SOP: Identification & typing of bacteria and yeast with MALDI-TOF MS (v1.4)

1. Subject

This protocol describes the workflow to identify and type cultured bacteria and yeast by means of MALDI-TOF MS in the routine laboratory P8.

2. Abbreviations

LBR	Laboratory Bacteriology Research
MALDI-TOF MS	Matrix-assisted laser desorption time-of-flight mass spectrometry
TFA	Trifluoroacetic acid
AU	Arbitrary units

3. Reagents

Reagent	Remark
Matrix (HCCA) working solution	Bruker, on bench in the post-PCR lab (room 110.012). Prepared from stock solution
Matrix (HCCA) stock	Bruker, box located in the door of 4 °C fridge (LBR T063) of the main lab (room 110.010)
OS solution	MALDI-MS, 50% ACN + 47.5% H_2O + 2.5% TFA, **Trifluoroacetic acid solution** Sigma, ref. 19182-250 mL, on bench in the post-PCR lab (room 110.012)
Ethanol solution 70 %	In-house preparation
Ethanol solution 96 %	VWR, ref. 20905.296. In fume hood in the post-PCR lab (room 110.012) or in PMGE (room 110.054)
Acetonitrile LC-MS (C_2H_3N)	Sigma, ref. 34967-250 mL, on bench or PMGE (room 110.054)
Formic acid (HCOOH = HCO_2H, dutch: mierenzuur)	Sigma, ref. 56302-50 mL, PMGE (room 110.054)
Trifluoroacetic acid, TFA ($C_2HF_3O_2$)	Sigma, ref. T6508-100 mL, PMGE (room 110.054)
Ultrapure water	Sigma, ref. 39253, main lab fridge (LBR T063, room 110.010)

4. Equipment, disposables and forms

Equipment/disposable/form	Remark
Target plates and holder	
Toothpicks	
Inoculating loops 1 µL (yellow)	MLS, Ref. Q30010
KimTech tissues	
Pipet and filter tips 1 µL	
Blanco malditof.xls	Dropbox\LBR_SOPs\MALDI-TOF\SOPs\blanc malditof.xls
Microflex LT/SH	Brucker, serial number 26994400235

5. Samples

Colonies on culture plates or ethanol preserved colonies.

6. Method

6.1. In advance

6.1.1. Preparing OS – solution (Room 110.012).

Normally, OS solution is used from Sigma (ref. 19182). Alternatively, OS solution can be prepared as described below. For all steps below, work in the fume hood.

1. In a 1.5 mL Eppendorf tube, make following solution: 475 µL ultra-pure water, 500 µL acetonitrile, and 25 µL TFA.
2. Store in fridge.

6.1.2. Preparing the matrix (HCCA) (Room 110.010).

1. Take a tube with lyophilized matrix from the box located in the 4 °C fridge of the main lab (LBR T063, room 110.010).
2. Add 250 µL OS stock solution (see 6.1.1.).
3. Vortex well.
4. Incubate 1 minute at room temperature.
5. Store on the bench in the post-PCR lab (room 110.012).

6.1.3. Preparing 80% TFA solution (Room 110.012, fume hood).

For all steps below, work in the fume hood.

1. Make 1 mL aliquots of 80%, by pipetting 200 µL water in the Eppendorf TFA solution first followed by pipetting 800 µL TFA in an Eppendorf tube.

   Importantly: always pipet the water first, and then the acid. Not the other way around!
2. Label as “TFA 80%” and store on the bench in the box “MALDI-TOF”.

6.1.4. Cleaning the target plates (Room 110.010).

1. Put the target plate in its holder and cover with 70% ethanol. Let the ethanol incubate for 5 minutes.
2. Take out the target plate and rinse with warm water. Try not to wet the backside of the target plate (magnet).
3. Clean with KimTech tissue and repeat steps 1 to 2.
4. In the hood (room 110.012): pipet 150 µL TFA 80% (trifluoroacetic acid) to cover the target plate. Thoroughly clean and remove all residual material using a cotton swab.
5. Rinse with molecular water. Try not to wet the backside of the target plate (magnet). Dry with KimTech tissue.
6. Target plate is now ready to use.

6.2. Procedure 'direct transfer method' (Room 110.010).

1. Leave your culture plates in the incubator until needed for identification/typing. You can spot your colonies onto the target plate and analyze them on the Bruker MALDI-TOF device, which is available in the routine lab Bacteriology (2P8) every day from 4 PM.
2. To register the sample names of a **large collection**: use the file '**blanc maldtof.xls**'. **Go to step 4**.
3. If it concerns a **small number of samples** (e.g. less than 10): keep track in your lab book which strain is spotted on which position on the target plate. **Go to step 5**.
4. Registration in case there are many samples to spot:
    a. Open the blanc Excel file on Dropbox/LBR_SOPs/MALDI-TOF/**blanc maldtof.xls**
    b. Copy and paste your sample names from your collection/work list or MaldiTOF worksheet.xlsx to the corresponding MALDI-TOF plate positions in column D in this Excel worksheet.
    c. Remove **all** empty positions in the file**, INCLUDING TITLES!**
    d. Save the new file on a USB stick:

       Name your file **YYMMDD_MALDITOF** and **Save as "CSV file (comma delimited) (*CSV)**" (or in Dutch version as "CSV-bestand 'gescheiden door lijstscheidingsteken)".
    e. Save the Excel file as a backup.
5. To spot a colony, pick up a colony or a piece of a colony with a toothpick or the back of an inoculation loop and gently distribute it on the target plate (try not to bring on too much material).

   In case of yeast or fungi: spot 1 colony in triplicate and add 1 µL of formic acid 70% on each spot. Let it dry.
6. After finishing spotting one row, pipet 1 µL matrix on each spotted colony (within 10 min after spotting). Vortex the matrix well before each use. Leave it to dry.
7. After addition of the matrix, the colonies are ready to be analyzed by the Bruker MALDI-TOF MS (see 6.4)

   Remark: maximum time between application of matrix on the target plate and analyzing on the MALDI-TOF MS is 24H for identification.

   The identification results will be reported in a PDF file, named 'MT_YYYYMMDD', and can be downloaded by USB. In this rapport, you will find your colony name followed by an identification with a MALDI-TOF score.

6.3. Procedure "ethanol/formic acid extraction method" (Room 110.010).

The "ethanol/formic acid extraction method" is used:

(i) When no satisfying identification is obtained,
(ii) When a new spectrum is to be made for inclusion in a database,
(iii) When bacteria or yeast need to be typed
(iv) When bacteria or yeast are preserved in ethanol for later identification.

1. Add several colonies (the suspension should appear turbid) into a 1.5 mL Eppendorf tube with 300 µL ultrapure water and homogenize by vortexing.
2. Add 900 µL ethanol 96% and homogenize by vortexing. This suspension can be stored at –20 °C for 6 months, or alternatively, a few days at room temperature.

3. Centrifuge for 2 minutes at 13,000 rpm (standard bench centrifuge), and gently decant the supernatant. Repeat the centrifugation step to remove the remaining supernatant by pipetting off any residual supernatant. Let the pellet air dry.
4. Add between 1 and 80 µL formic acid 70%. Homogenize by pipetting. Vortex only if sufficient fluid is present. For samples in which lots of material was suspended, 80 µL can be used. On the other extreme, when only a very small pellet is observed, suspend in 1 µL formic acid. For pellets of medium size, add an amount in between 20 and 40 µL formic acid. See table below.

	small single colony	large single colony	1 µL loop	10 µL loop
Formic acid 70%	1 – 5 µL	10 – 20 µL	20 – 40 µL	40 – 80 µL
Acetonitrile	1– 5 µL	10 – 20 µL	20 – 40 µL	40 – 80 µL

5. Add the same volume of acetonitrile in the bottom of the Eppendorf tube and close immediately.
6. Centrifuge 2 minutes at 13,000 rpm.
7. Pipet 1 µL supernatant on the target place and let air dry, it should appear homogeneous.
8. Pipet 1 µL of matrix on the dried supernatant. Do not forget to vortex the matrix well. Covering with matrix should be done within 10 minutes.

6.4. Starting a new IDENTIFICATION project on the MALDI-TOF MS (Routine lab 2P8)

1. On the MALDI-TOF MS, push the green open/close button, watch orange light next to “In progress” until it switches to the green light next to “Access” (long enough until you hear the machine start) to open the lock.
2. When the green light lights up (next to “Access”), the lock can be opened. Remove the target plate by pushing down the holder buttons on both sides of the plate from the previous run (**there should be a plate present in the machine at all times**).
3. Place the target plate for the new run in the MALDI-TOF MS, gently close the lock, and briefly push the same green button again. A project can only be started when the green light lights up.
4. Enter the sample names in the MALDI-TOF MS computer.

   A. In case samples names are saved on a .CSV file on a USB stick:

   1. Enter your USB stick with saved file in the MALDI-TOF MS computer.
   2. Click on “Data (D)” and open the data file on the MALDI-TOF MS computer.
   3. Open “CSV-backup procedure”.
   4. Copy the CSV file from the USB stick.
   5. Paste in “CSV-backup procedure”.
   6. Open MTB Compass on the MALDI-TOF MS computer
   7. Push on “+ RUN”
   8. Select the .CSV file
   9. Type the target plate Serial Number (SN) (When it is a new number, accept target)
   10. Push “Next”.
   11. Save run.

12. Select “Start acquisition”.

Note: before starting acquisition, check if the MALDI-TOF has reached its vacuum state: go to FlexControl software → On the bottom right select the “Status” tab → Double click on the word “Vacuum” → wait for the number corresponding “source high” is below 5.0E-06.

B. In case of manual input of sample names, for only a few samples:

1. On the MALDI-TOF MS computer, go to MBT compass to add the sample names.
2. Push on “+RUN”.
3. Select “Create an ID run”.
4. Type the target plate Serial Number (SN) and push “Next”.
5. Make sure that the correct spots are free on the target plate on the screen to add the sample name, otherwise: select ‘Reset target’ and select the first needed spot position.
6. Add the samples name and push enter to add the next sample names
7. Select “Start acquisition”.

Note: before starting acquisition, check if the MALDI-TOF has reached its vacuum state: go to FlexControl software → On the bottom right select the “Status” tab → Double click on the word “Vacuum” → wait for the number corresponding ‘source high’ is below 5.0E-06.

5. Wait for the run to start (goes automatically but can take up to ± 5 min).
6. Wait for the run to complete and check results in the MBT Compass program.
7. If: “no peaks detected”, “no identification possible”, or score < 1.7 (indicated as red); then re-shoot manually. Only re-shoot when run is complete.
8. Manual re-shooting at the end of the complete run:
    a. Go to the program “MBT Compass”. Under “Advanced: Remeasure samples”.
    b. Select “Remeasure”.
    c. Select the sample for re-shooting with a checkmark.
    d. Select “OK”.
    e. Push “Start measurement”.
    f. Open the program “Flex control”.
    g. Pause run and shoot manually (push “Start” and “Add”) with 40 shots until 240 is reached:
        - Click “Pause”.
        - If not on 40, increase shots to 40.
        - Navigate laser to region of image with material.
        - Click “Start”.
        - Check peaks: specify what is acceptable (intensity). If peaks are above 200 AU, click “Add”.
        - Navigate to other region and push start again and check peaks and so on.
        - When 240 is reached, you can click “Resume run” (but you can add more also).
        - The machine will go to the next samples, pause again and repeat all steps as described above.
9. Remove the target plate and always (!) put another target plate in (because the machine gets damaged without a plate for too long, if you don’t have another, routine lab has standard one next to machine to put in)

    If you remove your plate and put in another plate (see 1-2) **push the green button**!

6.5. Exporting data and of identification scores.

1. To open your run: select on the computer next to the MALDI-TOF machine in the MBT Compass home field, under "Latest ID runs", your run ID, e.g. 210915-1614-10103719.
2. Select "Report" in MBT compass
3. Adobe PDF viewer opens the file "Bruker Maldi Biotyper Identification Results" and can be saved on a USB stick. Put USB in the computer.
4. Select "File" and choose: "Save as". Save the page as "YYYYMMDD_LBR_Name" on the USB stick.
5. When you leave, always select "Home" (don't close the programs) and NEVER shut down the computer!

6.6. Interpreting results

According to the criteria recommended by the manufacturer, all identifications are reported with the following output score values:

Range	Interpretation	Remark
2.00 – 3.00	High-confidence identification	secure species identification
1.70 – 1.99	Low-confidence identification	identification to the genus leave
0.00 – 1.69	No organism identification possible	identification not reliable

As a rule of thumb, when the difference between the score of the first identification and the score of the first following identification, other than the first one, is > 0.2, the identification can be regarded as probable to the species level.
The same rule of thumb can be handled with prudence for red scores.

6.7. Typing

6.7.1. Creating spectra for typing experiments (Routine lab 2P8)

1. For each strain of your typing experiment, prepare eight spots of a column (= technical replicates) with the same MALDI-TOF extract, let dry, and cover with matrix as described in "6.3. Ethanol/formic acid method".
2. On the MALDI-TOF MS, push the green open/close button (long enough until you hear the machine start) to open the lock.
3. When the green light lights up (next to "Access"), the lock can be opened. Place the target plate in the MALDI-TOF MS, gently close the lock, and push again shortly the same button. A project can only be started when the green light lights up.
4. Go to the FlexControl software and click on "New" in the "AutoXecute" tab. Select "MSP96" in the next window and continue by clicking on "Next". In the next window click on "Cancel". Answer "Yes" on the warning pop-up window. A window appears showing a 96-spot sample setup window.
5. In the field "data directory", choose the appropriate folder under the folder "D:/Data/LBR/". Make one folder per species.

   Biological replicates (= cultures of the same origin and analysis comparison) and **technical replicates** (identical extracts analyzed on different timeslots) need to be named by strain taxonomic level otherwise the already present strain is excluded from Bionumerics (strains named identically are not possible).

Biological replicates: strainnameBR

Technical replicates: strainnameTR

6. Select all spots from a column by dragging the cursor around these spots or by clicking the column number (A to H). The spots of the column will be selected by a blue frame around every selected spot.
7. In the top right corner in the field "Sample name", name the selected spots as "YYYYMMDD_species_strainname". Raw files will automatically be named: "YYYYMMDD_species_strainname_0_A1_1.txt" by the software.
8. Click on the green "+" to add the sample name to the complete column.
9. Repeat steps 6 to 8.
10. Save the projects as "YYYYMMDD_LBR_species name" under the folder "D:/Data/LBR/". Click on "Save".
11. Select "OK".
12. In the "AutoXecute" tab, click on "Start Automatic Run". The laser will warm up (this can take up to a few minutes) and the spectra will be generated.

6.7.2. Exporting the spectra (Routine lab 2P8)

1. Make sure "ExportSpectrumSampleNameTXT.FAMSMETHOD" is installed on the MALDI-TOF MS computer. If not, location of the file: …\Dropbox\LBR_SOPs\MALDI-TOF\Script export spectra\ExportSpectrumSampleNametxt.FAMSMETHOD.
2. In FlexControl go to "Compass" and choose "FlexAnalysis" or open the FlexAnalysis software via the desktop shortcut.
3. Go to "Process" and choose "Batch process".
4. Go to "Folder…" and "Select all", choose "Open".
5. Under "Methods", next to "MS Method", click on "Browse". Select "ExportSpectrumSampleNametxt" and continue by clicking on "OK".
6. Click "Start". Wait for the process to finish.
7. Browse to the folder "D:/Data/LBR/". Use the search function with the filter ".txt" files selected to find your .txt files. Copy the files on an USB stick and place in the appropriate folder.

6.7.3. Creating your own database (Routine lab 2P8)

1. Open the FlexAnalysis software, open your files in the browser, select all spectra, and click on "Open". All spectra of the species will open in the FlexAnalysis software.
2. Select all spectra and click on "Baseline subtraction" and on "Smooth".
3. Click on the floppy disc icon to save and close the FlexAnalysis software. Click "No".
4. Open the "Maldi Biotyper Compass Explorer" software.
5. Open the files and click on "Add spectrum", choose the appropriate folder under the folder "D:/Data/LBR/", select all spectra and click on "Open".
6. Select all spectra, click on the right mouse button and choose "Create MSP". In the field "New name", enter the species name + MSP and click on "OK".
7. The spectra are now in the MSP database of the "Maldi Biotyper Compass Explorer" and can be used for identification of unknown species.

7. Literature and support

Wieser et al. 2012. MALDI-TOF MS in microbiological diagnostics—identification of microorganisms and beyond (Mini Review). Appl Microbiol Biotechnol 93:965–974.

8. Version history

SOP: Protocol identification and typing of bacteria and yeast with MALDI-TOF MS (v1.0)	
Formulated:	Piet Cools
Verification:	Leen Van Simaey
Approved:	Mario Vaneechoutte, 12102016.
Adaptation:	Piet Cools. Paragraph 6.3-5 was added; Tessa Gryp. Paragraph 6.6.3 was added.
SOP: Protocol identification and typing of bacteria and yeast with MALDI-TOF MS (v1.1)	
Adaptation, date	Piet Cools, 12042018. Paragraph 6.6. on the typing application was added.
Verification, date	Leen Van Simaey, 12042018.
Approved, date	Mario Vaneechoutte, 2042018.
SOP: Protocol identification and typing of bacteria and yeast with MALDI-TOF MS (v1.2)	
Adaptation, date	Tessa Gryp, 12052019; Paragraph 6.6.3. on the creation of database was added
Verification, date	Leen Van Simaey, 12052019.
Approved, date	Mario Vaneechoutte, 12052019.
SOP: Protocol identification and typing of bacteria and yeast with MALDI-TOF MS (v1.3)	
Adaptation, date	Nick Versmessen, 210705. Paragraph "6.1.4. Cleaning of the target plates".
Verification, date	Leen Van Simaey, 28022022.
Approved, date	Piet Cools, 16082021.
SOP: Protocol identification and typing of bacteria and yeast with MALDI-TOF MS (v2.1)	
Adaptation, date	Leen Van Simaey, 220228. Added in 6.2 how to register sample names in csv file. Added in 6.4 how to enter sample names in the MALDI-TOF MS computer.
Verification, date	Leen Van Simaey, 220228.
Approved, date	Piet Cools, 21March2022