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Anaerobic Media Preparation Protocol

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1 Works for me Share This protocol is published without a DOI.

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ABSTRACT

This is a generic protocol for the preparation of *any* strictly anaerobic media. The exact media composition is assumed to be known by the user of this protocol (for example, we routinely use this protocol together with the media sheet; OSM01_01, available from OSS Lab website).

ATTACHMENTS

Anaerobic_Media_Preparation_Protocol.pdf

PROTOCOL CITATION

Orkun Soyer 2021. Anaerobic Media Preparation Protocol. **protocols.io** https://protocols.io/view/anaerobic-media-preparation-protocol-bpq4mmyw

KEYWORDS

anaerobic media, anaerobic media preparation

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GUIDELINES

General remarks:

Within this document, we assume the media composition consists of the following parts; a **salt mix** in solid or liquid state, a **buffer solution** (e.g. bicarbonate), a **vitamin solution, Cysteine-HCl and Na2S** (as oxygen scrubber), **organic carbon source**, and **Rezasurin** (as redox indicator).

This protocol is for the preparation of 1 L. For smaller volumes, scale accordingly. Steps that are performed differently for the use of round neck flasks are indicated by italicized text.

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MATERIALS TEXT

Equipment

This protocol requires the following:

- A gassing manifold, including long, wide-bore, gassing canula
- Water bath, heating plate, and/or microwave
- pH and redox meter
- Anaerobic chamber

Glassware

- The procedure can be done in 500 mL long-neck round flasks, in which case 200 mL of medium should be prepared, or in 1 L Duran-Flasks for the preparation of 1 L of medium.
- Hungate tubes, used for dispensing the media into. Ideally, media should be transferred immediately to Hungate tubes, but can also be stored in the media vessel.

SAFETY WARNINGS

Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

BEFORE STARTING

Any material that is needed in the anaerobic chamber (Hungate tubes, stoppers, pipettes etc.) should be put in place the day before to allow degassing (i.e. “deoxygenation”) of the equipment overnight.

Prep. and mixing stage

1 In **900 mL milliq-H2O** dissolve the **salt mix** of the medium, while stirring the liquid on a stirrer plate.

This dissolved salt mix can be stored without any problem (e.g. contamination would not be expected as it does not contain any carbon source, most salts should be stable, etc.)

2 Add the **buffer solution** (e.g. $NaHCO_3$, Na_2CO_3).

3 Add the **trace metal solution.**

4 Add the **carbon source** (as defined by your media sheet and/or microorganism).

Depending on the nature of the C-source, you might want to add it after the degassing stage. For example, methanol, that can be used for M.barkeri, could have high volatility and be lost during degassing.

5 Add [M] **1 mg/ml Resazurin** , an indicator of redox level, if it was not already contained in the **salt mix** or **buffer solution.**

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6 Fill up to **1 L** with milliq-H_2O. Make sure you stop stirring, while topping up to **1 L** , as the stirring can cause you to misjudge the volume.

7 Place media flask on a pH-meter and adjust the pH with HCl / NaOH (or KOH in case of sodium-free media) to the level required by the specific media.

Once the pH is adjusted, we are ready for the degassing stage. At this point, the 1L media can be split into batches of smaller volume as desired. For example, degassing stage can be quicker for a smaller volume. Also, a 200ml batch can be used to fill 40 Hungate tubes with 5ml each.

Heating and degassing stage

8 Heat media close to boiling point but try to avoid boiling (as this would increase the loss of solvents). The heating step can entail;

- Placing the flask into a water bath that has been heated to **80 °C** (works well for Duran flasks).

- *Round neck flasks might be easier to heat on a hot-stirrer plate that has reached the* **100 °C** *heat-marking.*

Option: Place the media in the microwave with a stopper and bring it just below boiling temperature prior to placing it in water bath or hot stirrer plate. This can save time. Experience shows that there are no detrimental effects of microwave treatment, but note that this might change with specifics of the media composition. If using microwave, a guide is to use 1 minute per 100ml of media.

IMPORTANT: It must be noted that heating in the microwave can generate a vacuum in the flask, pulling in the stopper. To avoid the stopper being fully pulled in, make sure you loosen it well before putting the flask in the microwave and also pull the stopper out immediately when you get the flask out of the microwave– note that the flask will be hot!

9 Bring the heated flask next to the gassing manifold and set it on a heating/stirring plate.

10 Insert a redox and temperature probe into the media and the gas-line into the headspace. Secure a stopper in place, to minimize gas exchange between flask and atmosphere.

Option: The use of redox and temperature probes should be optional, however note that the use of Resazurin as redox indicator provides only an estimate of the success of degassing. Resazurin goes colourless at a redox value of -51mV and can interact with certain organics. Having a redox probe would provide an exact measure of redox and hence more information. In our experience, Rezasurin remaining pink correlates well with a media that will not support anaerobic growth but this might change with specifics of media and species. In general, we recommend use of redox probe where possible.

11 Turn on the gas cylinder and start gas flow at 1 liters per minute (LPM) using the appropriate gas mixture with your gassing manifold. As a rule of thumb, when the medium contains bicarbonate or carbonate, use a N_2/CO_2 (80/20)

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mixture, otherwise use 100% N_2.

A gas flow of 0.5 LPM will be sufficient for 200 mL in round-neck flasks. The gassing setup is shown in Picture 1.

Picture 1. Left: The degassing setup with a canula feeding gas into a 500 mL round-neck-flask on a heated stirrer. Note that before the addition of Cysteine- HCl and heat, the resazurin is actually a blue colour. Right: Degasing setup for 1L Duran flasks in a water bath. Image credits: Simone Zenobi.

12 When the medium has reached 80° C, push the gas-line down into the liquid, so it bubbles through the medium. If in step 10, no temperature probe was used, use the water bath or heating plate temperature and the time that the media spent on the heating device as a proxy to judge media temperature.

Note that pushing the gas-line into the liquid would not be possible for 200 mL in round-neck flasks, unless your gas-line/canula is long enough.

13 After **00:05:00** - **00:10:00** degassing at **80 °C** , turn off the heating system and add the **vitamin solution**, and **Cysteine-HCl** (it is assumed here that there are no additional compositional elements but this might change with the specifics of your media). 15m

14 Continue gas flow, while the medium is cooling and until a redox value of - 300 mV or less is reached AND the media turns colourless, i.e. the Resazurin dye shifts from pink to colorless.

15 Remove any probes that are still in the flask. This might cause the media to turn pink. In that case, continue degassing a bit more.

16 Remove the gassing line (while gas is still flowing), by pressing the stopper in place and carefully pulling the gas line (while minimizing any chance of air flow into the flask). Immediately push the stopper fully in place and tape it secure (this is important as moving into the anaerobic chamber in the next step might cause the stopper be displaced due to pressure changes).

Dispense and finalise stage 30m

17 Move the flask into the anaerobic chamber air lock and start the gassing sequence without any delay.

18 Once the airlock cycle is finished, move the flask into the anaerobic chamber. Ideally, you should let the flask in the chamber and wait a bit (**00:10:00** - **00:20:00**) before opening it. This would allow the anaerobic chamber to recover/equilibrate from the airlock sequence and reach low O_2 levels again. 30m

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19 If you have a hand-held probe in the chamber, open the flask and measure the pH and redox of the media and **record it in your lab book**.

20 Dispense medium into Serum flasks or hungate tubes as needed. If media shows any precipitation, place it on a stirrer during dispension, so to ensure homogenous media.

21 Seal tubes with butyl-rubber septa.

22 Place any remaining medium in a 1L Duran flask sealed with rubber butyl septa. **Label the flask with your initials, date, and the pH and redox measured in step 19**.

23 Take media out of the anaerobic chamber and place aluminium crimp seals on the tubes you used.

24 Autoclave media placed in flasks/tubes in step 20, as well as media to be stored (from step 22).

25 Store in the dark and at **Room temperature** .

IMPORTANT NOTE: Before using media for inoculation, the final component; Na_2S should be added. This component is used for additional reducing of redox potential and also as Sulfur source. Its addition at this late stage of inoculation is because it is quite unstable and not ideal for storage. When adding Na_2S, make sure you use the right amount for your culture volume (and given your stock solution concentration).

26 Any media that is older than 8 months should be discarded. Any stored media younger than 8 months can be used provided it has maintained its pH and redox in the required range.

27 If possible, measure pH and redox in the anaerobic chamber before using any stored media. If the measurements are not within range (redox, below -300mV and pH, as defined by specific media) discard media and prepare new one. Do not attempt adjusting these values with media additions.

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