ATCC medium: 7266 MWMM gradient plate medium

Top layer:

NOTE: (You will need an additional 20 ml of MWMM on top of the volume required for plates to test and adjust pH. Adjust concentrations of other reagents accordingly.)

16 ml MWMM (see below) per plate
5mM $NaHCO_3$
1 µl ATCC trace minerals (see below) per 1 ml MWMM
1 µl ATCC trace vitamins (see below) per 1 ml MWMM*
**add vitamins aseptically after autoclaving.*

Bottom layer:

4.25 ml MWMM per plate
4.25 ml FeS (see below) per plate
1.2% high melt agarose (Promega V3121)

Modified Wolfe's Mineral Medium (MWMM)

NH_4Cl .....1.00 g
$MgSO_4$. $7H_2O$ .....00.20 g
$CaCl_2$. $2H_2O$ .....0.10 g
K_2HPO_4 .....0.05 g
Distilled water.....1.00 L

Wolfe's Mineral Solution:

Available from ATCC as a sterile ready-to-use liquid (Trace Mineral Supplement, catalog no. MD-TMS.)

Nitrilotriacetic acid.....1.500 g
$MgSO_4$. $7H_2O$ .....3.000 g
$MnSO_4$. H_2O .....0.500 g
NaCl.....1.000 g
$FeSO_4$. $7H_2O$ .....0.100 g
$Co(NO_3)_2$. $6H_2O$.....0.100 g
$CaCl_2$.....0.100 g
$ZnSO_4$. $7H_2O$ .....0.100 g
$CuSO_4$. $5H_2O$ .....0.010 g
$AlK(SO_4)_2$. $12H_2O$.....0.010 g
H_3BO_3 .....0.010 g
Na_2MoO_4 . $2H_2O$.....0.010 g
Na_2SeO_3 (anhydrous) .....0.001 g
NA_2WO_4. $2H_2O$.....0.010 g
$NiCl_2$. $6H_2O$ .....0.020 g
Distilled water.....1.0 L

Add nitrilotriacetic acid to approximately 500 ml of water and adjust to pH 6.5 with KOH to dissolve the compound. Bring volume to 1.0 L with remaining water and add remaining compounds one at a time.

Wolfe's Vitamin Solution:

Available from ATCC as a sterile ready-to-use liquid (Vitamin Supplement, catalog no. MD-VS).

Biotin	2.0 mg
Folic acid	2.0 mg
Pyridoxine hydrochloride	10.0 mg
Thiamine . HCl	5.0 mg
Riboflavin	5.0 mg
Nicotinic acid	5.0 mg
Calcium D-(+)-pantothenate	5.0 mg
Vitamin B12	0.1 mg
p-Aminobenzoic acid	5.0 mg
Thioctic acid	5.0 mg
Distilled water	1.0 L

Ferrous sulfide (FeS):

- Heat 250 to 300 ml dH_2O to 50°C while stirring magnetically. Simultaneously heat another 300 ml dH_2O in a separate beaker; set aside.
- Measure 46.2 g $FeSO_4$*7H2O and 39.6 g Na_2S.
- Add the FeSO4 to the beaker first, followed immediately by the Na_2S. Stir until all Na_2S has dissolved.
- Decant into a clean 500 ml glass bottle. Rinse the first beaker with pre-warmed dH_2O and fill the bottle to the neck. *It is important to ensure that the equal molar amounts of the components are maintained.* Close the bottle with a rubber stopper.
- The precipitate will begin to settle immediately. Allow the precipitate to settle completely before the first wash.
- To wash, decant the supernatant carefully and add new dH_2O or MQH_2O to the neck of the bottle.
- Repeat this wash procedure five times before use in culture propagation.

If the solution does not settle within 24 hours, or if there is a strong sulfidic odor, check the pH. A pH higher than 10.0 is an indication that the ferrous sulfide will not be usable.

This stock ferrous sulfide solution may be maintained at room temperature, out of direct sunlight, for several months. You **must refill** the FeS with distilled or MQ water to the neck of the bottle every time you remove FeS from the bottle, or the stock will oxidize rapidly.

To pour the gradient plates:

Autoclave both layers separately for 20 minutes at 121°C. Immediately after autoclaving, place the top layer in an ice bath. Allow the bottom layer to cool slightly, though not to room temperature, to avoid the separation of FeS and agarose. Pipette 8.5 ml of the bottom layer into a standard Petri dish. Allow to set a minimum of 15 minutes, but no longer than 30 to avoid excessive abiotic oxidation of the iron.

While the bottom layer is setting, remove the top layer from the ice bath, and, provided it has cooled to at least room temperature, add the trace vitamin solution. Adjust the pH to between 6.0 and 6.4 by sparging with filter-sterilized CO_2. The top layer should be inoculated with two to three ml of freshwater iron-

oxidizing bacteria per 100 ml top layer. 16 ml of top layer is pippetted carefully over the solidified bottom layer. Place the plates in either GasPak jars with BD BBL CampyPak Plus Microaerophilic system envelopes with palladium catalysts or Mitsubishi AnaeroPack system jars with Pack-MicroAero gas generating envelopes adjusted for the volume of the container.