

ATCC Medium: 2469 MSH Medium

NaHCO ₃	8.4 g
DI Water.....	976 ml

To prepare the medium dissolve 8.4 g sodium bicarbonate in 976.0 ml of deionized water (100 mM Na₂HCO₃). The solution is bubbled with an oxygen-free gas mixture of N₂-CO₂ (70:30). While gassing, the stock solutions A and B are added:

Solution A (see below).....	10 ml
Solution B (see below).....	2 ml

The high initial pH of this solution may cause the minerals to precipitate. As the pH decreases as a result of equilibration of the CO₂:bicarbonate buffering system the minerals will go back into solution; at the same time O₂ is driven out of the medium. Dry ingredients are also added and dissolved in the order listed.

Yeast extract.....	2.0 g
Trypticase peptone.....	2.0 g
NaCl.....	29.95 g
MgCl x 6H ₂ O.....	1.6 g
KCl.....	0.5 g

The medium is allowed to gas until it has become clear, usually 30 to 60 minutes depending on the gas flow rate. The pH of the cleared medium should be between pH 7 and pH 7.3. At this point the solution C and trace minerals supplement can be added to the medium.

Solution C (see below).....	2 ml
Trace Mineral Supplement (ATCC MD-TMS).....	10 ml

The medium is then ready to be transferred to culture tubes. For this purpose, a gas-cannula system with at least three separate gassing ports should be used. A rack of Balch tubes should be made ready. The flask of medium is continuously gassed with one gas cannula; the other two cannula are placed, one each, in two sequential Balch tubes. A 10 ml pipette is used to pipette 10 ml of medium from the flask to the 1st Balch tube. The cannula is then removed from this tube and at the same time a butyl rubber stopper is pushed into the top; it is important to do this all in one motion to minimize contamination of the headspace of the tube with air. This cannula is then moved to the 3rd tube in sequence and the 2nd tube is filled with media and capped, and so on. After the all tubes are filled, capped, and crimped, the medium can be autoclaved at 121C. Following autoclaving a precipitate may form; with periodic mixing this should go back into solution within 48 hours.

Solution A (100X solution)

Ammonium chloride.....	100.0 g
Magnesium chloride hexahydrate.....	100.0 g
Calcium chloride dehydrate.....	40.00 g
DI Water.....	1000 ml

Adjust pH to 4.0 with HCl.

Solution B (500X solution)

Potassium dibasic phosphate trihydrate.....	200.0 g
DI Water.....	1000 ml

Solution C (500X solution)

Resazurin.....	0.5 g
DI Water.....	1000 ml

Note: Resazurin is often colorless after autoclaving the medium, probably because the yeast extract provides enough reducing power to cause complete reduction of the dye. Adding a reducing agent to the medium is still recommended.

Add the following reducing agent to the medium at least one hour before the medium is to be inoculated:

Co-enzyme M Solution (mercaptoethanesulfonic acid) (100 X solution):

Dissolve 5.0 g in 100 ml of deionized water. Distribute into screw cap test tubes, 5–6 ml per tube and seal with rubber stoppers under N₂ gas. Autoclave at 121C to sterilize. Excess tubes can be stored at room temperature for up to 2 months. Co-enzyme M is a compound produced by many methanogens. Some methanogens are sensitive to stronger reducing agents such as sodium sulfide. Co-enzyme M is the standard reducing agent we use when working with methanogens.

Sodium sulfide Solution (100 X solution): Dissolve 1.5 g in 100 ml of distilled water. Distribute into screw cap test tubes, 5–6 ml per tube, and seal with Hungate stoppers. Autoclave to sterilize. Excess tubes can be stored frozen for up to 6 months. Once thawed a tube of sodium sulfide should not be used for more than a week. CAUTION: if sodium sulfide comes into contact with a strong acid, hydrogen sulfide (H₂S), a very toxic gas is liberated immediately.

Cysteine Solution (100X solution): Dissolve 3.0 g in 100 ml of distilled water. Distribute into screw cap test tubes, 5–6 ml per tube, and seal with Hungate stoppers. Autoclave at 121C to sterilize. Excess tubes can be stored frozen for up to 6 months. Once thawed, a tube of cysteine should not be used for more than a week.