Protein fingerprinting and barcoding for bacterial identification and classification using MALDI BIOTYPER

1. **Matrix solution preparation:**
   - Saturated solution of α-HCCA in 50% acetonitrile (ACN), 2.5% trifluoracetic acid (TFA). The solution 50% ACN/2.5% TFA will be referred as organic solution (OS).
   - Transfer some α-HCCA crystals into an Eppendorf tube, add 200 to 500 ul of OS and vortex it for a couple of minutes at room temperature, until the solution is saturated.
   - Matrix can be stored and used at room temperature for up to 1 week.

2. **Sample preparation**
   a. Place 5-10 mg of bacteria into an eppendorf tube.
   b. Add 300 ul of water, vortex briefly.
   c. Add 900 ul ethanol, vortex briefly.
   d. Centrifuge at max. speed for approximately 2 minutes, remove supernatant.
   e. Repeat steps b, c and d.
   f. Add 50 ul 70% formic acid to the pellet and mix by vortexing.
   g. Add 50 ul pure CAN and mix it well.
   h. Centrifuge at max. speed for 2 minutes. Transfer supernatant to a new eppendorf tube immediately.
   i. Place about 1 ul of supernatant onto a steel target plate and air-dry it.
   j. Overlay with about 2 ul of matrix solution direct after drying.
   k. Air-dry the target plate (make sure that the plate is completely dried before placing it in the MALDI-TOF).
3. **MALDI-TOF MS measurement**

**Instrument calibration:** It is strongly recommended to prepare sample spots of *E. coli* DH5α on a target plate according to the above standard procedure to be used as positive control, prior to every MALDI-TOF MS measurement of bacteria of interest.

To avoid differences in spectral generation, the *E. coli* control and other bacterial samples should be prepared according to the same conditions and procedures.

To ensure effective calibration, it is important to generate and *E. coli* mass spectrum that includes the peak at 10299.09 Da. This is a characteristic peak for *E. coli* DH5α, and its presence indicates proper sample preparation, instrument tuning and calibration, and instrument operation.

After calibration, one sum spectrum of *E. coli* DH5α should be saved to calibrate the instrument at a later time or in case of doubt of the instrument operation. Calibration should be always done prior to sample analysis.

**Instrument settings (BioType.par protocol):** To best tune the instrument, it is recommended to find specific settings with a delay time (PIE) of approximately 300-500 ns. BioTyper.par protocol method (linear and positive mode) of flexControl is used for the procedure.

- Sample rate should be set to 0.5, electronic gain to 100 mV and real-time smooth to “high”.

- IS1 is set to 20 kV and IS2 is optimized based on the resolution of one peak of about 5000-7000 Da. A good starting point should be 18.65 kV. The best value of IS2 is relatively narrow. Even between 18.55 kV, 18.60 kV and 18.65 kV, noticeable resolution differences can be observed.

- Lens voltage should be only changed slightly at the beginning. After resolution optimization, the “electronic gain” should be carried to find optimal signal intensity. Final and stable resolution of the peak should be approximately 600-700.

- The whole tuning process is mainly a balance of parameters IS2, PIE and detector gain. The goal of tuning is to reach an average resolution of about 600-700 at optimal signal-to-noise levels within a mass range of 4000-10000 Da.

- It is important to measure at the desorption level. This will be achieved by firing some initial laser shots (10 to 20) with relative high energy to “clean matrix” (Matrix blast effect). Afterwards, the laser energy should be decreased until the signal disappear. Finding the optimal laser energy is possible by stepwise increasing the laser energy until signals with significant intensities are detected. For a new laser position of the target, the optimized laser energy level can be
used directly after “pre-shooting” (10-20 shots with high laser energy) to generate the desired spectrum.

4. **BioTyper software**

**Spectrum preprocessing (generation of peak lists):** load a Bruker mass spectrum by using the “load single spectrum” command. To load a collection of spectrum, use the “load spectra tree” command from the File menu.

**Construction of main spectra libraries:** Identification of unknown bacterial species on the basis of measured spectra requires a “Reference library”, also called main spectra library. Such a library contains characteristic patterns of each species. These datasets are adjusted peaklists with some additional information, such as peak frequency distribution. During main spectra library generation, 3 different BioTyper libraries are built: Raw databases (RDB), preprocessed databases (PDB) and main spectra libraries (MSP). They all can be saved for further applications.

**Identification, analysis and classification of spectra.** MALDI BioTyper offers three different statistical analysis: MSP (Main Spectra Projection), PCA (Principle Component Analysis) and Composite Correlation Index. For the separation of closely related bacteria from each other, it is recommended that one tries all available analysis tools. Furthermore, single peaks of the main spectra can be weighed to reach better separation of very similar spectra.

**REFERENCES:**