examples of suitable and unsuitable amounts of Gram-negative bacteria. **Row A**: ideal amount of biological material (between 10^4 and 10^7 cells per sample position); **Row B**: small but nevertheless sufficient amount of biological material; **Row C**: too much biological material.

Figure C-1 Ideal (row A) and suboptimal (rows B and C) amounts of biological material on a MALDI target plate

- If HCCA matrix solution is not added to samples within 30 minutes after they have dried, these positions cannot be tested.

After preparation of MALDI target plates

- Prepared MALDI target plates must be measured within 24 hours of preparation. If more than 24 hours have elapsed since preparation, the MALDI target plate must be cleaned and the sample preparation procedure repeated.
- After incubation, culture plates can be stored for up to 12 hours at room temperature. Storing culture plates in a refrigerator will adversely affect the quality of spectra.

C.9.2 Culturing samples

C.9.2.1 Culture media

The following isolation culture media have been tested for culturing samples to be identified using the MALDI Biotyper:

- Columbia blood agar with 5% sheep blood
- Trypticase soy agar with 5% sheep blood

- Chocolate agar
- MacConkey agar
- Columbia CNA agar with 5% sheep blood
- Brucella agar with 5% horse blood
- CDC anaerobe agar with 5% sheep blood
- CDC anaerobe 5% sheep blood agar with phenylethyl alcohol
- CDC anaerobe laked sheep blood agar with kanamycin and vancomycin
- Bacteroides bile esculin agar with amikacin
- Clostridium difficile agar with 7% sheep blood
- Sabouraud-dextrose agar
- Brain-heart infusion agar
- Campylobacter agar with 5 antimicrobics and 10% sheep blood
- Bordet-Gengou (BG) agar with 15% sheep blood

C.9.2.2 Culturing bacteria

Generally, bacterial cultures should be incubated for 18–48 hours at 37°C ±2°C.

Species-specific culture conditions:

- *Bordetella sp.*: Incubation on BG agar should not exceed 24 hours.
- *Campylobacter sp.*: Incubation can be prolonged for up to 72 hours.
- *Streptococcus pneumoniae*: To prevent autolysis, incubation should not exceed 24 hours.

After incubation, culture plates can be stored for up to 12 hours at room temperature. If more than 12 hours have elapsed since the culture plate was removed from the incubator, subculture the bacteria before starting MALDI Biotyper testing.

C.9.2.3 Culturing yeasts

Yeast cultures should be incubated for 18–48 hours at 29°C ±2°C.

After incubation, culture plates can be stored for up to 12 hours at room temperature. If more than 12 hours have elapsed since the culture plate was removed from the incubator, subculture the yeasts before starting MALDI Biotyper testing.

C.9.3 Direct transfer sample preparation procedure

Required reagents

- Dissolved BTS[1]
- Dissolved HCCA[1]

►►Direct transfer sample preparation procedure

1. For each sample, smear an isolated colony as a thin film directly onto a sample position using a sample applicator.
2. Deposit 1 µL of BTS onto each of the assigned **BTS QC** positions. Dry the spots at room temperature.
3. Overlay each sample position and **BTS QC** position with 1 µL HCCA matrix solution.

   Use a new pipette tip for each sample position to avoid cross-contamination.

   To minimize solvent evaporation, make sure that the screw cap tube containing matrix solution is tightly closed after use.
4. Air-dry the spots at room temperature.

   Make sure that spots are well-separated from each other and that none of the spots has bled into a neighboring position.

   If bleeding occurs on a reusable MALDI target plate, the MALDI target plate must be cleaned carefully as described in section C.6 and the entire sample preparation must be repeated.

   If bleeding occurs on a disposable MALDI target plate, the affected positions must be disregarded and sample preparation must be repeated on unused positions or a new disposable MALDI target plate.

[1] *Prepared as described in the relevant Instructions for Use.*

5. Load the MALDI target plate into the mass spectrometer.

C.9.4 Extended direct transfer sample preparation procedure

If MALDI Biotyper identification of the microorganism using the direct transfer sample preparation procedure does not result in a high-confidence identification with log(score) ≥2.0, testing can be repeated using the following extended direct transfer sample preparation procedure.

Alternatively, testing can be repeated using the extraction procedure in section C.9.5.

Required reagents

- Dissolved BTS[1]
- Dissolved HCCA[1]
- 70% formic acid
  - Transfer 300 µL of HPLC-grade water into a 1.5 mL Eppendorf tube. Carefully add 700 µL formic acid. Close the tube tightly and mix by inverting.

►►Extended direct transfer sample preparation procedure

1. For each sample:
   - Smear an isolated colony as a thin film directly onto a sample position using a sample applicator.
   - Overlay the sample position with 1 µL 70% formic acid.
   - Air-dry sample positions at room temperature.
2. Deposit 1 µL of BTS onto each of the assigned **BTS QC** positions. Dry the spots at room temperature.
3. Overlay each sample position and **BTS QC** position with 1 µL HCCA matrix solution.

   Use a new pipette tip for each sample position to avoid cross-contamination.

[1] *Prepared as described in the relevant Instructions for Use.*

To minimize solvent evaporation, make sure that the screw cap tube containing matrix solution is tightly closed after use.

4. Air-dry the spots at room temperature.

   Make sure that spots are well-separated from each other and that none of the spots has bled into a neighboring position.

   If bleeding occurs on a reusable MALDI target plate, the MALDI target plate must be cleaned carefully as described in section C.6 and the entire sample preparation must be repeated.

   If bleeding occurs on a disposable MALDI target plate, the affected positions must be disregarded and sample preparation must be repeated on unused positions or a new disposable MALDI target plate.

5. Load the MALDI target plate into the mass spectrometer.

C.9.5 Extraction sample preparation procedure

If a high-confidence identification with a log(score) ≥2.0 is not obtained for samples prepared using the direct transfer or extended direct transfer sample preparation procedure, testing can be repeated using the following extraction sample preparation procedure.

Required reagents

- Dissolved BTS[1]
- Dissolved HCCA[1]
- HPLC-grade water
- Absolute ethanol
- Acetonitrile
- 70% formic acid
  - Transfer 300 µL of HPLC-grade water into a 1.5 mL Eppendorf tube. Carefully add 700 µL formic acid. Close the tube tightly and mix by inverting.

[1] *Prepared as described in the relevant Instructions for Use.*

►►Extraction sample preparation procedure

1. Transfer 300 µL of HPLC-grade water into an Eppendorf tube.
2. Using a 1 µL inoculation loop, transfer isolated colonies from the culture plate into the water and mix thoroughly until the material is completely in suspension.

   Alternatively, the recommended Eppendorf micropestle can be used to generate a homogenous suspension.
3. Add 900 µL pure ethanol and mix the suspension.
4. Spin down the microbial material in a bench-top centrifuge for two minutes at 13,000 – 15,000 rpm.
5. Remove the supernatant using a pipette (avoiding contact with the microbial material).
6. Repeat step (4) and remove residual ethanol by pipetting (avoiding contact with the microbial material).
7. Air-dry the pellet for at least five minutes at room temperature.
8. Add 25 µL 70% aqueous formic acid and pipette the solution up and down until the pellet is resuspended.
9. Add 25 µL 100% acetonitrile to the tube and mix by pipetting the solution up and down two or three times.
10. Centrifuge the tube for two minutes at 13,000 – 15,000 rpm.
11. Deposit 1 µL of the supernatant onto a vacant sample position of a cleaned MALDI target plate.

    Use a new pipette tip for each sample position to avoid cross-contamination.

    Note *Sample extracts can be stored for up to 4 hours at room temperature before use. If more than 4 hours have elapsed since extraction, repeat the extraction procedure using fresh samples.*
12. Air-dry the MALDI target plate at room temperature.
13. Deposit 1 µL of BTS onto each of the assigned **BTS QC** positions. Air-dry the spots at room temperature.
14. Overlay each sample position and **BTS QC** position with 1 µL HCCA matrix solution.

Use a new pipette tip for each sample position to avoid cross-contamination.

To minimize solvent evaporation, make sure that the screw cap tube containing matrix solution is tightly closed after use.

15. Air-dry the spots at room temperature.

    Make sure that spots are well-separated from each other and that none of the spots has bled into a neighboring position.

    If bleeding occurs on a reusable MALDI target plate, the MALDI target plate must be cleaned carefully as described in section C.6 and the entire sample preparation must be repeated.

    If bleeding occurs on a disposable MALDI target plate, the affected positions must be disregarded and sample preparation must be repeated on unused positions or a new disposable MALDI target plate.

16. Load the MALDI target plate into the mass spectrometer.