

APPENDIX I: CRYOPRESERVATION

1. Prepare CPMB-2 Basal Solution (see recipe below).
2. Prepare L-Cysteine/Ascorbic Acid Solution (see recipe below).
3. Harvest cells from several cultures that are in peak density of growth and place on ice for 10 minutes.
4. Gently invert tubes 20 times and centrifuge at 200 × g for 5 minutes.
5. While cells are centrifuging, prepare the CPMB-5 Cryoprotective Solution:
 - a) Add 1 mL of DMSO to a 16 × 125 mm screw-capped test tube and place on ice until solidified.
 - b) Add 0.8 mL of 2.5 M sucrose, remove from ice, and invert until the DMSO is liquefied and return to ice bath.
 - c) Add 0.2 mL of the L-Cysteine/Ascorbic Acid Solution to the mixture and mix.
 - d) Add 6 mL of the CPMB-2 Basal Solution and mix.
 - e) Add 2 mL heat-inactivated bovine serum (HIBS) and mix.
6. Resuspend the cell pellets and pool to a final volume of approximately 10 mL with the supernatant.
7. Determine the cell density using a hemocytometer and adjust the concentration between 5 × 10⁵ and 1 × 10⁶ cells/mL using fresh media. If the cell concentration is lower than 5 × 10⁵ cells/mL, centrifuge the cell suspension, remove the supernatant, and resuspend the pellet in a volume that will yield a concentration between 5 × 10⁵ and 1 × 10⁶ cells/mL.
8. After the cell concentration is adjusted, centrifuge at 200 × g for 5 minutes.
9. Remove as much supernatant as possible and determine the volume removed.
10. Resuspend the cell pellet with a volume of the Cryoprotective Solution equal to the volume of the supernatant removed. Gently invert the tube several times to obtain a uniform cell density.
11. Dispense 0.5 mL aliquots into sterile plastic cryovials.
12. Place the vials in a controlled rate freezing unit. From room temperature, cool at -10°C/minute until the liquid begins to freeze; from this point until -40°C is reached, cool at -1°C/minute. At -40°C plunge the vials into liquid nitrogen. The cooling cycle should be initiated 15 to 30 minutes after the addition of DMSO to the cell preparation.
13. Store ampoules in a liquid nitrogen refrigerator until needed (-130°C or colder).

CPMB-2 Basal Solution

Yeast Extract	60.0 g
K ₂ HPO ₄	1.0 g
KH ₂ PO ₄	0.6 g
NaCl	2.0 g
Distilled water	1 L

Add the ingredients in the order listed above to the distilled water and mix. Adjust the pH to 6.8 and autoclave for 20 minutes at 121°C.

L-Cysteine/Ascorbic Acid Solution

L-Cysteine • HCl	1.0 g
Ascorbic Acid	0.1 g
10 N NaOH	~ 0.7 mL
Distilled water to	10 mL

Add 9.0 mL of distilled water to a 20 mL beaker and dissolve the first two components. While stirring, adjust the pH to 7.2 with 10 N NaOH (approximately 0.7 mL). Adjust the final volume to 10 mL with distilled water and filter sterilize using a 0.2 µm filter. The solution should be used soon after preparation. Discard any unused solution.

APPENDIX II: LIVER DIGEST – YEAST EXTRACT – IRON (LYI) *ENTAMOEB*A MEDIUM (ATCC® MEDIUM 2154)

1. Prepare the 10× Glucose Buffer Stock Solution (see recipe below) and filter sterilize using a 0.2 µm filter.
2. Prepare the LYI Base Stock Solution (see recipe below), by dissolving the dry ingredients of the LYI Base Stock in 600 mL of distilled water in the order indicated. Adjust the final volume to 780 mL with distilled water. Adjust pH to 6.8 with 1 N NaOH. Autoclave for 20 minutes at 121°C and allow to cool.

10× Glucose Buffer Stock Solution

K ₂ HPO ₄	1.0 g
KH ₂ PO ₄	0.6 g
Glucose	10.0 g
Distilled water	100 mL

LYI Base Stock

NaCl	1.0 g
Yeast Extract	25.0 g
Neutralized Liver Digest	5.0 g
L-Cysteine • HCl	1.0 g
Ascorbic Acid	0.2 g
Ferric Ammonium Citrate	22.8 mg
Distilled water to	780 mL

3. Prepare the LYI Broth (see recipe below), by aseptically adding 100 mL of the 10× Glucose Buffer Stock Solution to 780 mL of cooled LYI Base Stock Solution. Osmolarity should be 380 milliosmols/kg; adjust by increasing or decreasing NaCl. LYI Broth can be stored for at least 6 months at -20°C.

LYI Broth

LYI Base Stock Solution	780 mL
10× Glucose Buffer Stock Solution	100 mL

4. Prepare each of the three water-soluble vitamin stock solutions listed below:

Water Solution A

Niacin	62.5 mg
p-Aminobenzoic acid	12.5 mg
Distilled water to	150 mL

Dissolve solid ingredients in boiling distilled water and restore the final volume to 150 mL.

Water Solution B

Niacinamide	62.5 mg
Pyridoxine hydrochloride	62.5 mg
Thiamine hydrochloride	25.0 mg
Calcium pantothenate	25.0 mg
i-Inositol	125.0 mg
Choline chloride	1250.0 mg
Distilled water to	150 mL

Dissolve solid ingredients in 125 mL distilled water, then bring the final volume to 150 mL.

Water Solution C

Riboflavin	62.5 mg
Distilled water to	150 mL

Add riboflavin to 75 mL of distilled water and add 0.1 N NaOH dropwise until the riboflavin is fully dissolved. Bring the final volume to 100 mL with distilled water.

5. Prepare the Water-Soluble Vitamins Solution by combining each of the three water-soluble vitamin stock solutions prepared above. Bring the final volume to 500 mL with distilled water.

Water Soluble Vitamins

Water Solution A	150 mL
Water Solution B	150 mL
Water Solution C	100 mL
Distilled water to	500 mL

If the mixture appears turbid, it should not be discarded. Development of turbidity is an indication that an excess of NaOH has been used in the preparation of one of the stock solutions [Diamond, L. S. "Techniques of Axenic Cultivation of *Entamoeba histolytica* Schaudinn, 1903 and *E. histolytica*-Like Amebae." J. Parasitol. 54 (1968): 1047-1056. PubMed: 4319346].

6. Prepare the Biotin Solution following the recipe below:

Biotin Solution

Biotin	30 mg
Distilled water to	300 mL

Add biotin to 200 mL of distilled water and add 0.1 N NaOH dropwise until the biotin is fully dissolved. Bring the final volume to 300 mL with distilled water.

7. Prepare the Folic Acid Solution following the recipe below:

Folic Acid Solution

Folic Acid	30 mg
Distilled water to	300 mL

Add folic acid to 200 mL of distilled water and add 0.1 N NaOH dropwise until the folic acid is fully dissolved. Bring the final volume to 300 mL with distilled water.

8. Prepare each of the three Lipid Stock Solutions following the recipes listed below:

Lipid Solution A

Vitamin D ₂ (calciferol)	300 mg
Ethyl alcohol 9.5% (v/v)	63 mL
Vitamin A (crystalline alcohol)	300 mg

Dissolve vitamin D₂ in ethyl alcohol, then add vitamin A.

Lipid Solution B

Vitamin K (menadione sodium bisulfite)	60 mg
Tween 80 aqueous solution 5% (v/v)	300 mL

9. Prepare the Lipid-Soluble Vitamins A, D and K Solution following the recipe below by combining Lipid Solutions A and B prepared above:

Lipid-Soluble Vitamins A, D and K

Lipid Solution A	60 mg
Lipid Solution B	300 mL

10. Prepare the Vitamin E Stock Solution following the recipe below:

Vitamin E Stock Solution

Vitamin E (alpha tocopherol acetate)	25 mg
Distilled water	250 mL

11. Prepare the Diamond's Vitamin Solution 107 following the recipe below, and sterile filter using a 0.22 µm filter. The complete, clear solution may be stored at -22°C. Thaw and allow to adjust to room temperature before use.

Diamond's Vitamin Solution 107

Water-Soluble B Vitamins	500 mL
Biotin Solution	250 mL
Folic Acid Solution	250 mL
Lipid-Soluble Vitamins A, D & K	2500 mL
Vitamin E Solution	100.0 mL

12. Aseptically prepare the complete LYI *Entamoeba* medium following the recipe below:

LYI *Entamoeba* Medium

LYI Broth	88 mL
Diamond's Vitamin Solution 107	2 mL
Heat-Inactivated Bovine Serum (HIBS)	10 mL

Mix thoroughly and distribute into 13 mL aliquots to 16 × 125 mm screw-capped borosilicate glass test tubes. Store at 5°C to 9°C in the dark with the caps screwed on tightly. Use within 7 to 10 days.