General methods for culturing *Haemophilus influenzae*.

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1. Introduction

1.1 Nutritional requirements

The species *Haemophilus influenzae* is differentiated from other *Haemophilus* species primarily by its growth requirements for both hemin (called factor X in the old literature) and nicotinamide adenine dinucleotide (NAD or factor V). *H. influenzae* strains normally grow well in rich media such as brain heart infusion, suitably supplemented with hemin and NAD (sBHI), and on sBHI or chocolate agar plates. Many isolates classified as *Haemophilus influenzae* differ in their other nutritional requirements, for a purine and for specific amino acids [1, 2]. Thus, although a number of defined media have been described, their use often leads to frustration. The medium MMB described by Klein and Luginbuhl [2] is simplest to prepare; when supplemented with a small amount of casamino acids it reproducibly gives good growth.

1.2 Growth and viability

Standard laboratory strains of *H. influenzae* grow in rich medium with a doubling time of about 30 minutes, to a maximum density of $5x10^9$–$1x10^{10}$ cells per ml. Cells are not well adapted to survival under conditions that do not permit growth; viability of cells in liquid will decline continuously once the maximum density is reached. Colonies on solid medium will continue to contain viable cells for a week or more at room temperature or at 4°C, but the number of viable cells will decrease.

1.3 Storage

Cultures are best stored frozen in glycerol (see below).

1.4 Recognizing contaminants

1) *H. influenzae* colonies are grayish-buff and slightly translucent, with little odour. Colonies that appear opaque, white, pigmented or strongly smelling are probably contaminants.

2) If suspected contaminants produce colonies on an LB plate or other rich medium lacking hemin and NAD, the cells are not *H. influenzae*.

3) When examined microscopically, *H. influenzae* cells are very small rods, much smaller than typical bacteria such as *Escherichia coli*.

2. Materials

2.1 Media

1. Brain-heart infusion broth (BHI): 37 g/L Difco Brain Heart Infusion in distilled H_2O. Autoclave, store at room temperature. For plates, add 12-15g Bacto or MBI agar before autoclaving. (Note 1)
2. Hemin (1mg/ml): Place 4 ml Triethanolamine, 96 ml dH_2O, and 100 mg Hemin (Equine, Difco) inn a glass bottle. Cap loosely and heat at 65°C for 30 minutes (do not autoclave). The hemin will not dissolve, but will form a stable suspension upon heating. This stock is sterile and stable for many months at 4°C. If preparing more than one bottle of hemin stock, put the weighed hemin into each bottle separately, then add 100 ml of 4% triethanolamine and heat.
3. Nicotinamide adenine dinucleotide (NAD) stock: 10mg/ml in dH_2O. Filter sterilize. Stable for many months at –20°C, and for at least several weeks at 4°C.
4. Supplemented Brain Heart Infusion (sBHI): Add 10 ml hemin stock and 200 µl NAD stock per liter of BHI. Prepare as needed; use within 24 hr.
5. cMMB (MMB supplemented with casamino acids):

   Dissolve in dH_2O (1l),

L-arginine	300 mg
Glutamic acid	1.3 g
Glutathione	200 mg
Uracil	100 mg
Inosine	2.0g
Casamino acids	5 g
K_2HPO4	3.5 g
KH_2PO4	2.7 g
NaCl	5.8 g
$MgCl_2$	430 mg
$CaCl_2$	22 mg

   Autoclave.

   When cool, add:

Hemin	10 ml of 1 mg/ml stock
NAD	200 µl of 10 mg/ml stock
Thiamine	400 µl of 10 mg/ml stock (filter sterilized; store at –20°C)
Pantothenic acid	400 µl of 10 mg/ml stock (filter sterilized; store at –20°C)

6. Antibiotic stocks: Standard concentrations of antibiotics used for genetic manipulations in *H. influenzae* are given in Table I. Unless otherwise indicated, prepare in dH_2O, filter-sterilize, and store in 1 ml aliquots at –20°C. Stocks prepared in ethanol need not be sterilized.

2.2 Other

1. 80% glycerol stock: Combine 80 ml glycerol with 20ml H_2O. Mix well and autoclave. Store at room temperature. Keeps indefinitely.
2. Phosphate-buffered saline (PBS): 0.3 g KH_2PO_4, 1.1 g $Na_2HPO_4 \cdot 7H_2O$, 8.5 g NaCl, dH_2O to 1 L. Mix and autoclave. Store at room temperature. Keeps indefinitely. (Note 2)
3. Dilution solution: PBS supplemented with 5-10% BHI or sBHI.

2.3 DNA preparation

Chromosomal DNA minipreps:

1. Cell resuspension solution: 50mM Tris HCl pH7.4, 50mM EDTA.
2. 10% SDS
3. Phenol-chloroform 50:50:
4. 5M NaCl;
5. 95% EtOH,
6. Flame-sealed pasteur pipettes for DNA preparations.
7. 70% EtOH;
8. TE: 10mM Tris pH 7.4, 1 mM EDTA).

Lysed colonies for PCR:

9. 0.5M NaOH,
10. 1M Tris HCl pH 7.4

2.4 Cloning vectors

Many *E. coli* cloning vectors cannot replicate in *H. influenzae* (*e.g.* pGEM, pUC). The most convenient cloning vectors for genetic manipulation in *H. influenzae* are the pSU series (pSU8, pSU9, pSU18, pSU19, pSU20, pSU21, pSU23, pSU24) [3]. They are based on pACYC184, confer chloramphenicol resistance, have useful multi-cloning sites, and provide moderate copy number in both *E. coli* and *H. influenzae*.

2.5 Strains

1. Obtaining strains: The American Type Culture Collection has an extensive collection of H. influenzae isolates. Strains containing specific mutations are usually readily obtained from their creators.
2. Shipping strains: Cells may be grown on agar prepared in a plastic tube suitable for mailing, or a sterile spatula may be used to transfer colonies and agar from a fresh plate into a suitable sterile tube. Most strains will survive a week or more at ambient temperature, and so may be sent by mail if economy is an issue.

2.6 Equipment

1. Roller wheel or shaker at 37°C for small-volume liquid cultures in tubes (≤10ml).
2. Air or waterbath shaker at 37°C for larger volumes of liquid cultures.
3. Glass beads for spreading cells on plates (3mm diameter, autoclaved in glass screw-capped tubes) or alcohol-sterilized spreader.
4. Sterile loops or pipettes for streaking cells on plates and transferring colonies.
5. 37°C incubator for growing cells on solid media.

3. Methods

3.1 Liquid cultures

Small volume cultures: Prepare a test tube containing 5 ml of sBHI and any needed antibiotics. Inoculate with either a loopful of cells from a fresh well-isolated colony, a drop of a previous liquid culture, or scrapings from a frozen culture. Cap loosely and incubate at 37°C on a roller wheel or shaker overnight or until desired culture density is reached.

Moderate volume cultures: Instead of a test tube use a flask having at least five times the volume of the culture (e.g. 20 ml culture in a 100ml flask). Shake at 200 rpm at 37°C.

Large volume cultures: Inoculate from a fresh overnight culture if rapid initial growth is desired. Do not over-fill flasks; inadequate aeration is often the cause of low yields of cells or plasmids. Shake at 200 rpm at 37°C.

Monitoring growth: Read optical density at a wavelength of 600nm. Use sBHI as a blank. (Note 3) In dense cultures erroneously-low readings are caused by light scattering, so if the OD_{600} of the sample is higher than 0.3, dilute the sample five- or ten-fold with sBHI to get an accurate reading.

Diluting cultures for plating: Cultures may be diluted in medium or in phosphate-buffered saline (PBS) that has been supplemented with 5-10% BHI. Cells diluted in plain PBS rapidly lose viability.

3.2 Agar cultures:

1. Prepare BHI agar. Let agar cool to pouring temperature (approximately 50°C) before adding hemin, NAD and any antibiotics. (Note 4) Standard concentrations of antibiotics used for genetic manipulations are given in Table I. Allow surfaces of freshly-poured plates to dry before streaking or spreading cells, to prevent 'weeping' of the agar surface. For non-critical experiments, plates may be dried uncovered in a forced-air incubator for 1 hr; for critical experiments use a laminar flow hood.
2. To spread cells for colony counting, liquid may be spread on plates using a sterile spreader or sterile glass beads. To use glass beads, sprinkle 3-5 beads on each plate. Add liquid and shake gently to roll beads around, spreading the liquid. Once liquid is absorbed, tip beads into a container of disinfectant or ethanol for later washing and reuse.
3. To streak cells for isolation of single colonies, using a flamed loop or a sterile pipette.
4. Incubate plates at 37°C for 18-24 hr. Colonies are smaller than those of *E. coli*. They will grow slightly larger if left for 48 hr, but will not continue to grow beyond that time.

3.3 Freezing cultures:

Stock cultures of non-competent cells are prepared by inoculating cells into supplemented brain heart infusion broth (sBHI) and growing to an OD_{600} of 0.3- 0.4. Before frreezing add 0.25 ml 80% glycerol to 1 ml of culture, and mix well. Store at -70°C – -80°C. Competent cells in MIV may also be frozen this way for later use.

3.4 Preparation of DNA:

In general the methods developed for isolation of plasmids and chromosomal DNA from *E. coli* work well. Because the *H. influenzae* peptidoglycan layer is weak, pretreatment with lysozyme is usually unnecessary. For plasmid preparation, use the standard protocols developed for *E. coli*. [4]

Rapid small-scale preparation of chromosomal DNA: Pellet 1.5-3.0ml of a fresh overnight culture. Resuspend in 450µl of cell resuspension solution in a microfuge tube. Add 50µl of 10% SDS and mix until cells lyse and suspension becomes viscous. The suspension may be heated to 50°C to speed lysis. Extract twice with phenol:chloroform, each time transferring the supernatant to a fresh tube. Add sufficient 5M NaCl to give a final concentration of 0.15M (usually 12µl), then add two volumes of 95% EtOH (usually 0.8ml). Mix gently until a fibrous clump of DNA becomes visible, and retrieve the DNA by winding it onto the sealed tip of a pasteur pipette (Note 7). Rinse the spooled DNA by gently dribbling 1.0 ml of 70% EtOH down the pipette tip, held over a clean container. Store the pipette in an inverted position for at least 30 min to allow the DNA to air dry. Resuspend the DNA by swirling the pipette tip in 200µl of TE, and then allow several hours for the DNA to fully disperse, with occasional gentle vortexing or pipetting. Yield is about 20 µg of DNA, in 50-200kb fragments .

Single-colony DNA for PCR [5]: Transfer a large colony from a sBHI plate into 25µl 0.5M NaOH in a microcentrifuge tube. Completely suspend the cells by vigorous vortexing, and leave at room temperature for 30 min. Then add 25µl of 1M Tris buffer, pH 7.4 and 450µl of dH_2O. These lysates can be frozen at –20°C until needed. For use as a PCR template, 5-10µl of the lysate is added to a 100ul PCR reaction mixture.

4. Notes

1. We have not experienced any problems attributable to different sources of water. All deionized and distilled waters have worked well. However, attempts to economize by using less expensive brands of BHI and agar have been unsuccessful. Although their initial performances were often

satisfactory, all eventually caused problems with growth or plating that ruined expensive experiments, wiping out the original cost savings.

2. When large volumes of PBS will be needed, it is convenient to prepare the PBS as a 10X stock, and dilute with sterile water as needed.

3. OD_{600} readings for different batches of sBHI often differ substantially. Always use as a blank sBHI from the same bottle as is being used for the culture.

4. Fresh hemin must be added to plates older than 24 hours. Spread $1/100^{th}$ the agar volume of hemin stock on the plate (0.3ml on a 100mm plate). Be sure the hemin has soaked in and the agar surface is dry before using the plate.

5. Most kanamycin-resistance plasmids contain a Tn5-derived Kan^R gene, which functions poorly in *H. influenzae*. Most kanamycin-resistance transposons contain the Tn903-derived Kan^R gene, which functions well. Ribostamycin may be used in place of kanamycin.

6. Cells grow poorly in the presence of chloramphenicol, and 48 hr incubation may be required for sufficient growth of colonies. Do not assume that an experiment has failed because no chloramphenicol-resistant colonies are visible after 24 hr incubation; wait another day.

7. DNA collected by spooling in this way is much cleaner than DNA collected by centrifugation.

8. Most *H. influenzae* strains contain one or more restriction modification systems, and DNA extracted from them will carry the corresponding methylation and thus cannot be digested by these enzymes. *Hin*dII and *Hin*dIII will not digest DNA from the Rd strain.

References

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TABLE I

Recommended antibiotic concentrations for *H. influenzae*

Antibiotic	**Working concentration**	**Stock**
Kanamycin (Note 5)	7 µg/ml	70 mg/ml (10,000X)
Ribostamycin	15 µg/ml	150 mg/ml (10,000X)
Novobiocin	2.5 µg/ml	25 mg/ml (10,000X)
Spectinomycin	20 µg/ml	20 mg/ml (1000X)
Naladixic Acid	3 µg/ml	30 mg/ml (10,000X)
Chloramphenicol (Note 6)	1-2 µg/ml	20 mg/ml in EtOH (10,000X)
Tetracycline	5 µg/ml	10 mg/ml in EtOH (2000X)
Streptomycin	250 µg/ml	100 mg/ml (400X)
Ampicillin	5 µg/ml	50 mg/ml in 50% EtOH (10,000X)