Special Instructions

Cultivation of Anaerobes

The DSMZ holds a large collection of bacteria and archaea that thrive only under anaerobic conditions. In our experience beginners in culturing anaerobes or extremophiles encounter often difficulties in handling these cultures appropriately. This technical information should help everybody who is interested to start working with anaerobes. Please read it carefully, it will answer most frequently asked questions about culturing anaerobes!

You will find on this page information to the following topics:

General information about anaerobes

In the broadest sense **obligate anaerobes** can be defined as microorganisms which are unable to utilize molecular oxygen for growth. A further differentiation is possible based on their relationship to the presence of oxygen. Aerotolerant anaerobes are only slightly inhibited by significant levels of oxygen in the atmosphere. For instance *Clostridium intestinale* DSM 6191^T can grow well on the surface of agar plates incubated in air at atmospheric pressure.

The other extreme is represented by **strict anaerobes**, which die, or immediately stop growing, upon exposure to low levels of oxygen. It is therefore important to retain anoxic conditions during all steps of handling of these microorganisms. Most strict anaerobes require not only the absence of oxygen to initiate growth, but also a redox potential below -300 mV, which can be only achieved by the supplementation of media with reducing agents (see section on *Reducing agents and resazurin*). Between both extremes all kinds of adaptation exist.

The majority of anaerobic microorganisms are fastidious and require complex media with many supplements.

In the DSMZ catalogue of strains (https://www.dsmz.de/catalogues/catalogue-microorganisms.html) each DSM strain is linked with a specific medium number. It is strongly recommended to use the respective media formulations given for each strain, because only those media were tested at the DSMZ for culturing and a transfer to alternative media may cause a delay or complete failure of growth.

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Before ordering an anaerobe from the DSMZ it is advisable to have a look on the recipe of the medium necessary for growing this strain and to read relevant publications dealing with its cultivation.

It only makes sense to purchase a strain of interest, if you are convinced to be able to handle it properly!

A large number of strict anaerobes are available from the DSMZ only as **actively growing cultures**. We recommend using the **Hungate technique** to culture these strains. Some general remarks on this cultivation technique and required laboratory equipment follow below. Excellent descriptions of the Hungate technique can be found in the reviews of Hungate (1969) and Wolfe (1971), whereas the contribution of Breznak and Costilow (1994) contains more general information on anaerobiosis. However, please keep in mind, that even if described in detail, some steps of the handling of anaerobic cultures are frequently difficult to master without demonstration. For beginners in anaerobic microbiology it is therefore the best to visit a laboratory where anaerobic cultivation techniques are routinely in use.

Anaerobic strains that are available from the DSMZ as **lyophilized cultures** are normally not sensitive to a short exposure to low oxygen concentrations (**non-stringent anaerobes**). For instance, a majority of the clostridia and sulfate reducers, but not all of them, belong to this group of strains. If you have received an ampoule from the DSMZ with a vacuum dried sample of a non-stringent anaerobe please read also the instructions given in the section: *Handling of vacuum-dried anaerobic cultures*.

Further special instructions on difficult to handle microorganisms, like methanogens or hyperthermophiles are available at the DSMZ web pages.

Recommended vials for culturing strict anaerobes

Suitable containers for pre-reduced media are an important prerequisite for the successful culturing of strict anaerobes. For this purpose special glassware has been developed which enables the easy use of completely gas-tight closures. Of crucial importance is the material of the rubber stoppers. Only stoppers or septa made of **butyl rubber** can efficiently prevent permeation of air into the vial. Nevertheless, a repeated puncturing of stoppers with injection needles could make them become permeable to oxygen. As a rule, the thicker the stopper the more often it is possible to reuse it without loss of impermeability.

Two types of vials are commercially available for anaerobic culturing (Fig. 1):

The Hungate-type tubes are closed with a flange-type butyl rubber septum and a screw cap with 9 mm opening to allow puncturing of the septum with injection needles.

Balch-type tubes are more stable than Hungate-type tubes and recommended if an overpressure of 2 to 3 bar can be expected during culturing. They are closed with a thick butyl rubber stopper which is hold in place by sealing with an **aluminium crimp**. For sealing and removing of the aluminium crimp special devices (crimper/decapper) are necessary.

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Serum bottles which are available in various sizes can be used alternatively to Balch-type tubes. However, serum bottles are less stable than Balch tubes and should be handled with special care when strains are cultured that are expected to produce significant amounts of gas during incubation (see below).

Pre-reduced media can be stored in both types of vials at room temperature in the dark for several weeks without becoming oxidized.

Caution: Some microorganisms produce a considerable amount of gas during growth (e.g., clostridia by fermentation). The formed gas can lead to a substantial overpressure during growth in closed vials. Strains which are known to accumulate gas during growth should be incubated in vessels that are filled only up to 25% with liquid medium. In addition, cultures of fast growing strains should be vented at least on a daily basis to avoid overpressure. Wear protective goggles during handling of glass vessels that might have overpressure!

Suppliers of commercially available glassware and accessories for anaerobic culturing are for instance **Bellco Glass Inc.** (http://www.bellcoglass.com/) and **Ochs GmbH** (http://www.glasgeraetebau-ochs.de/).

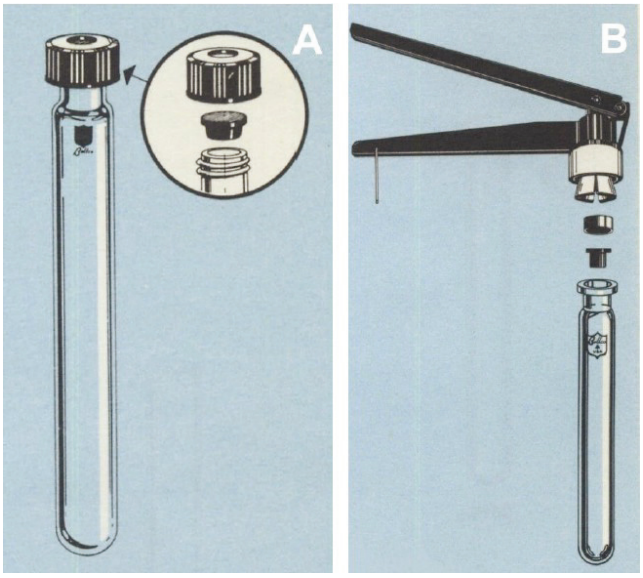

Fig. 1 Suitable vials for culturing strict anaerobes.

(A) Hungate-type tube with screw cap and butyl rubber septum.

(B) Balch-type tube with butyl rubber stopper and aluminium crimp seal to hold stopper in place. A crimper is necessary for sealing the vial.

Figures are courtesy of Bellco glass Inc.

Gassing of media and cultures with oxygen-free gas

When vials of pre-reduced media or anaerobic cultures are opened a constant flow of oxygen-free gas over the surface of the medium is necessary to avoid exposure to oxygen. The used oxygen-free gas should have the same composition as that used for medium preparation. We recommend to use oxygen-free gasses of high purity (containing less than 5 ppm oxygen), that are delivered as **compressed gas cylinders**. Normally, oxygen-free gasses of high quality do not require an additional oxygen removal system (e. g., heated copper column) and can be used directly for culturing a broad spectrum of anaerobes. Suppliers of compressed gasses are for instance Messer Griesheim GmbH (http://messergroup.com) or Linde AG (http://www.linde.com).

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The Hungate technique is based on the use of **gassing cannulas**. Usually, several cannulas are connected by butyl rubber tubing (e.g., Iso-Versenic) to a manifold supplying oxygen-free gas with an overpressure that should be adjusted to approx. 0.5 bars. At least two cannulas are needed: one for the vessel to be inoculated or filled with medium and one for the vessel containing the inoculum or the medium to be dispensed. When an aseptic gassing of media or cultures is necessary a barrel of a glass syringe is packed with cotton and fitted between the gassing needle and the butyl rubber tubing (Fig. 2A)

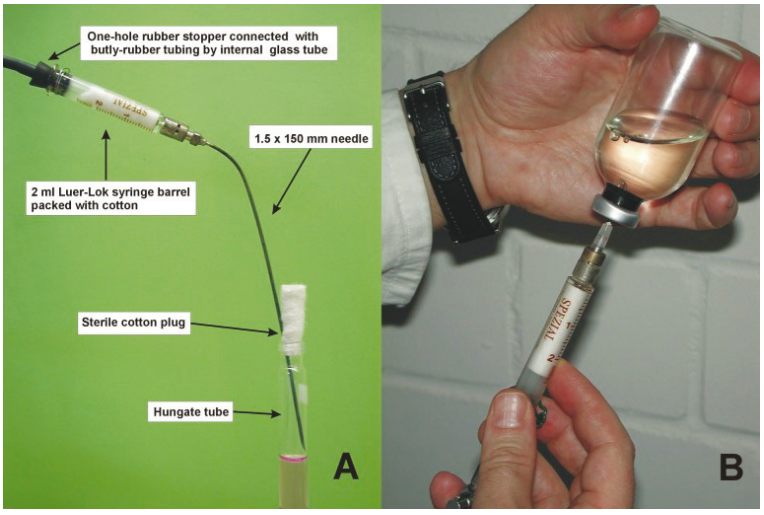

Fig. 2 Assembly of cannulas used in the Hungate technique for aseptic gassing.

(A) Cannula used for aseptic gassing of opened vials with oxygen-free gas.

(B) Overpressurizing of anaerobic cultures with sterile gas mixtures.

After assembly, autoclave the cotton-filled glass syringe and needle, dry in a drying oven at 100°C, allow to cool, and connect to the manifold. Prior to the first use flush the gassing cannula for approx. 15 min with oxygen-free gas to make it anoxic and then flame the needle to sterilize it.

Caution: Make sure that needles sterilized by flaming are cooled down prior to using gas mixtures containing H_2. Hydrogen gas is highly combustible and even only contact with hot surfaces may cause ignition. Wear protective goggles while overpressurizing vials.

For the **overpressurizing** of cultures with H_2 or H_2/CO_2 gas mixtures use disposable, sterile injection needles (i. d. 0.4 mm or 27G) connected to cotton-filled glass syringes as described above. To keep the pressure within the glass syringe barrel at a constant level during overpressurizing it is necessary to avoid an imbalance between the inflowing gas stream and the outflow. This can be achieved by puncturing the rubber stopper of the cotton-filled syringe with a steel needle (approx. 20G) which is connected to the rubber tubing by an appropriate valve with Luer-Lock fittings. Adjust the gas pressure to the desired value (in most cases 0.5 to 2 bar overpressure). Turn the vial with the culture upside down and puncture the sterilized septum with the injection needle (Fig 2B). A sputtering of gas bubbles indicates the inflow of gas into the medium and can be observed as soon as the tip of the cannula enters the liquid. When the flow of bubbles slows down the pressure within the vial reaches equilibrium with the external pressure of the gas supply. Withdraw gassing needle immediately when the gas flow stops.

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Preparation of anoxic media buffered with bicarbonate

Anaerobic microorganisms growing at a circumneutral pH usually require a buffered medium that keeps the pH at a suitable value. Otherwise fermentation products like butyrate could lead to an acidification of the medium, while for example ammonification or sulfate-reduction would lead to an alkalinisation.

Media prepared under 100% N_2 gas atmosphere often contain a biological buffer, e.g. HEPES or MOPS, to keep the pH in a suitable range. However, more often a **HCO_3^-/CO_2 buffer** is used, because CO_2 is required by many anaerobic microorganisms for growth. Therefore, if the medium recipe in our catalogue indicates the use of an 80% N_2 and 20% CO_2 or 80% H_2 and 20% CO_2 gas atmosphere, it cannot simply be replaced with a medium containing a biological buffer that has been prepared under 100% N_2 or 100% H_2 gas atmosphere.

In principal two options exist for preparing bicarbonate buffered anoxic media. Sometimes, it is indicated to add solid sodium bicarbonate or sodium carbonate to the anoxic medium prior to autoclaving. In this case you have to check the pH of the medium after the solid bicarbonate or carbonate has been completely dissolved. If the medium pH is above the desired value you have to continue sparging the medium with the CO_2 containing gas mixture until the correct pH value is reached.
In most cases it is however more favourable to add bicarbonate or carbonate to the autoclaved medium from **sterile anoxic stock solutions** prepared under 80% N_2 and 20% CO_2 gas mixture. The amount of bicarbonate or carbonate stock solution that has to be added to the medium depends on several factors, like for example the ratio of liquid medium to gas phase in the cultivation vessel, so that the amounts given in our medium recipes are only rough estimates. However, usually mineral media prepared under 80% N_2 (H_2) and 20% CO_2 gas atmosphere are buffered with **30 - 40 mM $NaHCO_3$** or 15 – 20 mM Na_2CO_3 to reach a pH around 7.0. In order to determine the exact amount of $NaHCO_3$ or Na_2CO_3 stock solution that is required for a distinct pH value, we recommend using one vial with the completed sterile medium and adding small aliquots of the stock solution (e.g., 0.1 ml of a 5% (w/v) Na_2CO_3 stock solution per 10 ml medium. Thereafter, check the resulting pH value by removing a small volume of medium while retaining anoxic conditions. These steps have to be repeated until the desired pH has been reached. The determined amount of stock solution can then be used for all other vials with the same volume of medium.

Reducing agents and resazurin

A **redox sensitive dye** is usually included in media used for culturing anaerobes to monitor the redox potential. The most commonly used redox indicator is resazurin, because it is generally non-toxic to microorganisms and is effective at very low concentrations of 0.5 to 1 mg/l. This indicator dye is dark blue in its inactive form and first has to undergo an irreversible reduction step to resorufin, which is pink at pH values near neutrality (the colour may change to blue under alkaline conditions). This first reduction step normally occurs when media containing an excess of organic nutrients are boiled for a few minutes or mineral media are heated under an oxygen-free atmosphere.
In a second reversible reduction step hydroresorufin is formed which is colourless. The resorufin/hydroresorufin redox couple becomes totally colourless below a redox potential of about **-110 mV** and regains a pink colour at a redox potential above -51 mV.

Please note, that some organisms require redox potentials lower than -110 mV and hence may not start to grow even if the medium is colourless. On the other hand, a pink colour of the medium does not automatically imply that it became oxidized by oxygen (e.g., through a gas permeable rubber septum). For instance, certain nitrate reducers produce nitrite during growth which acts as potent oxidant and so may raise the redox potential above -51 mV.

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Reducing agents are added to most anaerobic media to depress and poise the redox potential at optimum levels. The most common reducing agents are sodium thioglycolate, L-cysteine-HCl x H_2O, Na_2S x 9 H_2O, FeS (amorphous, hydrated), dithiothreitol and sodium dithionite.
Sodium thioglycolate is often used in combination with ascorbate and mainly incorporated in some traditional media for culturing anaerobes (e.g., Postgate's media for sulfate reducers, DSMZ medium 63). **Thioglycolate** as reducing agent has the advantage that it is relatively stable at room temperature and can be therefore included in media prior to flushing with oxygen-free gas. It is only activated by heating above 100°C and then efficiently removes oxygen. Hence, not so much care has to be taken in avoiding exposure to oxygen of the prepared medium prior to dispensing in anoxic vials. For this reason many commercially available media contain thioglycolate as reducing agent. However, the standard redox potential of thioglycolate alone (around -100 mV) is generally not low enough to allow initiation of growth of a majority of strict anaerobes which need highly reduced media.
For maximum effectiveness of reducing agents other than thioglycolate, stock solutions under nitrogen gas should be prepared. While preparing stock solutions of reducing agents avoid insertion of the gassing cannula into the liquid, because this can have negative effects on the reducing capacity. **Sodium dithionite**, which reacts extremely fast with oxygen, has to be dissolved in oxygen-free water and sterilized by filtration. Freshly prepared stock solutions of dithionite can be stored only for up 2-3 weeks at room temperature in the dark.
Add appropriate concentrations of reducing agents to the autoclaved medium just prior to use and allow the medium to sit until it becomes colourless (incubation at 37°C may accelerate this process). If the medium stays pink despite addition of reducing agent exchange the septum of the vial under a flow of oxygen-free gas, because it might have become permeable to oxygen. Finally, add a small amount of dithionite for final adjustment of the redox potential to a value below -300 mV. If this does not help discard the medium tube.

Handling of vacuum-dried anaerobic cultures

The DSMZ delivers lyophilized (freeze-dried) cultures of anaerobic strains exclusively in double-vial preparations, heat-sealed under vacuum. Double-vial preparations have the advantage that a contamination of the atmosphere by aerosols that can be produced by sudden release of the vacuum in single-vial preparations is efficiently prevented. In addition, the cell pellet is protected from contamination, because inflowing air filters through the sterile cotton plug of the inner vial. Before opening of the ampoule please identify the culture by the label on the inner vial which indicates the DSM strain number and date of preservation. Confirm that the ampoule is under vacuum by checking the colour of the desiccant at the bottom of the outer vial.

Note: The DSMZ has changed the indicator stain of the desiccant. From January 2002 on the used desiccant is red, instead of blue. The indicator stain will change its colour if the outer vial is damaged due to an increase of humidity inside the ampoule. The red colour changes to orange and the blue to pink.

It is important to retain anoxic conditions during all steps after the opening of ampoules with freeze-dried anaerobes. This can be achieved in several ways depending on the used anaerobic technique and available equipment in the laboratory. For general information about vacuum dried cultures please visit the following site Handling of cultures and opening of ampoules with freeze-dried material.

The freeze-dried pellet of most anaerobic strains available from the DSMZ is protected against short exposure to oxygen by amorphous ferrous sulfide (FeS) which confers a **black colour** to the pellet. However, certain nonstringent or spore forming anaerobes are suspended prior to lyophilisation in skim milk without addition of FeS, so that the ampoules display a white pellet.

If the **Hungate technique** is routinely used in the laboratory, open the ampoules as described in *Handling of cultures and opening of ampoules with freeze-dried material*.

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After opening keep the inner vial under a flow of oxygen-free gas by inserting a gassing cannula. Add approx. 0.5 ml of the recommended anoxic medium to the vial and resuspend the cell pellet completely (in some cases this may take several minutes). Transfer the cell suspension either by using a 1 ml syringe with hypodermic needle (length at least 38 mm) or a sterile Pasteur pipette, which was made anoxic by flushing with oxygen-free gas. If a Pasteur pipette is used, the Hungate tube containing the appropriate cultivation medium (5 to 10 ml) has to be opened and gassed with a second cannula during transfer of the inoculum. Please also have a look at our video tutorial on the opening of ampoules of anaerobes:

https://www.dsmz.de/support/video-tutorials.html#c2960

If an **anaerobic chamber** is available it is recommended to score the ampoule with a sharp file at the middle of its shoulder about one cm from the tip. Transfer the ampoule with the file mark in the anaerobic chamber and strike the ampoule with a file or large forceps to remove the tip. If necessary, wrap the ampoule in tissue paper and enlarge the open end by striking with a file or pencil, then remove the glass wool insulation and the inner vial. Gently raise the cotton plug and sterilize the upper part of the inner vial using an incandescent flaming device (alternatively wipe the upper part of the inner vial with tissue paper soaked in 70% ethanol). Add approx. 0.5 ml of anoxic medium to resuspend the cell pellet and transfer the suspension to a vial with the recommended cultivation medium (5 to 10 ml).
If possible the last few drops of the resuspended cell pellet should be transferred to an agar plate or slant of the recommended medium to obtain single colonies in order to check the purity of the strain. Anaerobic incubation conditions for agar plates can be achieved by placing plates in an anaerobic chamber or an activated

anaerobic Gas Pak jar or similar system (e. g., **Anaerocult®** bags available from Merck; http://www.merck.de).

We recommend preparing also 1:10 and 1:100 dilutions of the inoculated medium, because some ingredients of the freeze-dried pellet may inhibit growth in the first tube. Inoculation of only one tube may prevent successful resuscitation of certain lyophilized strains (e.g., ***Geobacter*** spp.).
In most cases freeze dried cultures of anaerobic strains exhibit a **prolonged lag period** upon rehydration and should be given at least twice the normal incubation time before regarding them as non-viable.

Handling of actively growing anaerobic cultures

For the aseptical injection and removal of samples from anaerobic cultures it is recommended to use the Hungate technique which is essentially based on the use of **disposable syringes** and has the advantage that it allows the use of defined, oxygen-free atmospheres for culturing. The anoxic removal of a sample from a Hungate tube is demonstrated in Fig 3.
First, sterilize the butyl rubber septum by flaming it using a drop of ethanol which has been placed on the septum. If overpressure in the vial can be expected due to microbial growth (e.g., gas production by fermentation) remove excess gas by puncturing the septum with a sterile injection needle. Then a sterile, disposable 1 ml syringe with a 25G to 23G **hypodermic needle** (i. d. 0.50 to 0.65 mm) is made anoxic by displacing the dead space with sterile oxygen-free gas or a reducing agent.

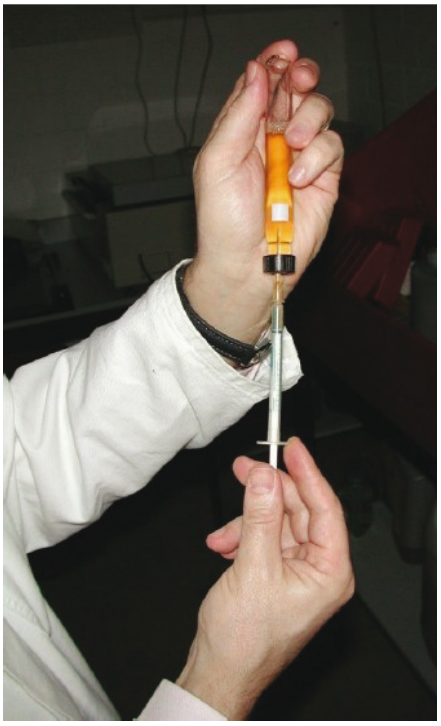

Fig. 3 Anoxic removal of a sample from a Hungate tube:
Penetrate the septum and inject the same volume of oxygen-free gas into the vial as will be subsequently removed as sample from the culture. By doing this the development of an underpressure in the culture tube will be prevented. Then, turn the vial with the culture upside down and fill the syringe with the needed amount of liquid. Finally, withdraw needle and filled syringe carefully.

Literature

Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes, pp. 117-132. *In* J. R. Norris and D. W. Ribbons (eds.), Methods in Microbiology, vol. 3B. Academic Press, New York.
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Notes

1. Abbreviations (excl. chemicals, reagents and measuring units):
   approx.= approximately
   fig= figure
   G= Gauge
   i. d.= inner diameter

2. Red colored information indicates an important subject regarding to the content given herein.

3. The information contained herein is offered for informational purposes only and is based on the present state of our knowledge. Recipients of our microorganisms must take responsibility for observing existing laws and regulations. DSMZ does accept no responsibility for the accuracy, sufficiency, reliability or for any loss or injuryresulting from the use of the information.

4. Have you any questions or comments to this page? Please send an e-mail to the following address: ssp(at)dsmz.de

