

Protocol for Preparing Nutrient Agar

[Modified from Hatcher, R. E.. 1965. Bryologist 68: 230-231.]

NB: Preparation of this Nutrient Agar is a two-day process.

Materials needed:

15 g	agar (we use Difco Bacto)
150 ml	Hatcher's Nutrient Stock A [see below]
4.5 ml	Hatcher's Nutrient Stock B [see below]
1.5 ml	MS Media Iron Stock C [see below]

Large ceramic Buchner funnel

Fisherbrand Filter Paper – Qualitative P4, 15 cm diameter

2, 2 liter Erlenmyer Flasks

Several liters ddH₂O

Glass funnel with red rubber tubing attached to base

Hose clamp

Large ringstand

1. Place a large Buchner funnel on top of a 2 liter Erlenmyer flask.
2. Place a disc of Fisherbrand filter paper into the Buchner funnel. Lay the filter paper down while pouring a bit of water over the paper to ensure there are no air bubbles under the paper.
3. Weigh out 15 g of Difco bacto agar on the digital scale and dump this on top of the pre-moistened filter paper in the Buchner funnel.
4. Slowly, pour approximately 1 L of ddH₂O into the Buchner funnel. Make sure the water doesn't lift the filter paper to prevent escape of the agar from the Buchner funnel.
5. When the water has almost entirely drained, fill the funnel top with ddH₂O again.
6. Leave this to drain overnight.
7. The next morning, place the clean, dry, 2 liter Erlenmyer flask on the digital scale and tare the scale to zero.
8. Using a plastic spoon, scoop the washed agar into the tared Erlenmyer flask and record the weight of the wet agar on a scrap of scratch paper. You will need this later.
9. Add 150 ml of Hatcher Nutrient Stock A to the flask containing the washed agar.

10. Using a 10 ml glass pipet and a green pipet pump, pipet 4.5 ml of Hatcher Nutrient Stock B into the agar containing flask.
11. Repeat for MS Iron Stock C, only add 1.5 ml of MS Iron Stock C.
12. Add the weight of the washed agar to the quantity of each nutrient stock solution added to the flask. For example:

	300	g	Washed Agar (1 g of H ₂ O = 1 ml of H ₂ O)
	150	ml	Hatcher Nutrient Stock A
	4.5	ml	Hatcher Nutrient Stock B
+	1.5	ml	MS media Iron Stock C
	456	ml	Total

Subtract this figure from 1500 ml:

$$1500 \text{ ml} - 456 \text{ ml} = 1046 \text{ ml}$$

This is how much ddH₂O to add to the flask to make a total of 1.5 L of Hatcher's Nutrient Agar. You should do the calculations each time as that the weight of the washed agar is variable.

13. Cover the top of the flask with a snug-fitting piece of aluminum foil.
14. Autoclave the flask for 20 minutes. On the department autoclave this is the Liquid 100 setting.
15. While the agar is autoclaving, prepare a tray full of clean baby-food jars leaving the tops off. The trays are on top of the paraffin oven.
16. Place the large ring-stand next to the tray of jars. Place the glass funnel with the piece of rubber tubing in the ring. Place the hose clamp, located on the drying rack, on the rubber tubing about 2 inches from the bottom of the tube.
17. When the agar is done autoclaving, pour agar into the funnel and use this to fill the jars with agar. Fill the jars with agar to a height of about 1" - 1.5". Use an already filled jar as a guide for height. Please, **WEAR AUTOCLAVE GLOVES WHEN REMOVING MATERIALS FROM AUTOCLAVE!**
18. Place clean lids on top of the jars. Ensure that the lids fit snugly on the jars.
19. Autoclave the now filled jars for 20 mins. This is the liquid 100 setting on the departmental autoclave.

20. Remove the tray of jars from the autoclave when the autoclave cycle is complete. Leave the jars on the bench or the cart overnight to solidify.
21. When jars have cooled and agar has solidified place these in the refrigerator.

Preparing Stock Nutrient Solutions for Modified Hatcher's Media

Hatcher's Nutrient Stock A (2 L)

MgSO ₄ • 7H ₂ O	14.7 g
Ca(NO ₃) ₂ • 4H ₂ O	5.2 g
Na ₂ SO ₄	4.0 g
NaNO ₃	1.6 g
KCl	1.3 g
KH ₂ PO ₄	3.3 g

Each salt must be added to solution individually. Do not add the next salt until the previous salt has completely dissolved.

If at any time the solution becomes cloudy or milky white then discard the solution and start over!

1. Add 500 ml of ddH₂O to a 1 L beaker. Add a magnetic stir bar and place on a stir plate.
2. Add MgSO₄ • 7H₂O to the beaker. Let it dissolve completely.
3. Add Ca(NO₃)₂ • 4H₂O to the beaker. Let it dissolve completely.
4. Add Na₂SO₄ to the beaker. Let it dissolve completely.
5. Add 500 ml of ddH₂O to the beaker.
6. Continue to add the rest of the salts, one at a time, allowing each salt to completely dissolve before you add the next one.
7. When the last salt has completely dissolved, measure out 1 L of solution in a 1 L graduated cylinder.
8. Pour this into the bottle in which the stock solution is kept.
9. Pour the remaining solution into the 1 L graduated cylinder.
10. Add ddH₂O to the graduated cylinder until a volume of 1 L is achieved.
11. Pour this solution into stock solution bottle and give the bottle a good shake. Label the bottle with the date the solution was made, and place in refrigerator.

Hatcher's Nutrient Stock B (1 L)

H ₂ SO ₄ (concentrated)	0.5	ml
MnSO ₄ • 4H ₂ O	3000	mg
ZnSO ₄ • 7H ₂ O	500	mg
H ₂ BO ₃	500	mg
CuSO ₄ • 5H ₂ O	25	mg
Na ₂ MoO ₄ • 2H ₂ O	25	mg
CoNO ₃	25	mg

Each salt/solution must be added to solution individually. Do not add the next salt until the previous salt has completely dissolved.

If at any time the solution becomes cloudy or milky white then discard the solution and start over!

22. Add 250 ml of ddH₂O to a 1 L beaker. Add a magnetic stir bar and place on a stir plate.
23. Pipet 0.5 ml of concentrated H₂SO₄ into the beaker of water. Use a glass pipet with a green pipet pump. **This step should be done in the fume hood! Make sure to wear nitrile gloves when handling strong acids!**
24. Add each salt individually to solution allowing the each salt to dissolve before the next one is added.
25. Once all salts have been added, pour the solution into a 1 L graduated cylinder and add ddH₂O until the volume of solution equals 1 L.
26. Pour this solution into stock solution bottle and give the bottle a good shake. Label the bottle with the date the solution was made, and place in refrigerator.

MS Media Iron Stock C (0.5 L)

MS Media iron stock solution is used instead of Hatcher's Iron Stock Solution. However if Ferric Citrate can be located then the Hatcher's Iron Stock Solution C should be prepared and used.

Na₂EDTA 0.9325 g

FeSO₄ 0.6950 g

- 5 You will need two 150 ml beakers with stir bars placed in the bottom each beaker. You will also need stir plates with heating capacity. Add approximately 100 ml of ddH₂O to each beaker. Place the beakers on the hot plates on a low to medium heat.
 - 6 Add Na₂EDTA to one beaker.
 - 7 Add FeSO₄ the other beaker.
 - 8 Gently stir each solution while heating.
 - 9 Heat and stir each solution for around 1 – 2hours.
 - 10 Mix the solutions together and continue to heat and stir for another hour.
 - 11 Pour the now mixed solutions into a 500 ml graduated cylinder and bring volume equal to 500 ml by adding ddH₂O.
 - 12 Pour into the Iron Stock Solution bottle.
 - 13 Allow to cool to room temperature before placing in the refrigerator.
- 27.