WCSB, Wageningen University	IBISBA-SOP-WU17
	Version 1.0

EPP - Standard Operating Procedure

(only for selected experiments intended to transfer results from one lab to the other)

Title: *Inoculating and sampling anaerobic cultures and CFU determination*

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Instruction

Inoculating and sampling anaerobic cultures and CFU determination

1. Introduction / Purpose

This protocol described the inoculation and sampling of anaerobic cultures. It also explains how to measure colony forming unit (CFU). This protocol is especially suited for *P. putida* and *E. coli*, since it makes use of pre-cultures that were grown aerobically.

Keywords: Anaerobic – inoculation – sampling - CFU

2. Equipment and chemicals

2.1. Equipment

- Gas exchanger (N_2/CO_2)

2.2. Chemicals

- Liquid culturing medium: Anaerobic (see Methods)
- Resazurin, Sigma-Aldrich: R7017

  L-cysteine, Sigma-Aldrich: C7352

2.3. Bacterial strains

The protocol has been applied to *Pseudomonas putida* KT2440 and *E. coli*.

2.4. Other materials

S*pecial consumables*

- Glass anaerobic crimp cap vials/bottles with volume of choice (can be filled with medium to only half of the total volume)
- Crimp caps / rubber stoppers
- Empty anaerobic crimp cap vials/bottles with N_2/CO_2
- 3 ml syringes (Thermo Fisher Scientific, Waltham, MA USA)
- 1.5” needles (BD Microlance)

3. Media and Buffers

- The media used for culturing anaerobically should contains the following:
- 0.75 g/l L-cysteine (Optional, advised for larger volumes)

  0.001 g/l resazurin

4. Procedures

Preparation of anaerobic medium

- The glass crimp cap vials/bottles for the cultures and medium components are prepared with water and the medium components will be added later to the anaerobic medium (rich medium like LB can be made completely and then gas exchanged as a whole).
- The anaerobic vials/bottles are closed by rubber stopper and crimp cap.
- Gas exchange the anaerobic vials/bottles with CO_2/N_2 by gas exchanger.
- The anaerobic vials/bottles are autoclaved.
- The rest of the (now anaerobic) medium components are added to the culture vials/bottles (only with minimal medium). The medium components are added with sterile N_2/CO_2 flushed syringes+needles through the rubber stopper.
- Between each addition or sampling, the anaerobic vials/bottles are sterilized using ethanol and flame.
- The anaerobic medium is likely still resazurin pink. This is needed for the first phase: aerobic growth until all oxygen is depleted to gain biomass and ensure full anaerobic conditions.
- The vials/bottles are now ready for inoculation. The anaerobic sterile medium can be stored for quite a long time at room temperature.

Anaerobic inoculation

- Inoculation is done with aerobically pre-cultured bacterial sample.
- Inoculate with a starting OD of ca. 0.1. using needle and syringe.
- Approx. 8 h after inoculation and strain growth, the resazurin will be completely colourless, indicating fully anoxic conditions.
- Anaerobic cultures are incubated in a non-shaking cabinet

Sampling

- Samples are taken using sterile CO_2 flushed 1.5” Needles (BD Microlance) and 3-5 ml syringes (ThermoFisher) to avoid O_2 exposure.

- Anaerobic conditions can be confirmed as the resazurin turns from colourless to bright pink within seconds in extracted samples.

- Extracted samples can be flash-frozen in liquid nitrogen for metabolomics, stored at -20˚C for sequencing or used directly for further analysis (e.g. CFU determination).

CFU determination

Survival rates are analysed by colony forming units (CFU) determination on the extracted samples.

- A 96-wells plate is prepared with 90 µl medium without antibiotics in all wells except the first column.

- The extracted sample is inserted into the first well of the first column.

- Using a 10 µl pipette, a 10x dilution series is made per row of the 96 wells-plate (10^0 – 10^{-7}).

- Per sample, five drops of 10 µl per dilution are applied onto 2 LB-agar plates without selection marker.

- The plates are incubated o/n.

- Colonies are counted manually per droplet the following day.

- Photos can be taken of the plates.

- Results can be analysed with Excel.

- Gram-staining on the plates can be performed to ensure culture purity, according to manufacturer's instructions.

5. Remarks / troubleshooting

- Depending on the purpose of the experiment you might want to start with some oxygen in your anaerobic vials or have them completely anaerobic. For this, a few things can be done:

- You can add (anaerobic start) or leave out (oxygen start) the L-cysteine

- You can add the medium components of minimal medium without preparing them in anaerobic vials to ensure presence of oxygen in the medium.

- Smaller volume (50 ml) anaerobic vials are easier to get anaerobic completely than 500 ml anaerobic bottles.

- It is advisable to always gas exchange your anaerobic vials: there is much oxygen left in the headspace and it takes really long to ensure headspace oxygen depletion.

6. Biosafety

During gas-exchange, bottles can break due to overpressure. Be careful and never leave the equipment unsupervised while gas-exchanging.

7. Acknowledgements


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