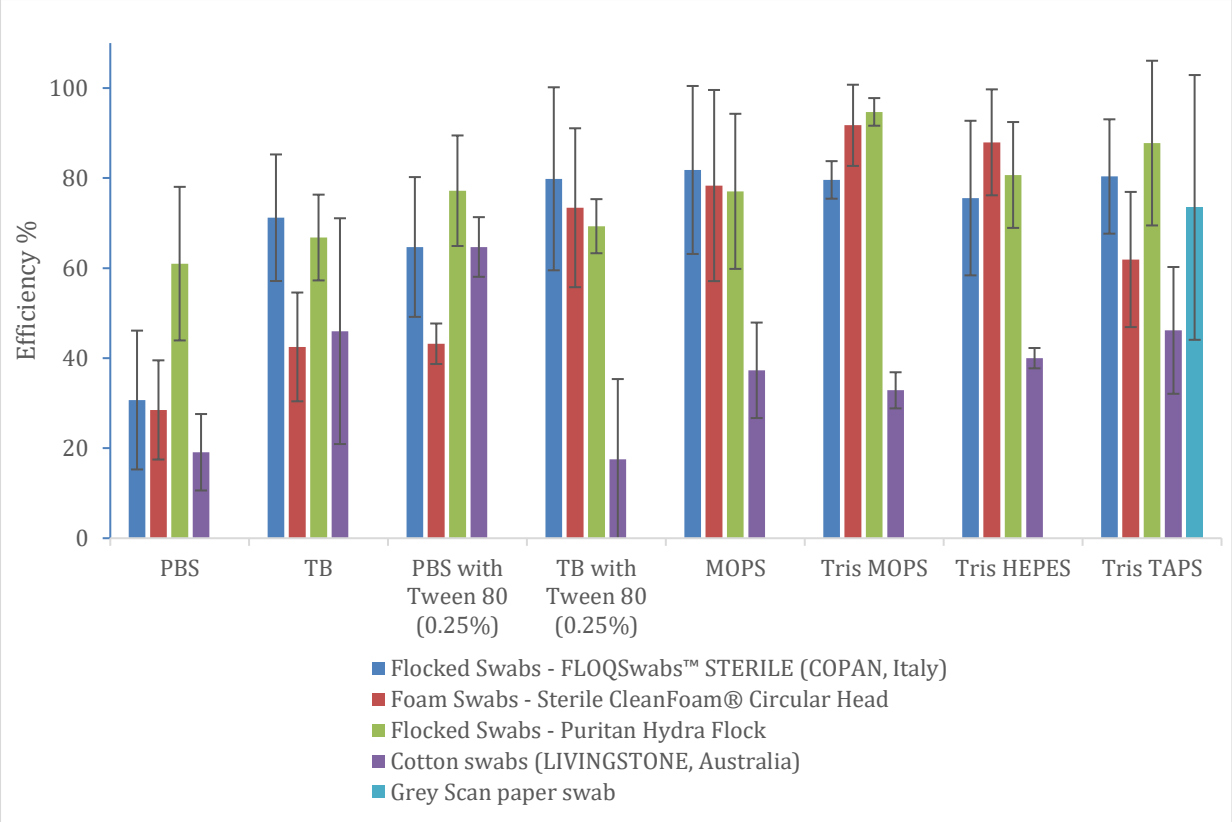

Figure 5: Release efficiency of four swab types with different releasing buffers. Microscopic method was used to determine the efficiencies (n=5).

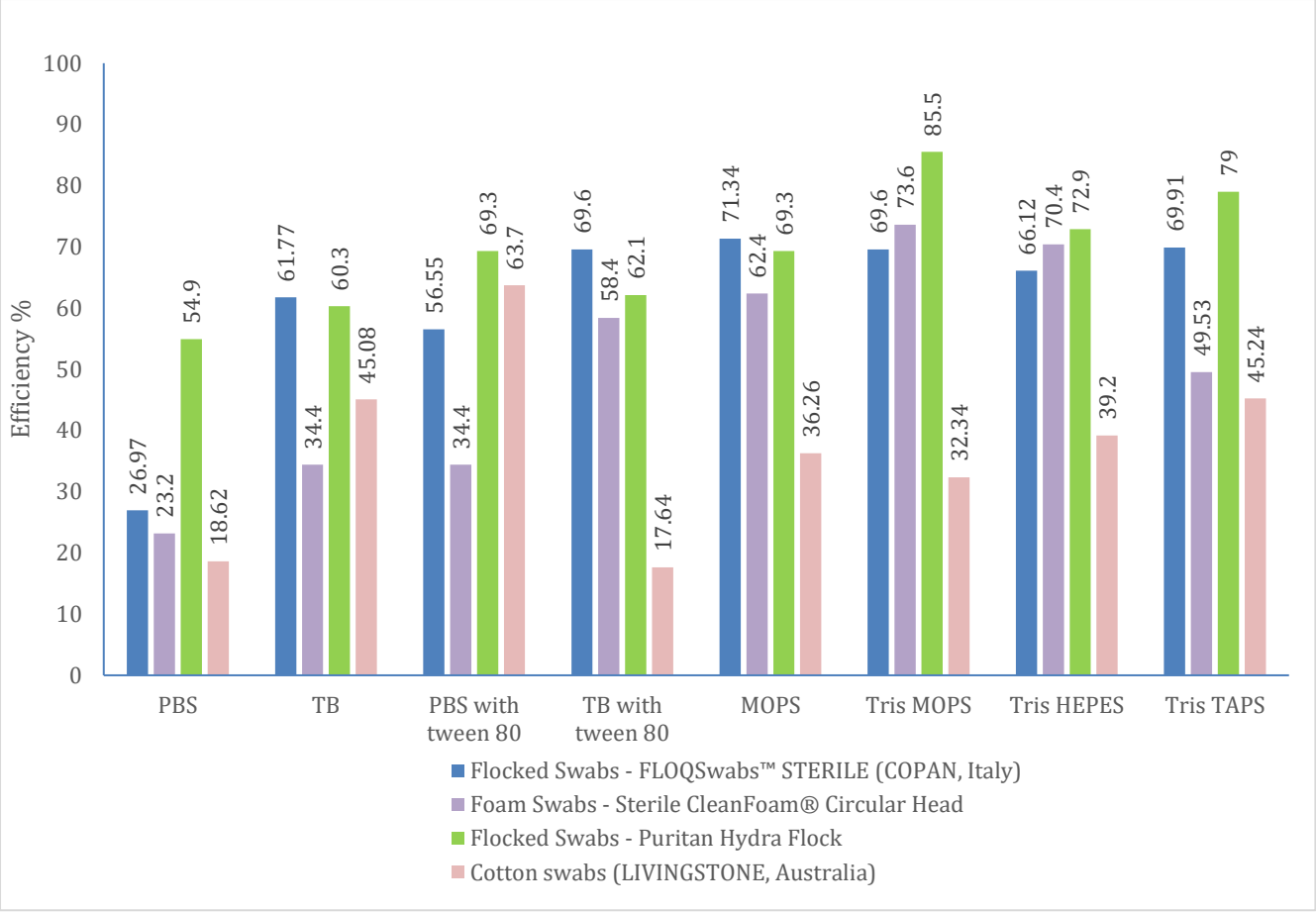

Figure 6: overall cell recovery efficiency

Flock swabs have maximum release efficiencies with Tris MOPS, Tris HEPES, Tris TAPS (figure 4). By combining data from Figures 4 and 5, the overall recovery of cells from surfaces was calculated (figure 6). The highest overall recovery efficiency was with flocked puritan swabs with Tris TAPS, Tris MOPS and Tris HEPES.

Prototype instrument with laser-induced fluorescence detector (milestone 3)

A prototype capillary electrophoresis instrument was constructed and is shown in Figure 7. The instrument comprises a series of miniaturized pumps and valves with custom circuit boards for operation and is powered through the USB port of a laptop computer. The instrument has been developed for out-of-field operation and is capable of operating for several weeks without refilling reagents. Its performance was tested by monitoring inorganic anions in water at a local freshwater dam.

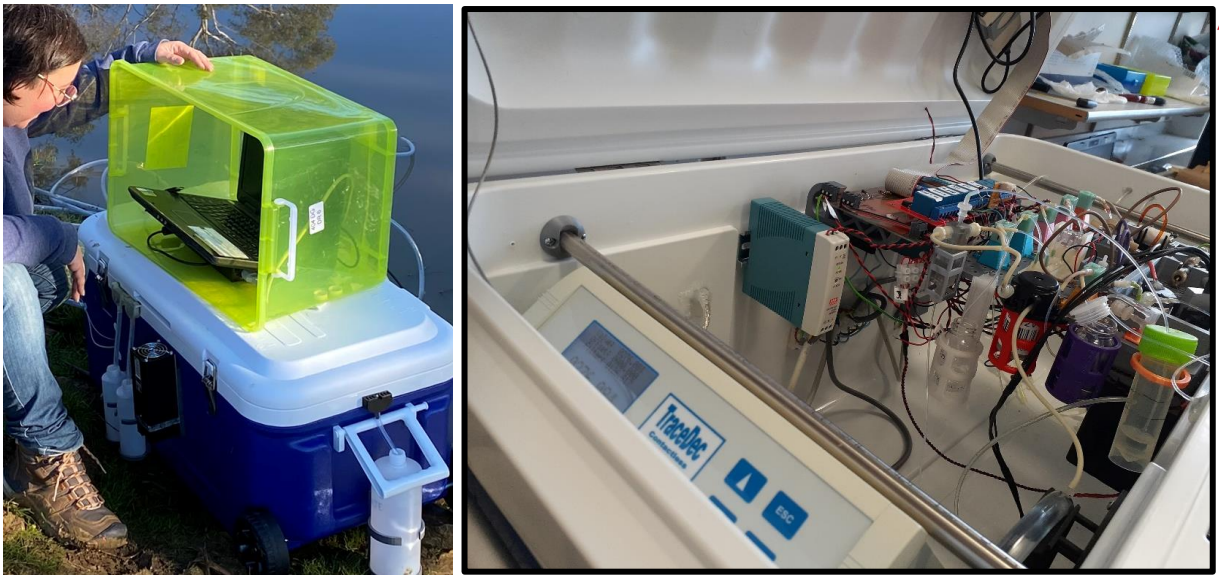

Figure 7: prototype capillary electrophoresis instrument in a self-contained portable enclosure suitable for out-of-laboratory operation.

A key part of the instrument is a laser-induced fluorescence detector which will be used to detect the cells after staining with a fluorescent dye. Two prototype detectors were constructed based on literature reports. The first (Figure 8) is an on-capillary detector. This was constructed using the interface from salvaged optical interface from a commercial instrument and relies on optical fibres to introduce the excitation light and collect the emission light. Optics and filters are used to reduce background noise and enhance the signal. The second (Figure 9) is a sheath-flow interface in which a flow of liquid is used to focus the effluent from the capillary allowing excitation and emission to occur at the end of the capillary avoiding the lensing effect and noise that arises when detecting through the capillary in the first prototype. The performance of these two detectors was compared with the outcome for a separation of fluorescein shown in Figure 10. The on-capillary detector provided a limit of detection of 1 x 10^{-8} mol/L while the sheath flow detector provided 1 x 10^{-10} mol/L. However, when benchmarked using the commerical Beckman MDQ instrument used for the chemistry development, the limit of detection was 5 x 10^{-12} mol/L, which is 20 times lower than the sheath-flow detecor. This difference in performance is due to either the different light source used and/or the different PMT used for detecton of the emitted light. Due to limited funding and the time spent constructing the portable unit, it was not possibe to puruse improvements to the detector for the portable instrument.

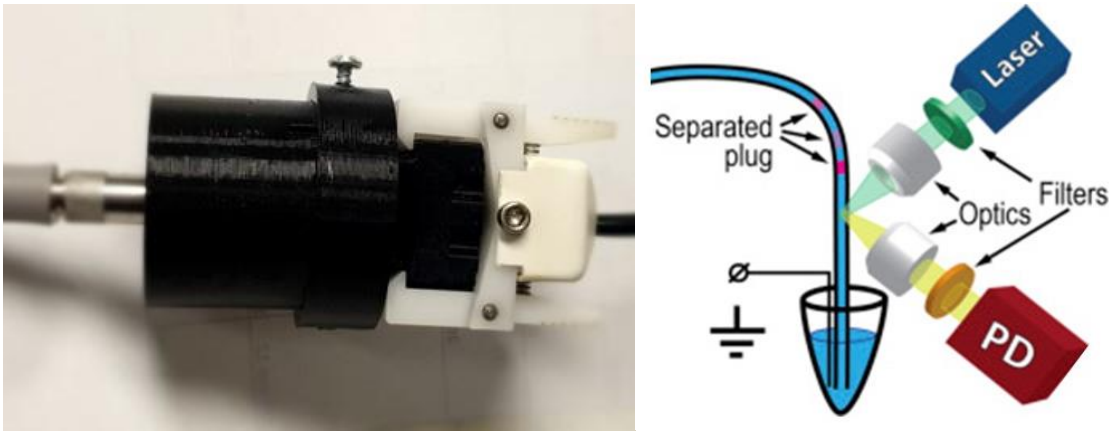

Figure 8: Prototype design of on-capillary fluorescence detector for detection of the labelled bacteria. Left is the prototype. Right is a schematic showing optical the optical configuration of the detector.

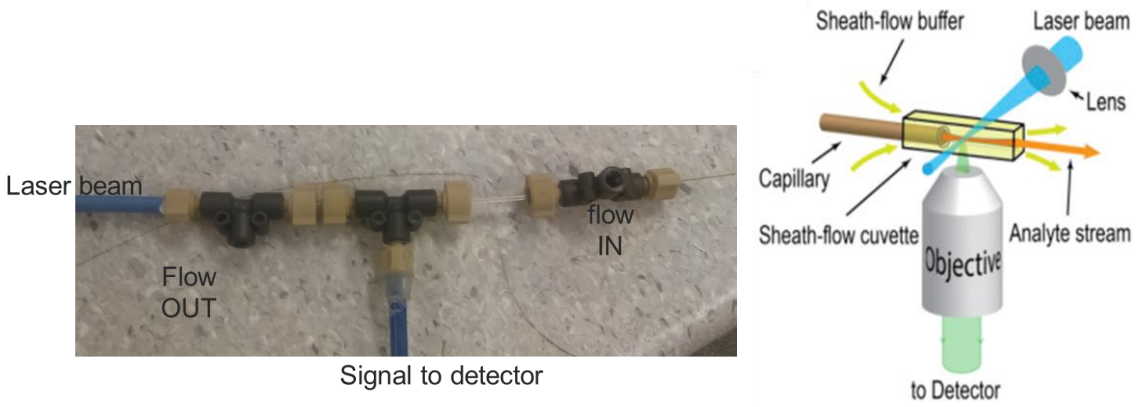

Figure 9: prototype design of the sheath-flow interface for fluorescence detection at the end of the capillary, removing optical aberations from the surface of the capillary. Left is a photo of the prototype. Right is the schematic of operation.

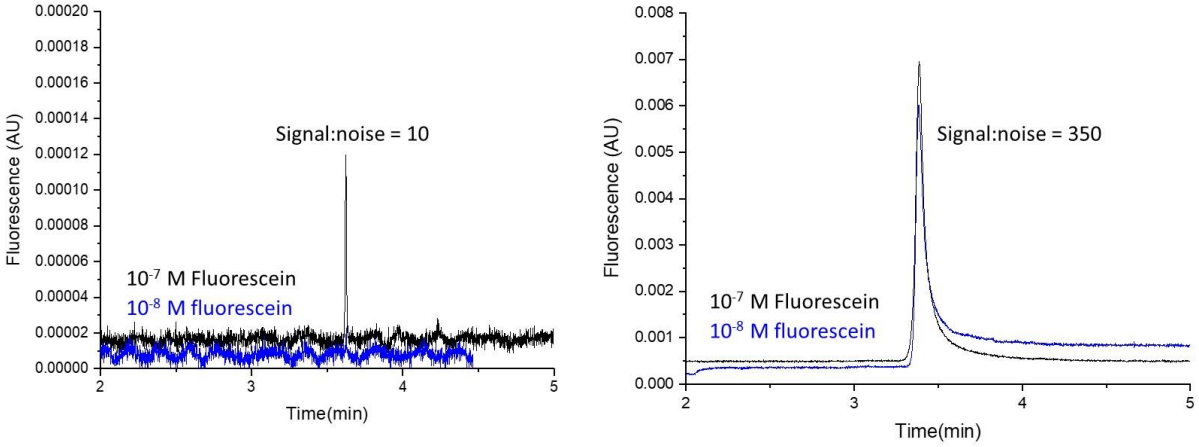

Figure 10: Electropherogram of Fluorescein (1 x 10^{-7} and 10^{-8} mol/L) using the on-capilalry detector (left) and the sheath-flow detector (right).

Optimisation of the Extraction chemistry for cell staining and separation (Milestone 6)

Preliminary isotachophoretic cell staining trials showed that Tris TAPS buffer is more compatible with ITP chemistry. While Tris MOPS buffer had the maximum overall cell recoveries for flock swabs, the overall cell recovery values for flock swabs with the Tris TAPS buffer are similar to the Tris MOPS values. Therefore, Tris TAPS buffer was selected as the releasing buffer/terminating electrolyte for further studies. Figure 11 shows separations of stained cells at different concentrations, with Figure 12 and Figure 13 showing the calibration plots. From these data, the detection limit is 38 cells/mL.

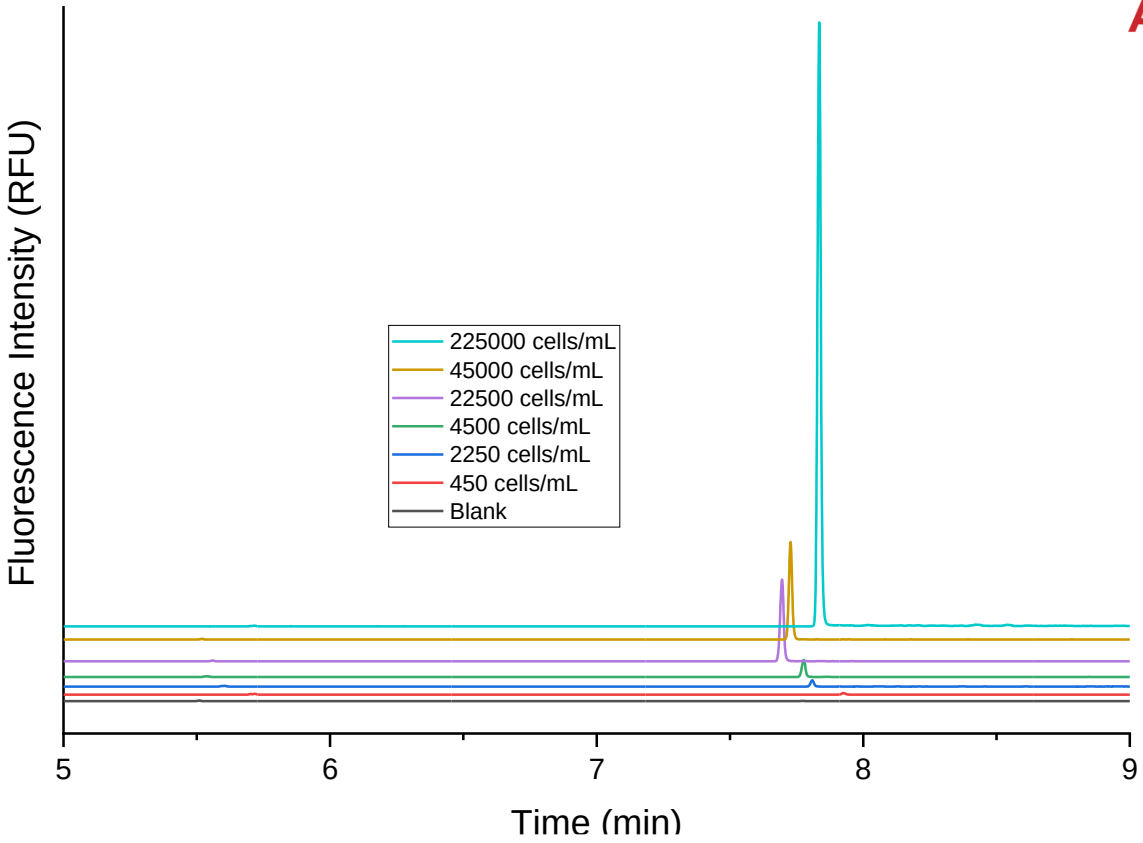

Figure 11: Isotachopherogram of different concentrations of E.coli M23

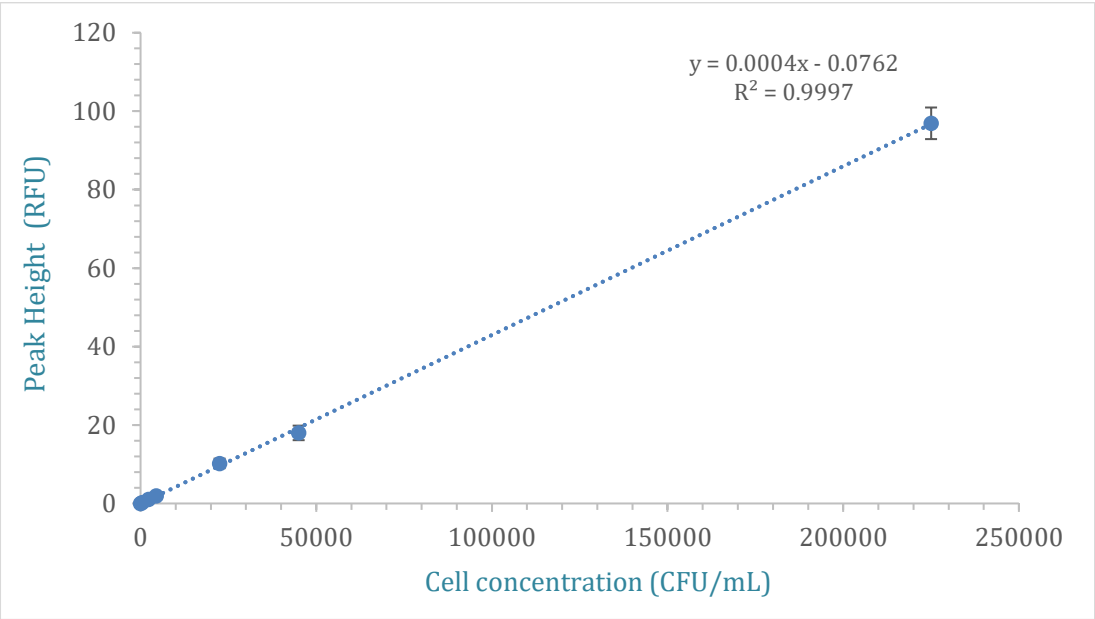

Figure 12: Peak Height calibration curve of ITP of E.coli cells(both dead and live cells) at concentrations of 450 – 225000 cells/mL. Each point is from 3 replicates. Experimental conditions Before analysis – flushed the capillary with 1% PVP in LE (Tris HCl 50 mM) at 45 psi for 1 min. And then flushed it with HEC (75 psi for 8 min) followed by flushing with LE (Tris HCl 50 mM) at 10 psi for 9 min). At each analysis, EKI injected the spacer (Tris MES 90 mM) at -16 kV for 0.5 min with counter pressure (1.3 psi) and then injected the cells in TE (Tris TAPS (10 mM) at -16 kV) at -16 kV

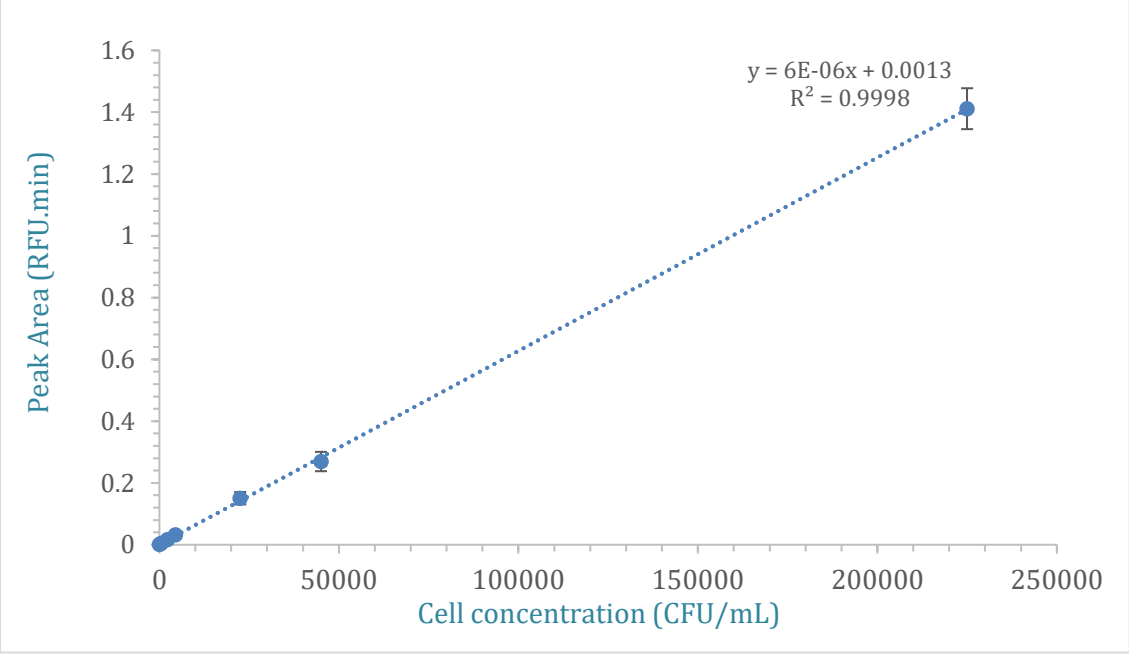

Figure 13: Peak Area calibration curve of ITP of E.coli cells(both dead and live cells) at concentrations of 450 – 225000 cells/mL. Each point is from 3 replicates. Experimental conditions Before analysis – flushed the capillary with 1% PVP in LE (Tris HCl 50 mM) at 45 psi for 1 min. And then flushed it with HEC (75 psi for 8 min) followed by flushing with LE (Tris HCl 50 mM) at 10 psi for 9 min). At each analysis, EKI injected the spacer (Tris MES 90 mM) at -16 kV for 0.5 min with counter pressure (1.3 psi) and then injected the cells in TE (Tris TAPS (10 mM) at -16 kV) at -16 kV for 2 min with counter pressure (1.3 psi) and changed the vial to TE and applied -16 kV at reversed polarity

Extraction system modification (Milestone 7)

At the beginning of the project, the anticipated pathway was to modify the existing extraction system designed and constructed during the development of the GreyScan trace explosives detection project that finished in 2014. This system featured an integrated sample extraction system for rapid solubilization of material collected on a swab, and mobilization of this to a portable Capillary Electrophoresis instrument. In 2019, we began using this extraction interface, but shortly after use, the computer with the software to control the system became dysfunctional. Discussion with the team from Grey Innovation who developed the extraction system indicated that the software was locked specifically onto that computer and was not able to be recovered and transferred to another computer. Furthermore, during the time since that software was developed, the company had changed to new software that was not compatible with the extraction system we already had, and they did not have capacity to help us make the system functional.

This has left us with two options, both of which were pursued concurrently. One of which is to construct our own new extraction interface based on the design of the previous system, using our in-house 3D printing capability (which we have used to make our portable system and our optical detection housing). Figure 14 shows a photograph of one of the prototype systems constructed. Unfortunately, while this was promising, we were not able to obtain a sufficient liquid seal in the extraction system around the swab, such that fluid leaks occurred during the extraction process. Several attempts to improve this failed, and re-design and re-consideration of material choice for the extraction system is required to successfully achieve this. Given resource limitations available at this point, it was not possible to pursue this further.

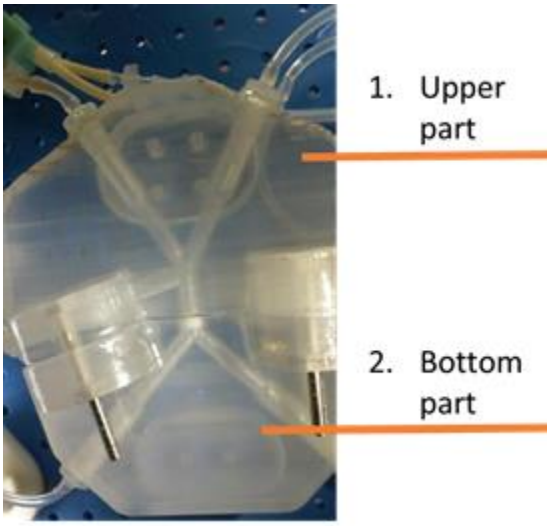

Figure 14: prototype home-built 3D printed extraction system to couple with the home-built CE instrument.

The second option is to work with GreyScan to adapt the current ETD-100 commercial system for trace explosive detection (Figure 15) to include a fluorescence detector. In May 2020, we began working with GreyScan on improvements to the ETD-100, including the development of first an absorbance detector to expand the potential applications and market for the base technology product. Swab experiments were subsequently performed with the swab used for the ETD-100, with the results shown for the ideal electrolyte Table 1 and Figure 2, showing a good (74%) recovery of the bacteria from the existing swab using the developed elution electrolyte. We anticipate further improvements in recovery using the existing swab with further optimization of the electrolyte should a modified ETD-100 become available to support the project. At the time of completion of this project, however, work with Grey Scan has not started on a fluorescence detector, and discussions are still underway with them and potential partners to pursue suitable market opportunities to realise this capability.

Figure 15: commercially available GreyScan ETD-100 trace explosive detection system developed on the research from the UTAS laboratory which is currently being modified to include different detection options.

Demonstrate detection of cells on surfaces (Milestone 10)

Using the developed method to quantify microbial load on food contact surfaces

Of all the tested swabs, only Copan Flock and Puritan Flock swabs were used for this experiment as these two swabs showed the highest cell recovery efficiency. A steel surface was used (Globe pharma Inc, New Brunswick, NJ, USA)(specifications; 12.7 × 12.7 cm^2, #7 finish) to test cell recovery from surfaces. Other surfaces, such as plastic, will be evaluated for cell extraction efficiency in the future.

There was a complication with the puritan Flock swab as the fibers came off the swab during the release step and interfered with the analysis. It is suspected that the swabbing procedure might have loosened the fibers from the shaft. The current was not steady and fluctuated during the separation most likely as a result of injecting small fibers with cells into the capillary tube. Cells are negatively charged (at pH 8) and during the electrokinetic injection, the negatively charged particles are injected into the capillary tube. If the cells are tightly attached to the fibers, this can result in injecting them together thus changing the capillary electrophoretic chemistry/system. Therefore, the Copan flock swab was selected as it has the second-best recovery efficiency and does not lose fibers as easily.

Steel surfaces are durable, easy to clean, and easy to sterilize which makes them a suitable and common option for food preparation surfaces. Meat processing facilities often contain many steel surfaces including steel blades, steel equipment, steel vessels, and steel conveyor belts. Therefore, for this surface efficiency study, we used a 10cm × 10cm steel surface. A grid was used to separate the area into 100 1cm × 1cm small squares. This helped to spread the bacteria evenly across the surface area. The required microbiological safety limit for food contacting surfaces is 10 CFU per $1cm^2$, above this limit confirms that there is a hygiene problem in the facility which needs immediate attention. Our preliminary study shows that we can detect these levels within a short time (30 min compared to a minimum of 24h for culture-based methods). and the whole procedure, which involves two fluorescent stains SYTO9 and Propidium Iodide, is simple to perform. Figure 16 shows the electropherograms of the E.Coli placed on stainless steel surfaces with the calibration plots shown in Figure 17 and Figure 18. The calibration is curved, unlike the previous calibrations which were linear. This is due to the new calibration including both the uptake onto the swab and release into the solution, as well as the measurement in contrast to the previous data. The linear range of the calibration curve for *E. coli* in solution is 0 - 10^4 CFU/ mL which covers the range of approximately 140 - 7000 CFU/mL when swabbing the surface. The different response indicates a limitation of the swabbing process. While this will cause errors at high cell concentrations, it will allow accurate estimations at low cell numbers, as illustrated in the low-level calibration upto 80 CFU/cm^2 shown in Figure 19.

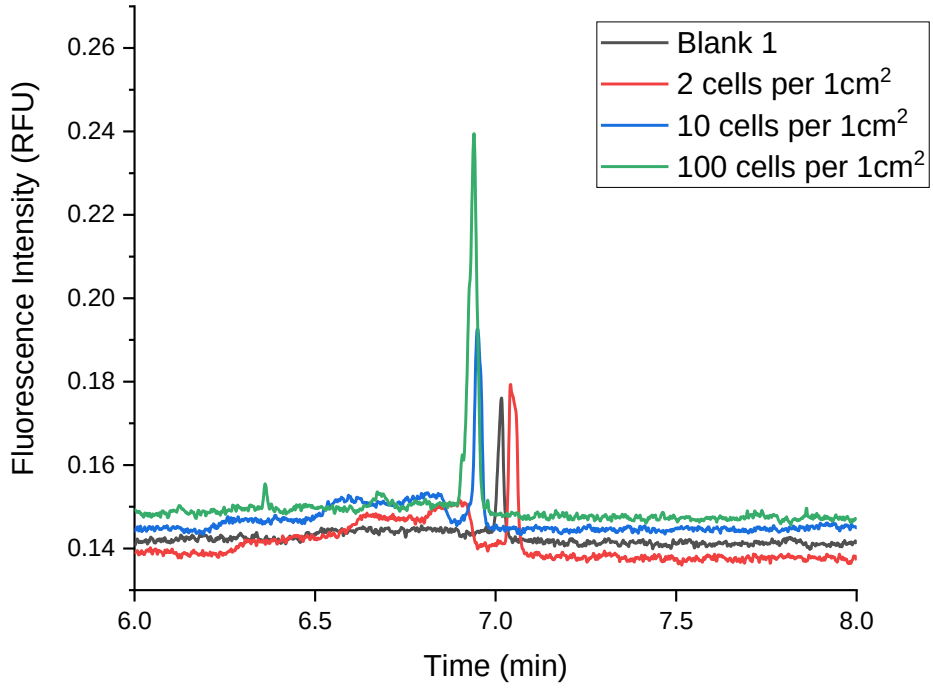

Figure 16: Figure 16 : isotachopherograms of the bacterial cell (E.coli M23) swab-extraction sample from steel surface (2 cells/cm² - 100 cells/cm²).

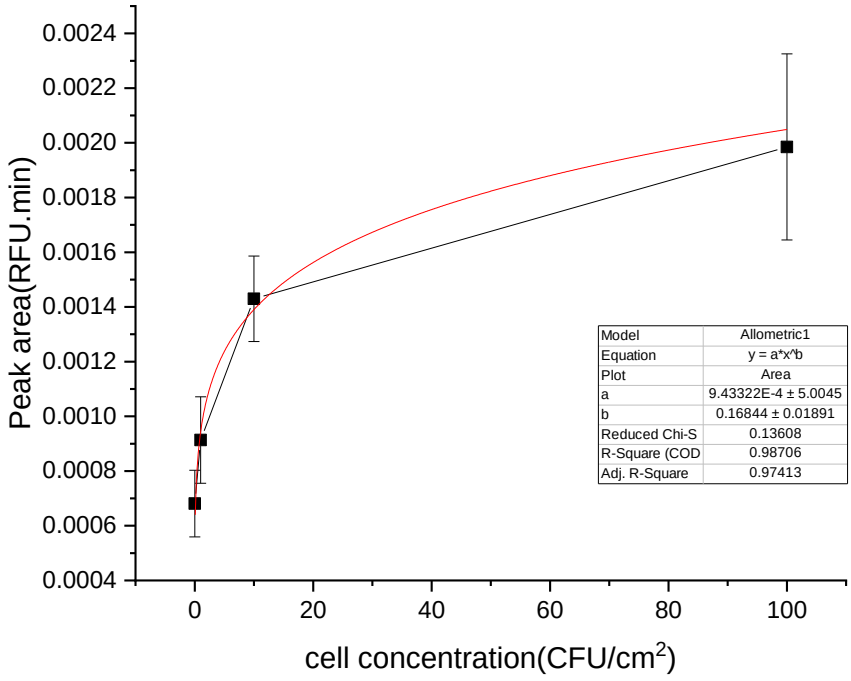

Figure 17: Peak area calibration curve for different cell concentrations recovered from the steel surface using Copan flock swab (each point is from 3 replicates)

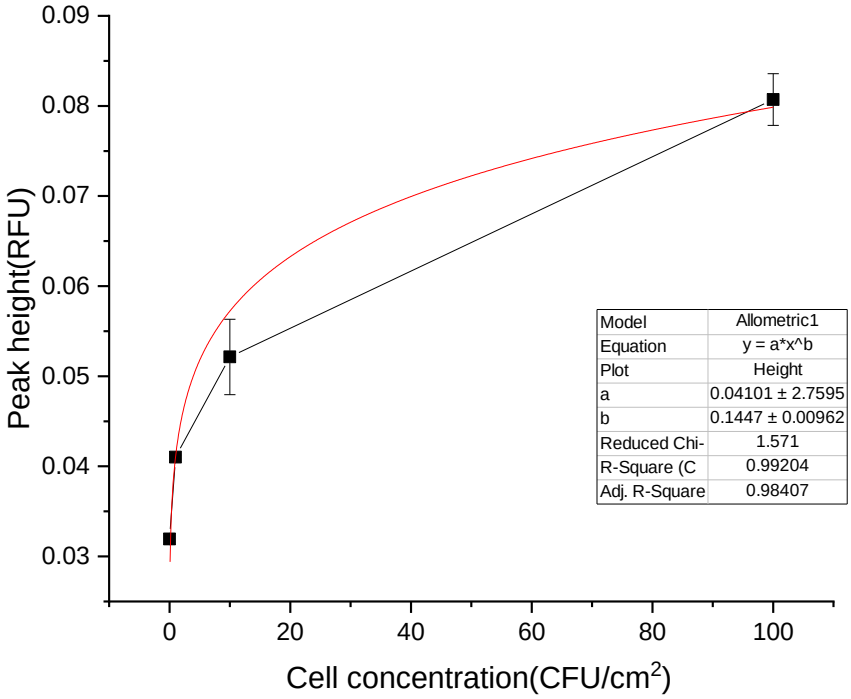

Figure 18: Peak height calibration curve for different cell concentrations recovered from the steel surface using Copan flock swab (each point is from 3 replicates)

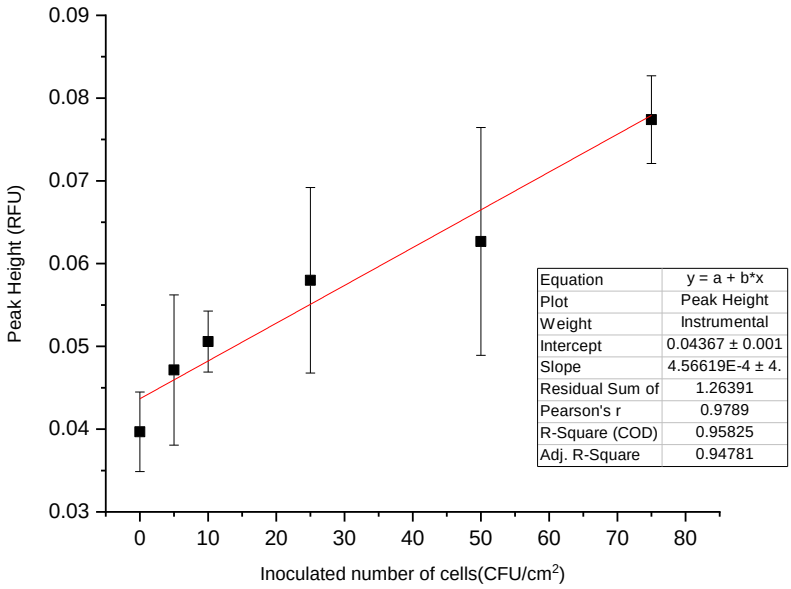

Figure 19: Linear range of the calibration curve(peak height) for different cell concentrations recovered from the steel surface using Copan flock swab (each point is from 3 replicates)

Another experiment was performed to examine the cell extraction efficiency from a plastic surface. Cells were deposited as described for the stainless steel surface. The calibration curve was similar to the calibration curve of the steel surface (Figure 20). The constructed calibration curve with different cell concentrations (6 CFU/cm^2 – 578 CFU/cm^2) shows that the required hygiene levels can be detected on plastic surfaces as well.

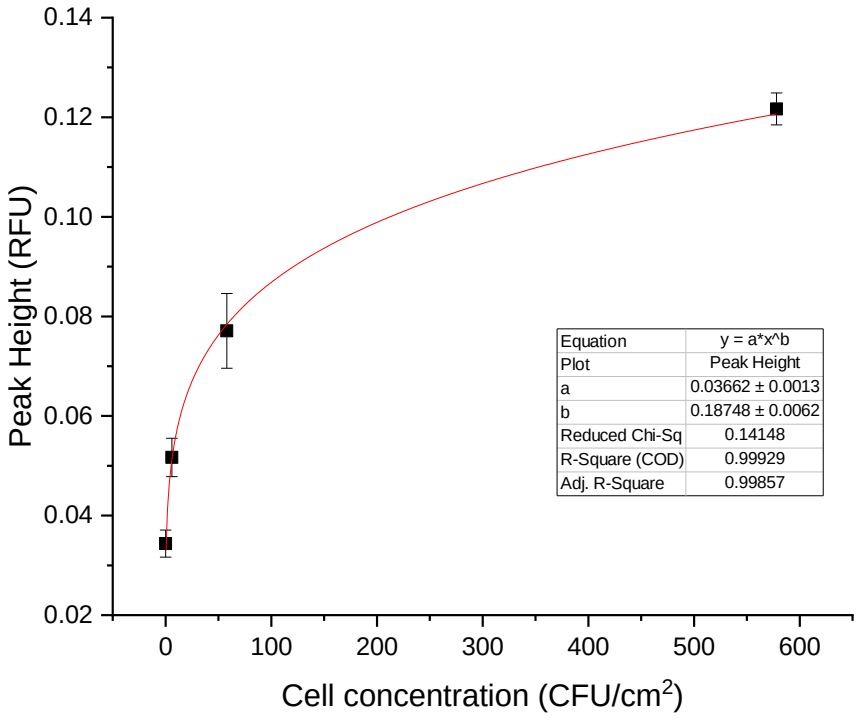

Figure 20: Peak height calibration curve for different cell concentrations recovered from the plastic surface using Copan flock swab (each point is from 3 replicates)

Validation of the CE method was performed by comparison with plate counts. Figure 21 shows the linear range of the calibration curve ranging from 5 CFU/cm^2 to 75 CFU/cm^2 of E.coli cells inoculated on the steel plate. The Copan swabs were used in the entire experiment. The selection was made as it had the minimum adverse effect on the ITP process and had the second-best cell recovery. As can be seen from the figure, there is a similar trend and similar error in both measurements.

According to the European and Australian standards, surfaces which exceed the 10 CFU/cm^2 limit are not acceptable for food processing purposes (NSW Food Authority 2013). The standard deviation of the blank does not overlap with the standard deviation of the 10 CFU/cm^2 data point, which means the method can detect the required, acceptable standards. The standard deviations of the data points are high as we are working with the biological samples. Different reasons may have affected this, such as the changes in swabbing, variations in deposited cell numbers, and the differences in swabs. Since all levels over 10 CFU/cm^2 are not acceptable, the accuracy of each data point above that level is not that significant, which means if the device detects any signal above the blank, that sample is not acceptable. Therefore this method can be considered as a semi-quantitative method. But when compared to culturing techniques(which take a minimum of 24 hr), the ITP technique only takes tens of minutes and only requires

inexpensive chemicals/buffers. So it can be employed as a fast preliminary quality check of surfaces in a meat processing facility. Cross-contaminations can lead to product recall and profit loss in food processing, and early detection of contaminated surfaces can save the products. And this is why having a rapid, cheap technique can be beneficial for the food industry.

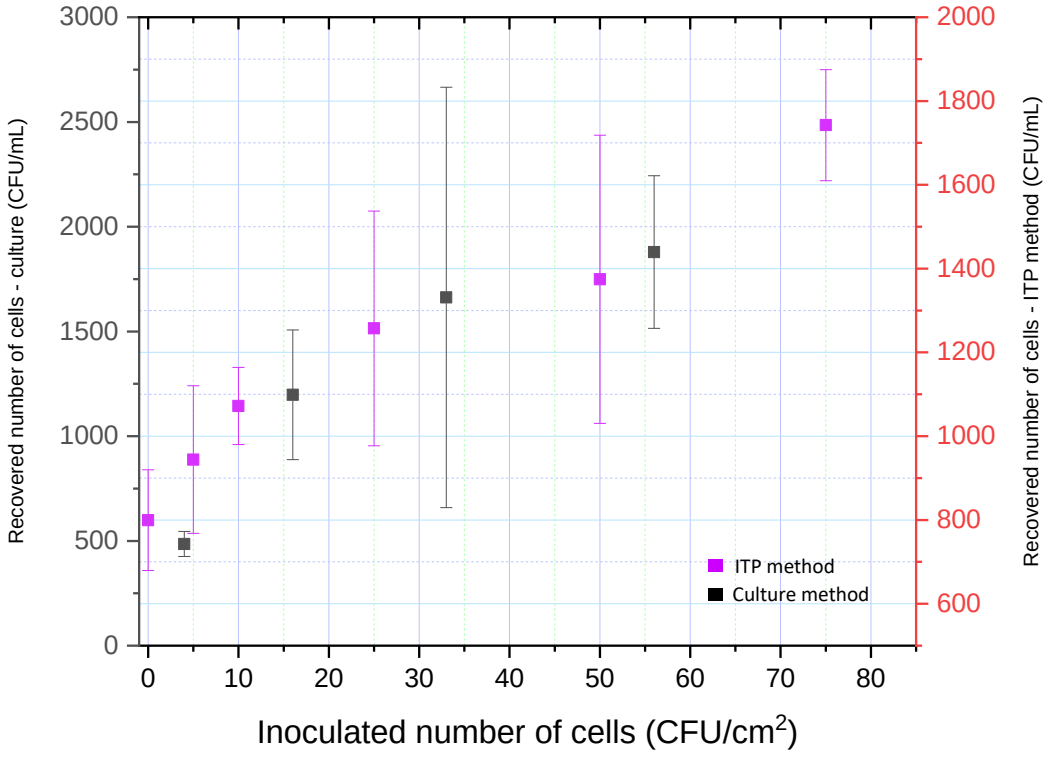

Figure 21: comparison between the culturing technique and the ITP technique. 100cm² area was swabbed

High standard deviations that can be seen with the ITP method can imply potential problems with the new technique, such as pressure defects, issues with the injection. However, this can also be due to the other previously discussed causes like inconstancies of deposited cell numbers, inconsistencies of swabbing techniques, etc. To confirm this, we performed an experiment parallel to the ITP experiment. The 100 cm^2 steel plate inoculated with E.coli cells was swabbed with a Copan swab, and recovered cells in PBS were plated onto LB agar plates. Then the agar plates were incubated at 37°C for 24 hr. Similarly, the inoculated steel plate with *E.coli* was swabbed with another swab, and recovered cells in TE (Tris TAPS) were quantified using the ITP method. Figure 22 shows considerably high variations even with the culturing method. This proves that the data points are spread on a broader range, not because of the possible defects in the ITP method but the inconsistencies related to the swabbing process.

Using the developed method to quantify microbial load on red meat carcasses.

The swab-approach was next evaluated for the detection of bacteria directly on meat surfaces. The isotachopherograms of consecutive runs obtained from injecting extracted bacterial cells from red meat using Puritan flocked swabs are shown in Figure 22. As can be seen, they were not consistent for the same cell concentration, as cell peaks appeared at different migration times with different peak heights. The current profile of the first few runs followed the same trend, but after 3 runs the current started to vary (Figure 23 and Figure 24). One possible explanation for this phenomenon is that something else(molecule/eukaryotic cells/meat fat) has injected with the cells into the capillary and may have attached to the capillary walls changing the electroosmotic flow, hence modifying the migration time. There is a possibility of picking up eukaryotic cells (meat cells) with the bacterial cells during the extraction procedure (swabbing). Therefore, meat cells (which are bigger than bacteria) might be interfering with the analysis by attaching on to the walls of the capillary. To test this, a small drop of the cell extraction taken from the meat surface was placed on a glass slide and stained with SYTO9 and propidium iodide. Then it was checked under the fluorescent microscope (Figure 25). Big cloudy particles were observed along with small *E.coli* M23 cells. Assuming they could be the reason for the unstable current profile, the extraction was filtered using 5 µm syringe filters and again observed under the microscope. And as shown in Figure 26, the large cloudy particles were removed using filtering.

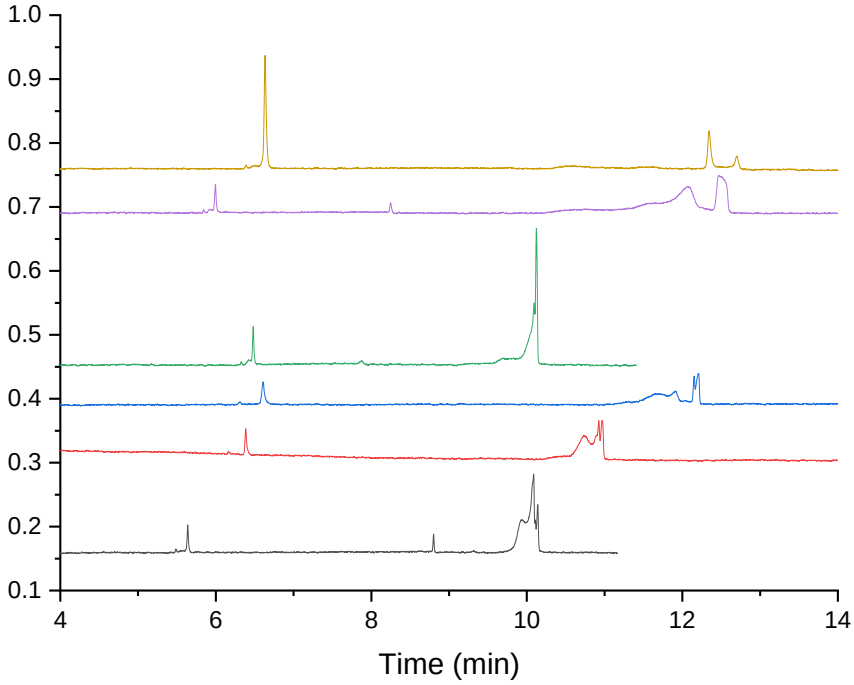

Figure 22: isotachopherograms of the bacterial cell (E.coli M23) extraction sample from meat carcass with 788 cells/mL

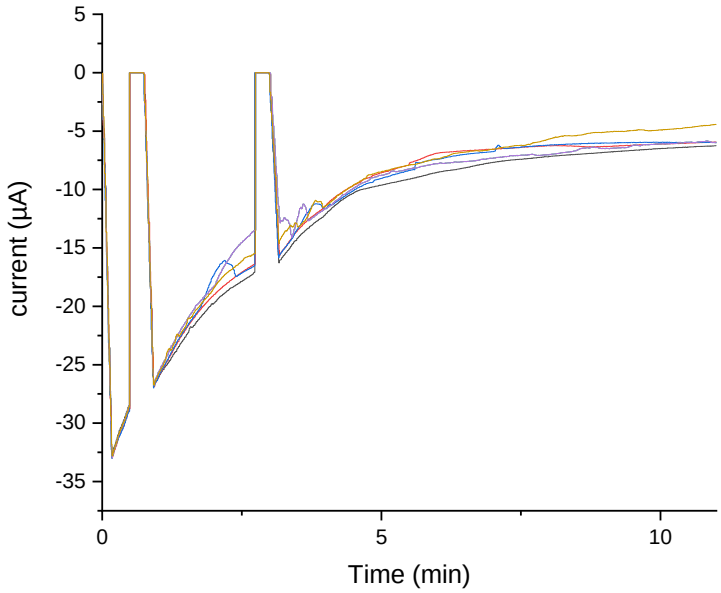

Figure 23: Current profiles of consecutive injections of extracted cells from meat

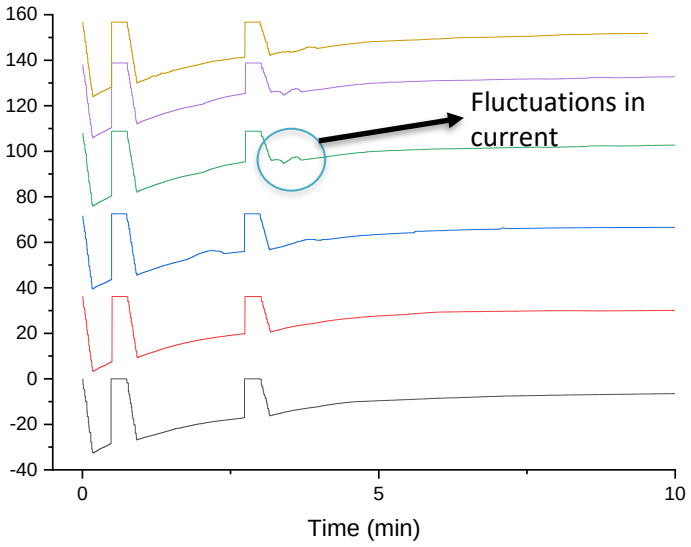

Figure 24: Current profiles of consecutive injections of extracted cells from meat within the same day as in figure 12 in stack up arrangement

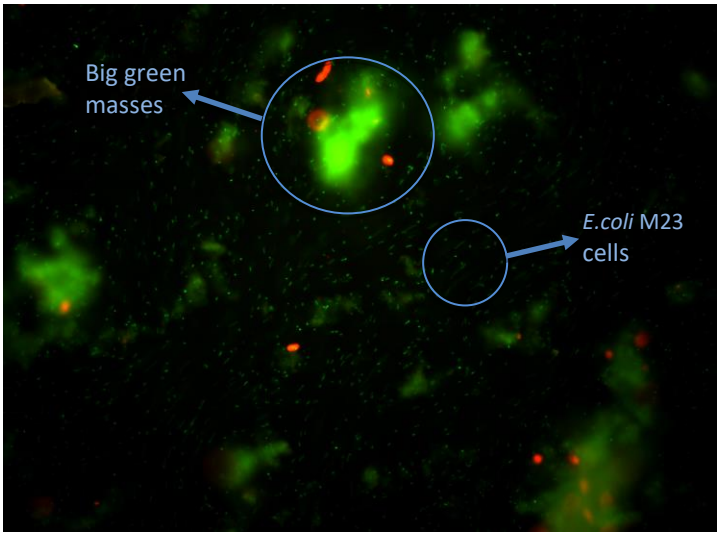

Figure 25: extract from meat surface stained before filtering the extract

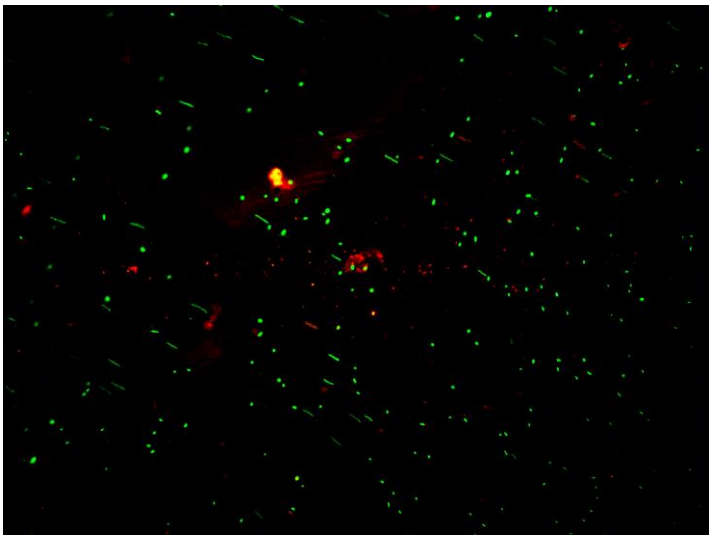

Figure 26: meat extracted stained and visualized after filtering

As explained above, the meat's fat particles interfered with ITP separation. This has led us to include an extra sample preparation step to filter the sample before analyzing it using ITP. The Isotachopherogram (Figure 27) of cell recoveries from meat surface samples shows that the technique works well after the filtration step; no current fluctuations and peaks appear at the same time. Also, the method can detect different cell concentrations easily.

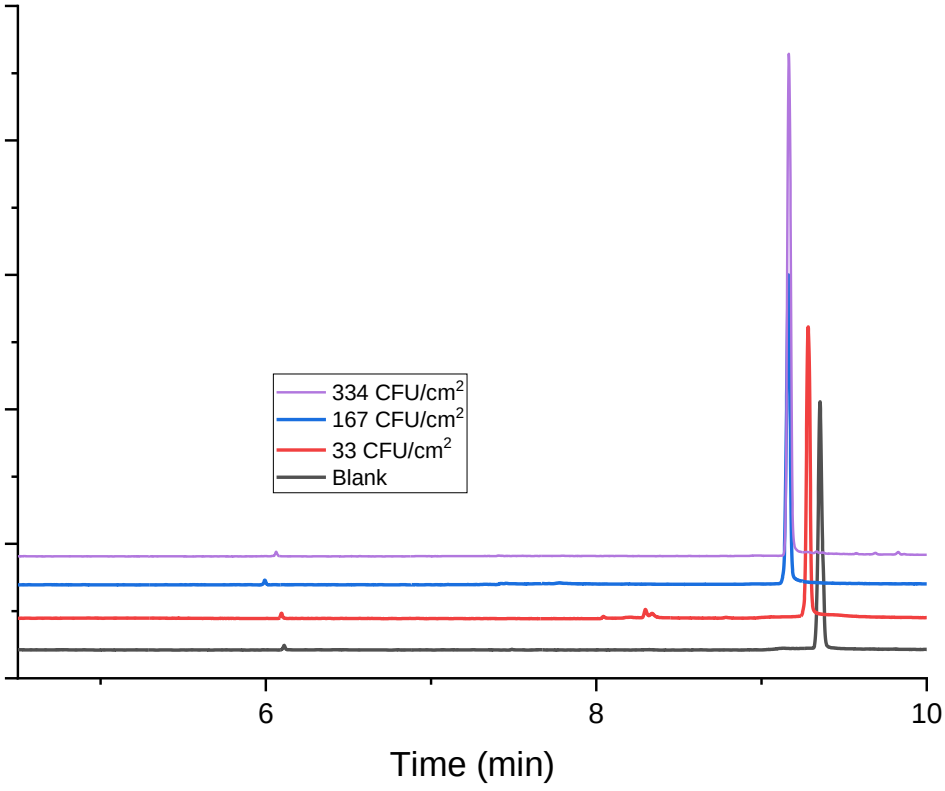

Figure 27: Isotachopherogram of different cell concentrations recovered from the meat surface using Puritan flock swab

According to the standards, cell concentrations over 3.1625×10^4 CFU/cm^2 are unacceptable for red meat surface samples (Australian Government 2021). Detection of these high levels is possible as the technique is sensitive enough to detect low cell numbers such as 100 cells/mL. Figure 28 shows the standard addition calibration curve of the different cell concentrations on red meat samples recovered using Puritan swabs. Puritan swabs have loose fibers, which interfered in the ITP analysis of the steel surface study. Therefore we used Copan swabs instead of Puritan swabs to avoid this issue. But with meat samples, to get rid of the fatty bits, we added the filtering step, which also helps to eliminate the loose fibers of the puritan swabs. Thus the study was carried out using the Puritan swabs, which had the best cell recovery. The figure shows a correlation of signal response to bacteria level deposited on the meat.

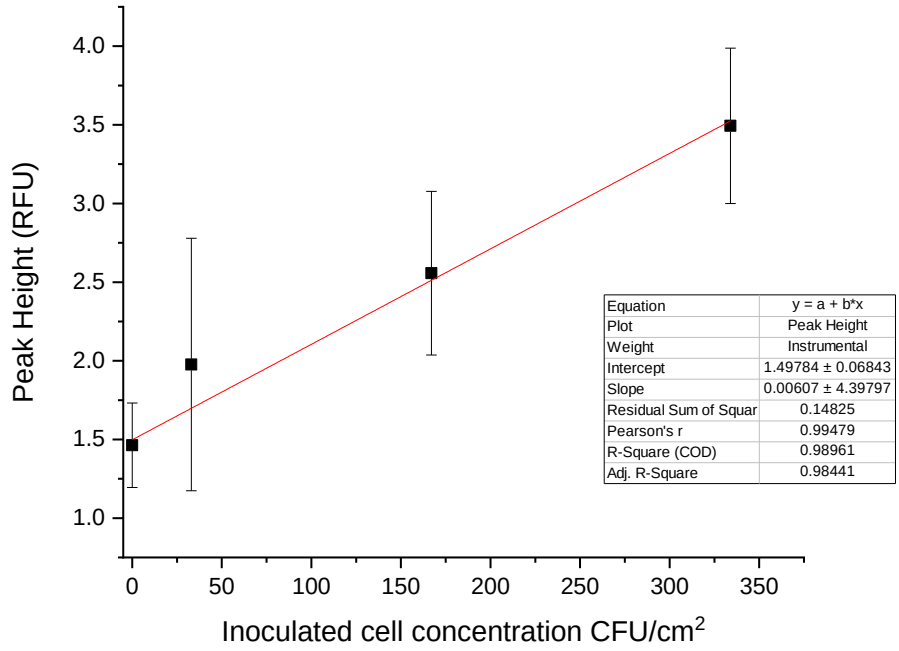

Figure 28: standard addition calibration curve of different cell concentrations recovered from the meat surfaces using puritan swabs

Meat that is available in the market is not sterile. There are always a certain amount of non-harmful bacteria (lactic acid bacteria) present on meat. The acceptable level of the bacteria present on meats surfaces is log 4.3 CFU/cm^2. Therefore, roughly 1000 bacteria on a 1 cm^2 area are still acceptable for nutrient-enriched food such as meat. As we used non-sterilized meat samples for this study, the meat blank contained a considerable amount of bacteria. Using the spiked concentrations added in Figure 28 the concentration of bacteria on the meat was calculated to be 247 CFU/cm^2 by the CE method.

To verify this data, a culture experiment (50 cm^2 area of meat surface was swabbed before depositing cells using a Puritan swab and recovered in PBS and plated on LB agar plates), the number of bacteria on the meat was measured to be 371 CFU/cm^2. This is similar to that obtained by the CE method with the difference likely due to the heterogenous nature of swab sampling.

Selective cell detection

The above chemistry stains all cells based on a nucleic acid stain. Viable cells can be targeted by using live/dead dyes to give a result that directly correlates to CFU. This approach may be useful to determine a total viable count, but is not sufficient for the detection of specific bacteria. Specific bacteria require the use of a more selective dye. Previously, we achieved this with small nucleic acid probes that penetrated the cell and bound selectively to the RNA, however, a loss of sensitivity by 1000 times was observed. To address that problem here, we instead use antibodies modified with Quantum Dots (QD) to target the proteins on the cell surface. QDs are brighter than standard fluoropres and should facilitate more sensitive detection.

Two QDs were purchased, one emitting green light (~520 nm) and one emitting red light (~640 nm). These were both evaluated by electrophoresis with superior results obtained for the red QD. This QD was then modified with an antibody targeting the Enterobacteriaceae family. This was conjugated to the QD via streptavidin/biotin linkages.

E.coli stained with Ab-QD_{645} conjugates.

E.coli stained with only QD_{645}.

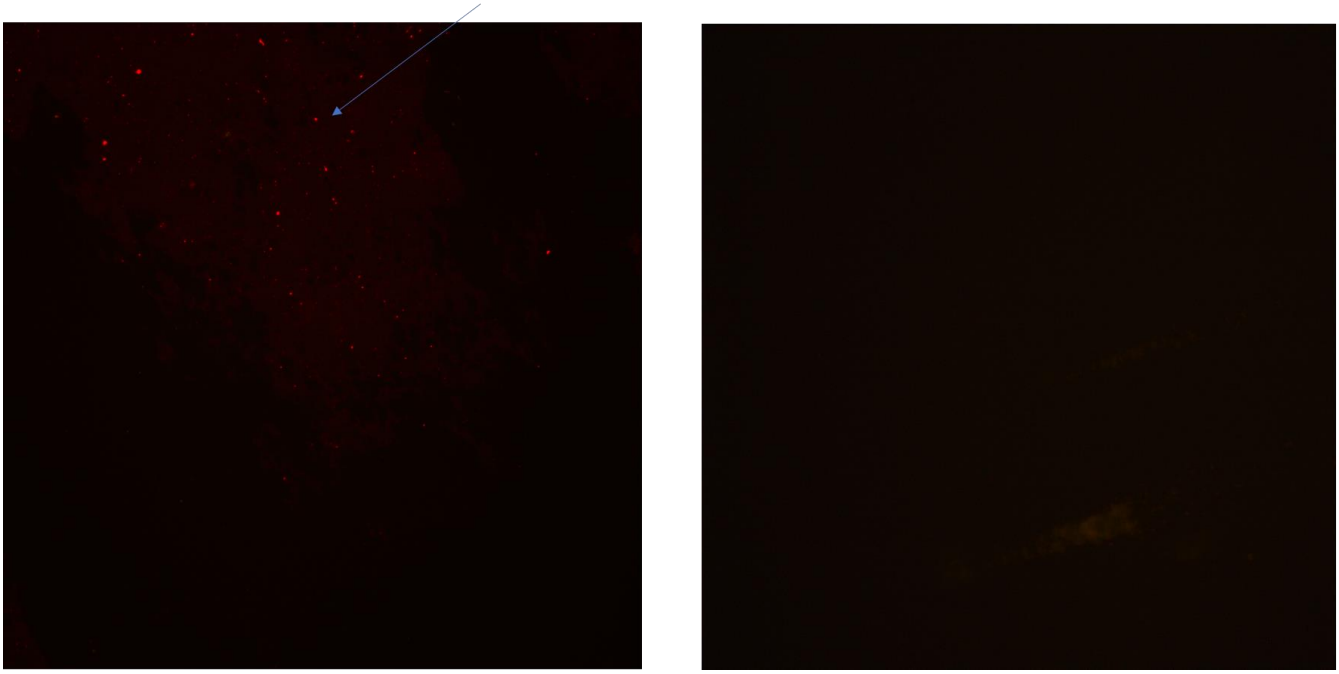

Figure 29: E. Coli cells stained with the AB–QD_{645} label (left) and QD_{645} (right). Bacteria can be seen fluorescing red in the left image only

Injection of the labelled *E. Coli* cells into the CE instrument yielded peaks with a size proportional to the cell number (Figure 30). The calibration plot is shown in Figure 31, which shows a linear response. The system shows an ability to detect 2 cells injected onto the capillary.

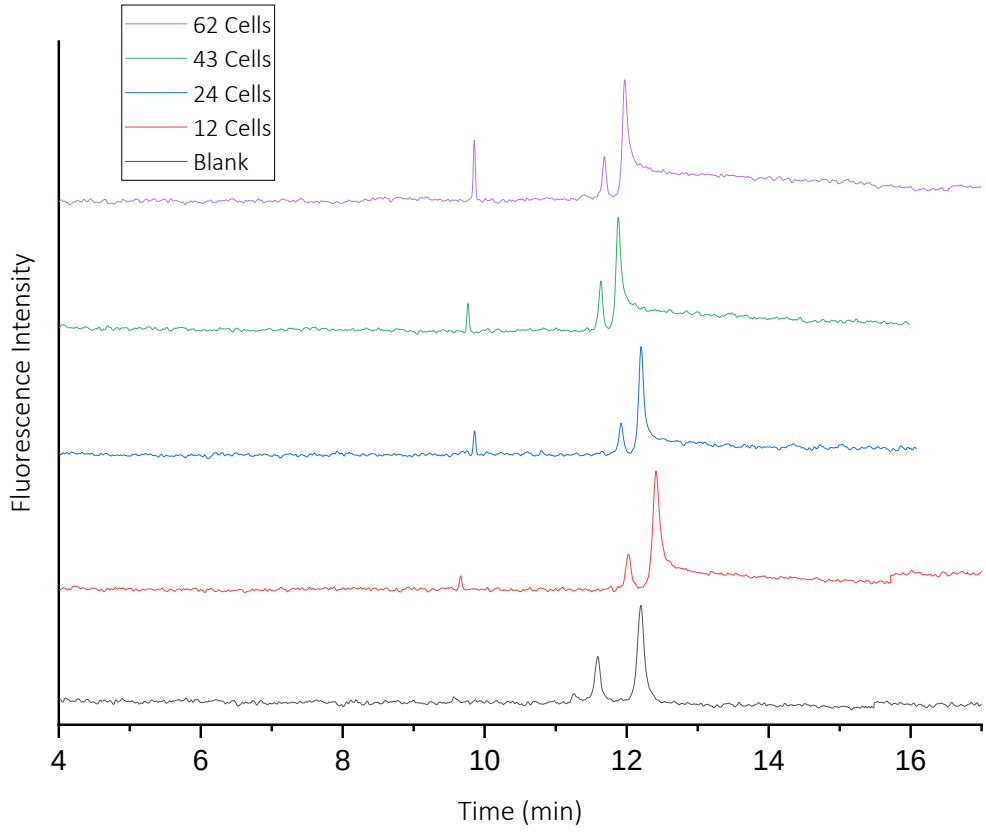

Figure 30: electropherograms of E. coli stained with AB-QD$_{645}$.

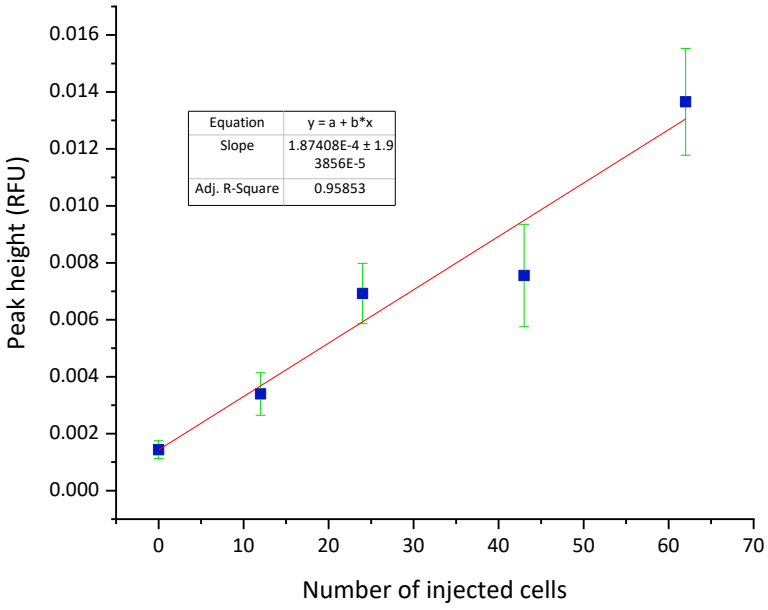

Figure 31: Calibration plot of E. Coli stained with AB-QD$_{645}$.

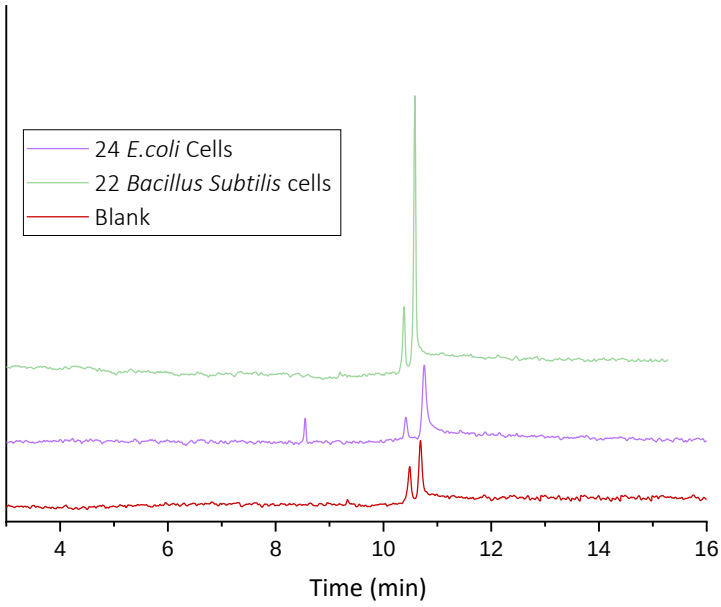

Figure 32: Staining of E. Coli and B. Subttilis with AB-QD$_{645}$. A peak is sean at 8.5 min only for the E. Coli sample.

6.0 OVERALL PROGRESS OF THE PROJECT

The project did not track to the original schedule due to a delay in student recruitment. As the initial contract was not signed until the 15th of September 2017, the Ph.D. student that had been selected and agreed to the project in Jan 2017 decided to pursue studies elsewhere when he was presented with his offer. This required a new search for a graduate student. The position was advertised both nationally and internationally, with only international applicants (this is not unusual from my experience over the past 5 years). The best candidate was an international student and there was nearly a 12-month wait for her visa, as is currently the scenario with a lot of international Ph.D. students. The Ph.D. student (Thisara) began her Ph.D. on the 17th of September 2018.

In October 2020, new milestone dates were agreed, essentially factoring in this 12-month delay in the start of the project.

The chemistry aspects of the project proceeded well and according to plan once the delayed start was factored in. A method developed by Sui Phung was modified to quantify bacterial cell count (Phung, Nai et al. 2015) and to separate stained bacteria from a background interference. This required redevelopment of the chemistry: a spacer electrolyte (90 mM, pH-8) and a sieving matrix (1.6% w/v hydroxyethyl cellulose). When combined with SYTO 9, a nucleic acid stain to stain both dead and live bacterial cells (*E.coli* M23) the method was sensitive to detect 28 cells/mL, which with a sample volume of 200 µL, translates to a detection limit of 5-6 cells.

In order to detect total viable bacterial count (the above label detects both live and dead cells), a combination of two nucleic acid dyes was used. The bacterial cell membrane act as a permeability barrier and It allows the SYTO 9 to penetrate through the live bacterial cell membrane. Therefore SYTO 9 stains both dead and live bacteria. In contrast, Propidium bromide cannot pass through the live cell membrane, hence it only stains dead cells where the cell membrane is not functional. As Propidium iodide has more affinity to DNA than SYTO 9, it replaces the SYTO 9 in dead cells. This means the green channel gives only the quantity of live bacterial cells in the sample. The limit of detection of the method with the two stains is 131 viable cells/mL, which again with a volume of 200 µL, translates to a detection limit of 25 cells/mL.

The European Community(NSW Food Authority 2013) has established the following limits for hygiene surface samples from meat or poultry abattoirs or cutting rooms. The standards apply to surfaces that have been cleaned and sanitized and are dry and smooth. Samples are taken before production starts.

Table 2: Mean values for the number of colonies(CFU) for testing of surfaces

	Acceptable	**Unacceptable**
Total viable counts	**0-10 CFU/cm^2**	**>10 CFU/cm^2**
Enterobacteriaceae	**0-1 CFU/cm^2**	**>1 CFU/ cm^2**

The acceptable level of total viable count for food contacting surfaces is 10 CFU/cm^2. By assuming there are 10 bacterial cells on 1 cm^2 on the surface, swabbing 10*10 cm2 would give 1000 bacterial cells/mL. However, cell recovery from swabs is not 100%. Maximum cell recovery was observed with Puritan Flock swab which is 79% with Tris TAPS buffer (figure 4). Therefore, only 790 cells/mL are available for analysis. This is still considerably higher than the LOD method limit of is 131 Cells/mL. Therefore the method can successfully detect above standard limits, and was shown to produce results similar to that observed with a plate count.

The sensitivity is currently not suitable for Enterobacteriaceae levels on surfaces. Swabbing 10*10 cm2 would give 100 bacterial cells, which with 80% recovery into 0.2 mL, gives a concentration of 400 CFU/mL. The CE system can currently detect 2 bacteria, but this is from a small injection volume (~10 nL). Method development is required to implement similar strategies to those employed for the total viable cell count to improv the signal. This has not been undertaken at this point due to the PhD Candidate reaching the end of her time and research.

The hardware development did not proceed according to plan. Home-built fluorescence detectors were found to be at least 50 times worse than the commercial detector on the lab-built instrument. The lab-instrument was capable of reaching suitable detection limits, but would not be functional with the lower sensitivity of the home-built detector. Improvements in optics, filtering and data acquisition are needed to improve the detector further.

The sample-extraction system envisaged to be used for this project became dysfunctional during the project and did not allow suitable competion of this part of the project. An alternatve in-house extraction system was designed, constructed and tested, but could not be made leak-free. Improvements in the extraction system are also required.

8.0 CONCLUSIONS/ RECOMMENDATIONS

The chemistry for a total viable count detection of bacteria on surfaces and on meat has been demonstrated with sufficient sensitivity for implementation in a laboratory instrument. With further development, it is possible that this can be implemented on a portable instrument for rapid (~10 min) field detection of bacteria in meat processing facilities.

9.0 BIBLIOGRAPHY

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