MP 519

# Automated bacterial sample preparation from agar to MALDI target for routine bacterial identification

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## 1. Introduction

Over the past decade, MALDI-TOF mass spectrometry has established itself as a routine, rapid method for microbial identification at the species level. The typical sample preparation is referred to as the *smear technique* and involves transferring a small amount of cultured material to a MALDI target using a small inoculation loop or toothpick and then smearing the deposited sample on the sample position to create a more uniform deposit. Finally, a small volume of MALDI matrix solution is added and mixed with the sample before being allowed to dry.

Due to the manual nature of the sample transfer and subsequent deposition onto the MALDI target, the sample preparation step is prone to variation which can introduce differences in the quality of sample spots prepared by the same operator or between samples prepared by different operators. Sample reproducibility can be improved with practice but the sample preparation still remains a time consuming, laborious and variable step. Here, we evaluate the use of a colony picking robot to improve the sample preparation reproducibility for microbial identification by MALDI-TOF mass spectrometry.

## 2. Methods and Materials

*Escherichia coli* (*E.coli*) was used for method development and *Bacillus subtilis* (*B. subtilis*), *Cellulomonas uda* (*C. uda*) and *Pantoea agglomerans* (*P. agglomerans*) were used to test the optimised sample preparation method. Samples were cultured on Columbia agar containing 5% horse blood (Biomerieux, UK) and incubated for 18-24 hours before use. Prior to use, the samples had been frozen on porous beads in microbank cryovials and underwent 3 passage cycles. CHCA matrix solution was prepared at 40 mg/mL in acetonitrile/ethanol/water containing 3% TFA. Samples were prepared on disposable FlexiMass-DS slides (Shimadzu). For manual sample preparations, culture material was prepared using a 1 µL inoculation loop (smear technique) and 1 µL CHCA matrix solution was manually added and allowed to air-dry. For automated sample preparation, samples were picked using a PIXL colony picking robot (Singer Instruments, UK) and deposited onto a FlexiMass-DS slide using a smear-like technique. Different matrix deposition techniques were evaluated (single or double transfer). Samples were analysed on an iD*plus* Performance MALDI-TOF mass spectrometer (Shimadzu) and submitted to a SARAMIS database for identification.

## 3. Results

### 3-1. Preliminary experiments

The PIXL robot is shown in figure 1 (left). Figure 1 (right) shows the internal layout of the robot including the arm and colony picker tip, filament cutter, the MALDI target holder and the agar plate.

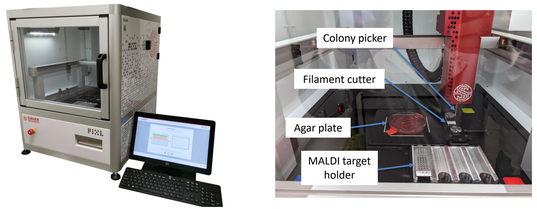





Figure 1. (Left) PIXL colony picking robot and (right) internal layout showing colony picker tip, filament cutter, target holder and agar plate.

Preliminary experiments were performed to test different sample preparation methods using the colony picking robot. These included: (i) mixing picked material in an MTP well containing various volumes (10, 20, 30, 40 and 50 µL) of MALDI matrix and subsequent deposition onto the MALDI target and, (ii) automated deposition of picked material using different deposition methods (pinning vs. smear method) followed by automated deposition of matrix solution from an MTP well. See figure 2.

Of the preliminary methods tested, two methods showed promising results based on the MALDI-TOF based IDs: (i) mixing picked culture in an MTP well containing 50 µL of matrix solution and subsequent deposition onto the MALDI target and; (ii) the smear deposition of picked material onto the target followed by automated transfer of matrix solution onto the deposit. A third method (automated smear plus manual matrix addition) was also chosen for further evaluation.

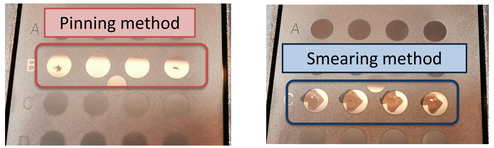


Figure 2. Examples of automated bacterial transfer. (Left) the pinning method i.e. 'dab' picked material onto target surface and, (right) the smear method. As can be seen, the pinning method was not as reproducible as the smearing method. Pictures in the figure show bacteria deposited before addition of matrix solution.

### 3-2. Further optimisation of selected methods

Three methods were chosen for further evaluation using *E.coli*:

**A. Mix bacteria in MTP well containing 50 µL matrix solution and single/double deposition onto the MALDI target:** In this method, the PIXL robot was used to pick bacteria which was then mixed by the robot in a MTP well containing 50 µL of CHCA matrix solution. Aliquots of the bacteria/matrix suspension were deposited onto the MALDI target using either single or double deposition.

**B. Automated smear deposition onto target with manual matrix addition**: The PIXL robot was used to deposit picked material using a smear-like method directly onto the MALDI target. Subsequently 0.5 µL or 1 µL of matrix solution was *manually* added to the deposited material

**C. Automated smear deposition onto target with automated single/double deposition of matrix solution:** The PIXL robot was used to deposit picked material using a smear-like method directly onto the MALDI target. The robot was then used to deposit matrix solution from an MTP well onto the deposited bacteria. One and two matrix depositions were tested.

Although the PIXL robot can perform automatic colony detection, for the purposes of the sample prep optimisation, the positions of the bacterial colonies were manually selected in the software. Furthermore, for each of the 3 methods (A-C (section 3.2)), bacteria were selected from 3 different positions on the cultured material (see figure 3): centre of the culture growth (referred to as **Deep),** edge of the culture growth (referred to as **Edge**) and centre of culture growth and subsequent 'dab' onto a blank area of agar not containing bacteria (referred to as **Deep + blank dab**). The results of the 3 methods are shown in figure 4.

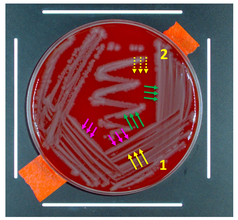


Figure 3. Examples of the three areas where bacteria were picked:

**Green** arrows: central, thickest part of the culture growth 'streak'. Referred to as **Deep**

**Pink** arrows: edge of culture growth 'streak'. Referred to as **Edge**

**Yellow** arrows: material picked from central, thickest part of the culture growth 'streak' (solid arrows, 1) then dabbed onto an area of agar not containing any culture to remove excess material prior to depositing onto the MALDI target (dashed arrows, 2). Referred to as **Deep + blank dab**

Of the 3 methods tested, methods B and C resulted in largely >99.9% (**dark green**) confidence IDs for *E. coli* and good data counts (>200) i.e. number of detected peaks. SARAMIS results obtained for method C are shown in figure 4. From the observed data counts, there did not appear to be a significant difference relating to the area from which the bacteria were selected (see figure 3). Data counts were slightly lower for the single matrix dip (Av. = 172) compared with the double addition of matrix (Av. = 214). One position (2C2) produced no ID and which we suspect was due to lack of material picked from the edge of the growth streak.

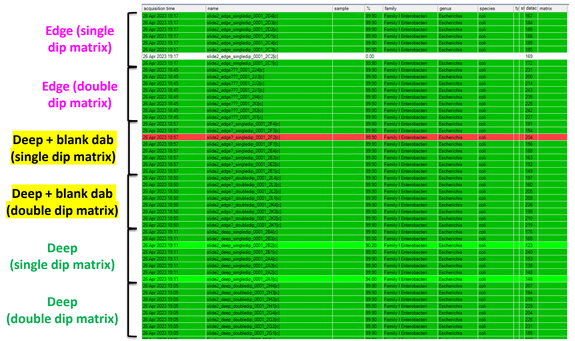


Figure 4. SARAMIS results for *E.coli* samples prepared using Method C i.e. automated smear deposition and automated single or double dip matrix deposition. Dark green = >99.9% confidence, light green = 90.0 – 99.8% confidence, red = mixed ID, white = no ID.

The only method which failed to produce any results was method A (i.e. mix in MTP with 50µL matrix then deposit onto target). Although the method was successful in the preliminary experiments, in the further experiments, all spectra were dominated by a polymer distribution (*m/z* 2000-8000), dM = ~100 Da. We suspect these may be plasticisers/contamination from the MTP plate used to hold the matrix in these experiments although, interestingly, the same MTP plate was used for the matrix in method C without issues. Due to time limitations, it was not possible to repeat method A using a different MTP.

### 3-3. MALDI-TOF based identifications (SARAMIS)

As method C was the most automated method (automated sample *and* matrix deposition), this method was chosen to prepare 3 full slides using the other standard bacteria. For the optimised method, samples were selected from the deep part of the culture growth streak and double application of matrix solution was applied to each sample from an MTP well. Sixteen (16) spots of each bacteria were prepared on each of 3 slides. Example results obtained are shown in figure 5. The red IDs for *B. subtilis* are due to a mixed ID (Bacillus_atrophaeus/subtilis). The same mixed IDs were also obtained for *B. subtilis* for the manually smeared control samples (data not shown). The results obtained are consistent with results expected for good manually prepared samples. Example spectra for manually prepared and automated sample preparation samples are shown in figure 6.

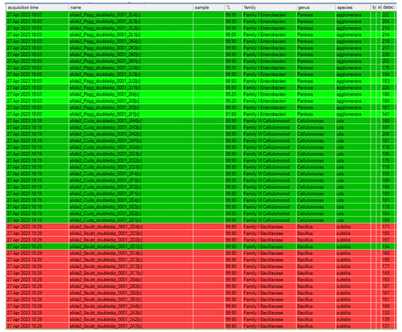

Figure 5 (left). SARAMIS results for bacterial standards *B. subtilis*, *C. uda* and *P. agglomerans* prepared using optimised Method C i.e. automated smear deposition and automated double dip matrix deposition. Dark green =

>99.9% confidence, light green = 90.0 – 99.8% confidence, red = mixed ID.

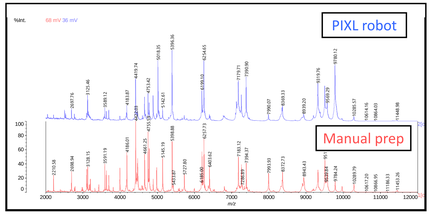


Figure 6 (right). Example MALDI-TOF MS spectra obtained for *P. agglomerans*: red spectrum: manually prepared smear sample; blue spectrum: smear sample prepared using the PIXL robot using Method C.

## 4. Conclusions

The aim of this work was to determine whether a colony picking robot could be adapted to prepare samples suitable for MALDI-TOF MS-based identification. The PIXL robot used for this work is typically used to pick colonies and generate arrays for library construction and high-throughput screening. **The results obtained demonstrate that the PIXL colony picking robot is capable of producing samples suitable for confident microbial identification by MALDI-TOF MS.** The results obtained were similar to those expected for good quality manual sample preparations. The automated approach should improve the reproducibility of sample preparation, particularly for less experienced users.