Published in final edited form as:

Methods Mol Biol. 2019 ; 1997: 217–231. doi:10.1007/978-1-4939-9496-0_14.

Generation of metal-depleted conditions for *in vitro* growth of *Neisseria gonorrhoeae*

Cynthia Nau Cornelissen^{1,2}

¹Department of Microbiology and Immunology, School of Medicine, Virginia Commonwealth University, Richmond, VA, USA

²Institute for Biomedical Sciences, Center for Translational Immunology, Georgia State University, Atlanta, GA, USA

Abstract

Neisseria gonorrhoeae employs high affinity metal acquisition systems to obtain necessary nutrients, such as iron (Fe) and zinc (Zn) from the environment. Because growth and replication depend upon successful metal acquisition, these high-affinity uptake systems are important virulence factors. Expression of metal acquisition systems is tightly controlled and preferentially expressed under low-metal conditions. Therefore, in order to optimally produce these transport proteins and study them *in vitro*, growth media must be deployed that mimic low-metal conditions. This chapter describes the chelators, media and culturing conditions that can generate low-metal *in vitro* growth conditions.

Keywords

Iron; Zinc; Transferrin; Calprotectin; *Neisseria gonorrhoeae*; Chelator

1. Introduction

Neisseria gonorrhoeae is a human specific pathogen that does not naturally infect any other animal (1). This selectivity is, in large part, due to the specificity that this pathogen demonstrates for many nutrients and receptors during the pathogenesis process (2–5). In an effort to thwart bacterial growth and virulence, human hosts deploy a strategy known as “nutritional immunity” (6–8), a term used to describe the phenomenon of metal sequestration by the host in order to inhibit microbial replication. To overcome nutritional immunity, pathogens, including *N. gonorrhoeae*, produce high affinity metal acquisition systems to compete with the sequestration proteins for the necessary metals (for review, see (9)). *N. gonorrhoeae*, as a human-adapted pathogen, has evolved an even more simple, yet elegant, strategy than that deployed by other bacteria. *N. gonorrhoeae* directly binds to and extracts the metals from human nutritional immunity proteins (for review, see (9)). Nutritional immunity proteins that sequester iron (Fe) include transferrin and lactoferrin. Proteins that prevent microbial access to zinc (Zn) include calprotectin and other S100

proteins (10). Receptor/transporters that enable *N. gonorrhoeae* to use these human proteins as metal sources are *bone fide* virulence factors as they directly contribute to the virulence of the pathogen (11,12). The metal uptake systems are only produced when metal concentrations are low, as in the host (13,14). Thus, to study these virulence factors *in vitro*, we must mimic these low-metal conditions to maximize production. Conditions for growing metal-deprived *N. gonorrhoeae* can be generated by adding specific chelators to complex medium or by growing in a defined medium reconstituted with water lacking contaminating metals. This chapter discusses the chelators, media and culturing conditions that can mimic a low-metal growth environment.

2. Materials

2.1. Acid-washed glassware

1. 1% (v/v final concentration) Aquet detergent.
2. Concentrated nitric acid (see Note 1).
3. Aluminum foil or metal caps for glassware.
4. Hot air oven (temperature controlled up to 220°C).

2.2. Growing *N. gonorrhoeae* under low Fe or low Zn conditions in GC broth

1. GC broth: dissolve 15 g of proteose peptone #3, 4 g of K_2HPO_4 , 1 g of KH_2PO_4 and 5 g of NaCl in deionized H_2O , up to a final volume of 1 L.
2. 50 mM Desferal (deferoxamine mesylate) stock solution in deionized H_2O (see Note 2). Desferal is a ferric (Fe) chelator.
3. 40 mM TPEN (N,N,N',N'-tetrakis-(2-pyridylmethyl)ethylenediamine) stock solution in 100% ethanol. TPEN is a zinc (Zn) chelator.
4. Supplement I stock solution (15): add 600 g of glucose, 10 g of L-glutamine, 20 mg of cocarboxylase dissolved in deionized H_2O , up to a final volume of 1 L.
5. 12 mM Supplement II stock solution: dissolve 1.25 g of Fe(III)nitrate [$Fe(NO_3)_3$] nonahydrate in deionized H_2O , up to a final volume of 250 ml.
6. *N. gonorrhoeae* stored in freezing medium (see Note 3) at $-80^\circ C$.
7. GC medium base (Difco) prepared as indicated by manufacturer. Dilute Supplement I 1/100 and Supplement II 1/1000 into molten agar medium before pouring plates.
8. 50 mM zinc sulphate ($ZnSO_4$) stock solution in deionized H_2O .

¹ Always add nitric acid to H_2O , not H_2O to concentrated nitric acid. Use appropriate personal protective equipment (PPE) including long lab coat, goggles and neoprene (acid-resistant) gloves.

² Use deionized H_2O for all steps rather than distilled or ultra-pure H_2O . In-house stills contain metal pipes and therefore re-introduce metals into the H_2O . Ultra-pure H_2O systems (for example, Milli-Q water systems) restrict metal levels too severely and *N. gonorrhoeae* will not grow in media made with this H_2O source.

³ Freezing medium for *N. gonorrhoeae* contains 3 g of trypticase soy broth, 25 mL of glycerol and make up to 100 mL with deionized H_2O . Distribute 500 μL per 1.5 mL freezer vial and autoclave to sterilize.

9. 125 mL side-arm flasks with baffled bottoms; flasks should be acid washed and sterilized in a hot air oven before use.
10. Hot air oven (temperature controlled up to 220°C).
11. Klett Summerson Colorimeter (Thomas Scientific).
12. Autoclave capable of sterilization at 122°C, 3 Kg/cm², and slow exhaust.
13. Stir platform and stir bar.
14. Sterile Petri plates (90mm).
15. Class II Biosafety cabinet.
16. Benchtop microcentrifuge (*e.g.* Eppendorf).
17. Shaking incubator capable of reaching a temperature of 35°C. Atmosphere must be supplemented with CO₂ through a port into the incubator. A gas regulator between the CO₂ tank and the port regulates the flow into the incubator.
18. Humidified CO₂ incubator for incubation of GC medium plates.

2.3. Preparation of liquid CDM (chelexed-defined medium)

1. Ingredients for liquid CDM (16) are listed in Table 1.
2. 0.45 µm filter sterilization units accommodating up to 500 mL of liquid.
3. Chelex-100 (Bio-Rad).
4. pH meter.
5. 1N HCl.
6. Sterile plastic containers.

2.4. Growing *N. gonorrhoeae* under low Fe or low Zn conditions in CDM in flasks

1. *N. gonorrhoeae* stored in freezing medium (see Note 3) stored at –80°C.
2. GC medium base (Difco) prepared as indicated by manufacturer and autoclaved. Dilute Supplement I 1/100 and Supplement II 1/1000 into molten agar medium before pouring plates.
3. Liquid CDM at room temperature.
4. 50 mM ZnSO₄ stock solution in deionized H₂O.
5. 12 mM Supplement II: Fe(NO₃)₃ 9H₂O (final concentration of stock).
6. 125 mL side-arm flasks with baffled bottoms; flasks should be acid washed and sterilized in a hot air oven before use.
7. Hot air oven (temperature controlled up to 220°C).
8. Klett Summerson Colorimeter (Thomas Scientific).

9. Shaking incubator capable of reaching a temperature of 35°C. Atmosphere must be supplemented with CO₂ through a port into the incubator. A gas regulator between the CO₂ tank and the port regulates the flow into the incubator.
10. Humidified CO₂ incubator.
11. Class II Biosafety cabinet.

2.5. Growing *N. gonorrhoeae* under low Fe or low Zn conditions in microtiter plates

1. *N. gonorrhoeae* stored in freezing medium (see Note 3) stored at –80°C.
2. GC medium plates.
3. Liquid CDM at room temperature.
4. 50 mM ZnSO₄ stock solution in deionized H₂O.
5. 12 mM Supplement II: Fe(NO₃)₃ 9H₂O (final concentration of stock).
6. Phosphate buffered saline (PBS), pH 7.4.
7. 125 mL side-arm flasks with baffled bottoms; flasks should be acid washed and sterilized in a hot air oven before use.
8. Hot air oven (temperature controlled up to 220°C).
9. Sterile, flat-bottomed 96-well microtiter plates.
10. Microtiter plate reader capable of reading absorbance (Optical Density, OD) at $\lambda_{600\text{nm}}$.
11. Humidified CO₂ incubator, with a shaking platform.
12. Class II biosafety cabinet.

2.6. Washed agarose

1. Ultra-pure agarose (Invitrogen).
2. 100% Ethanol.
3. 100% Methanol.
4. Centrifuge capable of attaining a speed of at least 3000 x g.
5. Centrifuge bottles of composition able to withstand exposure to 100% ethanol and 100% methanol.
6. Metal free, acid-washed glass tray and bottle for storage.
7. Stirring platform and stir-bar.
8. Fume hood.

2.7. Solid, low Fe or low Zn medium using GC medium base

1. GC medium base (Difco).
2. Supplement I.

3. 12 mM Supplement II: $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (final concentration of stock).
4. 50 mM Desferal stock solution.
5. 40 mM TPEN stock solution.
6. 50 mM ZnSO_4 stock solution in deionized H_2O .
7. Autoclave.
8. Acid-washed flasks.
9. Stirring platform and stir-bar.
10. Water bath.

2.8. Solid, low Fe or low Zn medium using CDM

1. 4x stock solution of liquid CDM (Table 1).
2. Stock solutions 6, 7 and 8 (Table 1).
3. Washed agarose (see Section 2.6. above).
4. Potato starch.
5. 12 mM Supplement II : $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and/or 50 mM ZnSO_4 stock solution for Fe- and Zn-replete conditions, respectively.
6. Other possible Fe metal sources: partially-saturated human transferrin (Calbiochem or Sigma Aldrich) (2,17), partially-saturated human lactoferrin (Calbiochem or Sigma Aldrich) (18,19), hemin (Sigma Aldrich) (20), hemoglobin (Sigma Aldrich) (21,22).
7. Other possible Zn source: calprotectin (10, 23).
8. Stirring platform and stir-bar.
9. Autoclave.
10. Water bath.
11. Sterile Petri plates (90mm).
12. Class II Biosafety cabinet or other laminar flow hood to ensure sterility.

3. Methods

3.1. Acid-washed glassware

1. Dilute concentrated nitric acid with four volumes of deionized H_2O . For example, in a large tub, add 1 gallon of concentrated nitric acid to 4 gallons of deionized H_2O (see Notes 1 and 4).

⁴Generating the nitric acid solution as described results in a solution of greater than 3N. However, H_2O from the glassware will dilute the acid bath solution over time. The nitric acid will take on a yellow color as it ages and accumulates Fe.

2. Wash glassware (see Note 5) with Aquet detergent diluted to 1% (v/v) in deionized H₂O.
3. Rinse 4-5 times with tap H₂O.
4. Rinse 4-5 times with deionized H₂O.
5. Drain in dish drying rack.
6. Place clean glassware in nitric acid bath at least overnight. Make sure the entire inside surface of the glassware is coated with acid.
7. Carefully remove glassware from acid bath and rinse 5 times with deionized H₂O.
8. Drain glassware until thoroughly dry.
9. Cap glassware or cover with aluminum foil.
10. Place glassware upside down in rack and bake overnight in a hot air oven set to 220°C (see Notes 6 and 7).

3.2. Growing *N. gonorrhoeae* under low Fe or low Zn conditions in GC broth

1. Pour GC medium plates. Dissolve GC medium base (Difco) in 1 L of deionized H₂O. Add a stir bar to the flask. Autoclave the medium for 20 minutes on slow exhaust. Cool the medium to 56°C in a waterbath; place flask on stirring plate and add Supplements I and II. Per 1 L of medium, use 10 mL of Supplement I (1/100) and 1 mL of Supplement II (1/1000). After Supplements have been added, pour medium into petri dishes and allow to become solid. Dry the plates briefly in laminar flow hood or class II Biosafety cabinet before use.
2. Two days prior to the experiment, inoculate *N. gonorrhoeae* strains from freezer stock (see Note 3) stored at -80°C, onto GC medium plates (see Note 8).
3. One day prior to the experiment, pass a few colonies of *N. gonorrhoeae* from GC medium plates to fresh GC medium plates. Streak for isolated, single colonies.
4. On the day of the experiment, add the desired volume of GC medium to acid-washed flasks. Add Supplement I to all flasks. Add Supplement II to all flasks *except* those that will be Fe-depleted.
5. Inoculate the flasks with *N. gonorrhoeae* from 24-hour old GC medium plates. A 125 ml side-arm flask with baffles on the bottom, containing no more than 25 mL of GC medium, works best for aerated growth of *N. gonorrhoeae*. Use a

⁵Plastic storage containers and disposable tubes, etc. cannot be acid washed. These do not carry contaminating metals but virtually all glassware harbors additional metals that must be removed by acid washing.

⁶Some laboratory glassware dishwashers have a final acid wash cycle. After acid washing and a final rinse, glassware should be washed additionally a minimum of 3 times with deionized H₂O. Glassware can then be air dried and sterilized in a hot air oven.

⁷Except for media, avoid autoclaving for sterilization of any materials used for metal-depleted growth. Steam is generated from in-house H₂O systems, which contain adventitious metals. These metals will be deposited upon glassware that is autoclaved. Therefore sterilization is instead accomplished by heating in a hot air oven at 220°C.

⁸When handling *N. gonorrhoeae* cultures, use appropriate PPE including gloves and lab coat. If there is a risk of aerosolization, as in opening tubes, do so inside a Class II Biosafety cabinet.

cotton swab to transfer growth from plate to liquid medium. Inoculate to a density of approximately 20 Klett Units (KU), quantified using a Klett-Summerson Colorimeter (see Note 9).

6. Grow cultures in a gyratory, shaking incubator with vigorous shaking (225 rpm) at 35° in an atmosphere of 10% (v/v) CO₂.
7. When the culture density doubles (20 KU increases to 40 KU in 1-2 hours), add the appropriate metal chelators. To initiate Fe stress, add Desferal to a final concentration of 50 µM. To initiate Zn stress, add TPEN to a final concentration of 10 µM.
8. Add Supplement II for Fe-replete cultures and no Desferal.
9. To generate Zn-replete conditions, add ZnSO₄ to a final concentration of 15-20 µM. No TPEN should be added to the Zn-replete cultures.
10. Grow all cultures with shaking (225 rpm) for 3-4 hours.
11. At the end of the incubation period, harvest the cells by centrifugation (see Note 10) for the desired assay (Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), Western blot, binding assay, etc.). For most applications, collection of cultures in sterile 1.5 mL Eppendorf tubes followed by microcentrifugation at 16,000 x g for 5 min yields sufficient cell mass. Standardize the individual cultures by density reading (KU) or by protein assay.

3.3. Preparation of Liquid CDM (chelexed defined medium)

1. Prepare stock solutions 1, 4 and 5 as described in Table 1. Ingredients should be added in the masses listed and dissolved in deionized H₂O (see Note 2).
2. Prepare stock solution 2 by adding the appropriate masses of the ingredients listed in Table 1 to a solution of 50% (v/v) ethanol.
3. Prepare stock solution 3 by adding the amino acids listed in Table 1 (see Note 11) in indicated masses for 1 L or 4 L of stock solution. Dissolve L-cysteine and L-cystine in a small amount of 1N HCL before addition to solution 3. After ingredients are nearly dissolved in deionized H₂O, pH the solution to above 10. At this pH, all constituents should fully dissolve.
4. Sterilize solutions 1-5 in disposable 0.45 µm filter units. Store individually in plastic tubes or bottles in the indicated volumes to be used for preparing the 4x medium (column 2, Table 1).
5. Store solutions 1-5 long-term at -20°C.

⁹. 20 Klett Units corresponds to approximately 1.8×10^8 cells per mL of culture.

¹⁰. Centrifugation of *N. gonorrhoeae* cultures risks creating an aerosol; therefore, open centrifuged tubes inside a Class II Biosafety cabinet.

¹¹. Reduced glutathione and cystine should be stored under desiccation as they are both easily oxidized in ambient air.

6. Prepare solutions 6, 7 and 8 by adding the indicated mass of each ingredient (Table 1) to deionized H₂O. Solutions 6, 7 and 8 should be sterilized by filtration and stored at 4°C until used to prepare the 1x medium.
7. To prepare a 4x concentrated solution, mix filter-sterilized solutions 1-5 with 20 g of solid HEPES (Table 1). The volumes of solutions 1-5 are as follows: 50 mL of solution 1, 20 mL of solution 2, 250 mL of solution 3, 50 mL of solution 4 and 20 mL of solution 5. Mix these pre-measured solutions with solid HEPES and stir until dissolved. Adjust the pH of this solution to pH 7.4 and bring up the final volume to 500 mL with deionized H₂O (see Note 2).
8. In a separate container, add 50 g (per 500 mL of 4x concentrate to be prepared) of Chelex-100 to 1 L of deionized H₂O. Stir to wash the Chelex-100 to remove residual preservatives.
9. Filter out washed Chelex-100 into the top chamber of a 0.45 µm filter unit.
10. Add the washed Chelex-100 from step 9 above to the 4x concentrated solution from step 7 above. Stir slowly at room temperature for 90 minutes (see Note 12).
11. Remove and discard the used Chelex-100 by filtration through a 0.45 µm filter unit.
12. Store the deferrated 4x concentrated stock at 4°C in a sterile plastic container.
13. Immediately prior to use, dilute the 4x concentrated stock 1:4 with deionized H₂O. Before the final volume is reached, add appropriate volumes (column 3 in Table 1) of solutions 6, 7 and 8. Bring the solution up to the final volume with deionized H₂O (see Note 2) and sterilize by filtration.

3.4. Growing *N. gonorrhoeae* under low Fe or low Zn conditions in CDM in flasks

1. Two days before the experiment, inoculate GC medium plates (containing both Supplements I and II) with *N. gonorrhoeae* strains from freezer stocks stored at -80°C (see Note 8).
2. One day before the experiment, pick a few colonies from GC medium plates and streak for isolation onto fresh GC medium plates.
3. On the day of the experiment, dilute the 4x CDM down to 1x, if necessary, or warm 1x medium to room temperature, if it has been stored at 4°C.
4. Add desired volume of CDM to acid-washed side-arm flasks. 5 mL of CDM for initial inoculation is recommended.
5. Inoculate the medium with *N. gonorrhoeae* colonies from 24-hour old plates, to a density of 20 KU, quantified using a Klett Summerson Colorimeter.
6. Grow for 1-2 hours at 35°C with 10% (v/v) CO₂ with vigorous shaking (225 rpm).

¹²Do not incubate the medium with Chelex-100 for more than 90 minutes, as metals will become too limited and *N. gonorrhoeae* will not grow, even if sufficient Zn and Fe are supplied.

7. When cultures have doubled in density (from 20 KU to 40 KU), back-dilute cultures by adding 3 volumes of fresh CDM (pre-warmed). This should result in a culture at a density of approximately 10 KU. For example, if the starting volume was 5 mL, add 15 mL of fresh CDM at room temperature.
8. At the time of back-dilution, for Fe-replete controls, add Supplement II to a final concentration of 10-20 μ M.
9. For Zn-replete controls, add ZnSO₄ stock solution to a final concentration of 15-20 μ M.
10. Fe-depleted and Zn-depleted cultures require no further additions.
11. Grow the bacteria for 3-4 hours in a shaking incubator at 35°C with 10% (v/v) CO₂ with vigorous shaking (225 rpm). The final density should be 60-80 KU for metal-depleted cultures. Metal-replete cultures can reach as high as 120 KU.
12. At the end of the incubation period, harvest the cells (as described in Section 3.2. step 11) for the desired assay (RT-PCR, Western blot, binding assay, etc.). Standardize the cultures by density reading (KU) or by protein assay.

3.5. Growing *N. gonorrhoeae* under low Fe or low Zn conditions in microtiter plates

1. Two days before the experiment, inoculate GC medium plates (containing both Supplements I and II) with *N. gonorrhoeae* strains from freezer stocks stored at -80°C (see Note 8).
2. One day before the experiment, pick a few colonies from GC medium plates and streak for isolation onto fresh GC medium plates.
3. On the day of the experiment, dilute the 4x CDM down to 1x, if necessary, or warm 1x medium to room temperature, if it has been stored at 4°C.
4. Add 10 mL of CDM to acid-washed side-arm flasks.
5. Inoculate medium with *N. gonorrhoeae* from 24-hr old plates, to a density of 20 KU, quantified using a Klett-Summerson Colorimeter.
6. Incubate the flasks at 35°C with 10% (v/v) CO₂ with vigorous shaking (225 rpm).
7. Cultures should double from 20 KU to 40 KU in approximately 1 hour. Measure the OD of the culture by placing 100 μ L of each culture into wells of the microtiter dish. Measure the OD $\lambda_{600\text{nm}}$ using a microtiter plate reader.
8. Dilute each culture with fresh, warm 1x CDM to a standardized density of OD $\lambda_{600\text{nm}}$ = 0.02 (see Note 13).

¹³The microtiter format enables testing of multiple strains and growth conditions simultaneously. Manipulation and dilution of *N. gonorrhoeae* liquid cultures must be accomplished quickly as the pathogen is fragile and dies quickly at room temperature without CO₂.

9. Add 90 μ L of each diluted culture to a microtiter well. Dilute desired Fe or Zn sources in filter-sterilized PBS and add a volume of these supplements so that the final culture volume in the microtiter well is 100 μ L.
10. Swirl the microtiter plate to mix the culture with the supplements. Take an initial OD $\lambda_{600\text{nm}}$ reading before incubating the plate. Affix the plate onto a rotating platform in a stationary incubator. Incubate at 35°C with 10% (v/v) CO₂ with the platform rotating at 200 rpm. Remove the plate at 1-2 hour intervals to monitor growth via OD $\lambda_{600\text{nm}}$ readings taken with a microtiter plate reader. Monitor growth over 6 to 8 hours.

3.6. Washed agarose

1. Weigh out 50 g of ultra-pure agarose in a plastic weigh boat.
2. Add agarose to 1 L of deionized H₂O. Add a stir bar and stir for 1 hour. Transfer to a centrifuge bottle and centrifuge for 15 min at 300 x g at room temperature. Discard H₂O by decanting carefully.
3. Add enough deionized H₂O to the agarose pellet to transfer all of the agarose to a 1 L flask. Bring volume up to 1 L with deionized H₂O. Stir for 1 hour at room temperature. Transfer to centrifuge bottles and centrifuge to obtain a pellet as in step 2. Discard H₂O by decanting carefully.
4. Add 100% ethanol to the agarose pellet in the centrifuge bottle. Transfer to a 1 L flask. Bring up the volume to 1 L with 100% ethanol. Wash for 1 hour at room temperature. Transfer to centrifuge bottles and centrifuge to obtain a pellet as in step 2. Discard ethanol by decanting carefully.
5. Repeat step 4 above.
6. Add 100% methanol to each agarose pellet. Transfer to 1 L flask. Bring up the volume to 1 L with 100% methanol. Wash for 1 hour at room temperature. Transfer to centrifuge bottles and centrifuge to obtain a pellet as in step 2. Discard methanol by decanting carefully.
7. Repeat step 6 above.
8. Prepare a glass metal-free tray by acid-washing as described in Section 3.1. Transfer the washed agarose to the metal-free tray. Dry in a fume hood (see Note 14).
9. Transfer the completely-dried agarose to a sterile, acid-washed glass bottle for long-term storage.

¹⁴. If the agarose is added to the tray in a thin layer, incubation in the fume hood to remove the residual alcohols may take as little as 24 hours. If the agarose layer is thick, complete evaporation of the alcohols may take more than 24 hours. Make sure that all residual alcohols are evaporated from the washed agarose before adding to medium to be poured into plates. Any contaminating alcohol will inhibit the growth of *N. gonorrhoeae*.

3.7. Solid, low Fe or low Zn medium using GC medium base.

1. Weigh out 36 g of GC medium base per 1 L of medium in a plastic weigh boat. Add the powdered medium base to a 2 L flask. This flask need not be acid washed since chelators will be added to remove residual metals.
2. Bring volume up to 1 L with deionized H₂O.
3. Add a stir bar to the flask before covering with foil. Autoclave for 20 minutes with slow exhaust.
4. Cool the sterilized medium to 56°C in a water bath.
5. Remove the flask from the water bath when cool and place on stirring plate.
6. Using sterile technique, add Supplement I to all flasks: 10 mL per 1 L of medium.
7. Add Supplement II to any plates that are to be Fe-replete: 1 mL per 1 L of medium.
8. Add Zn stock solution to any plates that are to be Zn-replete: 375 µL of 50 mM stock solution per 1 L of medium.
9. Add Desferal to a final concentration of 5 µM to any plates that are to be Fe-depleted: 100 µL of 50 mM stock solution per 1 L medium.
10. Add TPEN to a final concentration of 10 µM to any plates that are to be Zn-depleted. 250 µL of 40 mM stock solution per 1 L medium.
11. Using sterile technique, pour approximately 25 mL molten medium into each sterile petri dish.
12. Allow agar to set at room temperature.
13. For long-term storage, store at 4°C.

3.8. Solid, low Fe or Zn medium using CDM

1. Per 100 mL of media for plates, mix 1 g of washed agarose with 0.5 g of potato starch in 73 mL of deionized H₂O. Add a stir bar to the flask. Cover with foil and autoclave the solution for 20 minutes with slow exhaust.
2. Cool the sterilized agarose to 56°C in a water bath.
3. To the cooled, molten agarose add 25 mL of 4x CDM, 25 µL of solution 6, 107 µL of solution 7 and 2 mL of solution 8.
4. For Fe/Zn-replete conditions add 10-20 µM of ferric nitrate (Supplement II), 2.5 µM of transferrin or lactoferrin, 15-20 µM of ZnSO₄ or 5-10 µM of calprotectin.
5. Distribute approximately 20 mL of molten agarose medium into each petri dish: 100 mL of medium results in approximately 5 plates.
6. Allow the agar to set at room temperature and store long-term at 4°C.

7. Dry plates briefly in a laminar flow hood or class II Biosafety cabinet before inoculating with *N. gonorrhoeae* (see Note 15).

Acknowledgements

This work was supported by the National Institute of Allergy and Infectious Diseases grants R01 AI 125421 and AI 127793. The author also thanks the members of her lab for critical feedback on the manuscript.

5. References

1. Weyand NJ (2017) *Neisseria* models of infection and persistence in the upper respiratory tract. *Pathog Dis* 75 (3). doi:3078547 [pii] 10.1093/femspd/ftx031
2. Cornelissen CN, Sparling PF (1996) Binding and surface exposure characteristics of the gonococcal transferrin receptor are dependent on both transferrin-binding proteins. *J Bacteriol* 178 (5):1437–1444 [PubMed: 8631722]
3. Ngampasutadol J, Ram S, Blom AM et al. (2005) Human C4b-binding protein selectively interacts with *Neisseria gonorrhoeae* and results in species-specific infection. *Proc Nat Acad Sci USA* 102 (47):17142–17147 [PubMed: 16275906]
4. Ngampasutadol J, Ram S, Gulati S et al. (2008) Human factor H interacts selectively with *Neisseria gonorrhoeae* and results in species-specific complement evasion. *J Immunol* 180 (5):3426–3435. doi:180/5/3426 [pii] [PubMed: 18292569]
5. Sintsova A, Wong H, MacDonald KS et al. (2015) Selection for a CEACAM receptor-specific binding phenotype during *Neisseria gonorrhoeae* infection of the human genital tract. *Infect Immun* 83 (4):1372–1383. doi:IAI.03123-14 [pii] 10.1128/IAI.03123-14 [PubMed: 25605771]
6. Kochan I (1973) The role of iron in bacterial infections, with special consideration of host-tubercle bacillus interaction. *Curr Top Microbiol Immunol* 60:1–30 [PubMed: 4197776]
7. Weinberg ED (1977) Infection and iron metabolism. *Am J Clin Nutr* 30 (9):1485–1490 [PubMed: 409273]
8. Weinberg ED (1978) Iron and infection. *Microbiol Rev* 42 (1):45–66 [PubMed: 379572]
9. Cornelissen CN (2018) Subversion of nutritional immunity by the pathogenic *Neisseriae*. *Pathog Dis* 76 (1). doi:4553517 [pii] 10.1093/femspd/ftx112
10. Zackular JP, Chazin WJ, Skaar EP (2015) Nutritional Immunity: S100 Proteins at the Host-Pathogen Interface. *J Biol Chem* 290 (31):18991–18998. doi:R115.645085 [pii] 10.1074/jbc.R115.645085 [PubMed: 26055713]
11. Anderson JE, Hobbs MM, Biswas GD et al. (2003) Opposing selective forces for expression of the gonococcal lactoferrin receptor. *Mol Microbiol* 48 (5):1325–1337 [PubMed: 12787359]
12. Cornelissen CN, Kelley M, Hobbs MM et al. (1998) The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers. *Mol Microbiol* 27 (3):611–616 [PubMed: 9489672]
13. Ronpirin C, Jerse AE, Cornelissen CN (2001) The gonococcal genes encoding transferrin binding proteins (Tbp) A and B are arranged in a bicistronic operon but are subject to differential expression. *Infect Immun* 69 (10):6336–6347 [PubMed: 11553578]
14. Yu C, McClure R, Nudel K et al. (2016) Characterization of the *Neisseria gonorrhoeae* Iron and Fur Regulatory Network. *J Bacteriol* 198 (16):2180–2191. doi:JB.00166-16 [pii] 10.1128/JB.00166-16 [PubMed: 27246574]
15. Kellogg DS Jr., Peacock WL Jr, Deacon WE et al. (1963) *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J Bacteriol* 85:1274–1279 [PubMed: 14047217]
16. West SEH, Sparling PF (1987) Aerobactin utilization by *Neisseria gonorrhoeae* and cloning of a genomic DNA fragment that complements *Escherichia coli* *thiB* mutations. *J Bacteriol* 169 (8):3414–3421 [PubMed: 3112120]

15. When inoculating CDM plates, collect *N. gonorrhoeae* cells from GC medium base plates containing both Supplements I and II. Strains will not grow reproducibly on this chelexed, defined medium if inoculated directly from freezer stocks.

17. Cornelissen CN, Biswas GD, Tsai J et al. (1992) Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. *J Bacteriol* 174 (18):5788–5797 [PubMed: 1325963]
18. Biswas GD, Anderson JE, Chen C-j et al. (1999) Identification and functional characterization of the *Neisseria gonorrhoeae lbpB* gene product. *Infect Immun* 67 (1):455–459 [PubMed: 9864256]
19. Biswas GD, Sparling PF (1995) Characterization of *lbpA*, the structural gene for a lactoferrin receptor in *Neisseria gonorrhoeae*. *Infect Immun* 63 (8):2958–2967 [PubMed: 7622218]
20. Strange HR, Zola TA, Cornelissen CN (2011) The *fbpABC* operon is required for Ton-independent utilization of xenosiderophores by *Neisseria gonorrhoeae* strain FA19. *Infect Immun* 79 (7):267–678 [PubMed: 21041493]
21. Chen CJ, Elkins C, Sparling PF (1998) Phase variation of hemoglobin utilization in *Neisseria gonorrhoeae*. *Infect Immun* 66:987–993 [PubMed: 9488386]
22. Chen C-J, Sparling PF, Lewis LA et al. (1996) Identification and purification of a hemoglobin-binding outer membrane protein from *Neisseria gonorrhoeae*. *Infect Immun* 64 (12):5008–5014 [PubMed: 8945539]
23. Gilston BA, Skaar EP, Chazin WJ (2016) Binding of transition metals to S100 proteins. *Sci China Life Sci* 59 (8):792–801. doi:10.1007/s11427-016-5088-410.1007/s11427-016-5088-410.1007/s11427-016-5088-410.1007/s11427-016-5088-4 [pii] [PubMed: 27430886]

Table 1.

Composition of chelexed defined medium (CDM)

Component	Conc. (g/L) of stock solution		Vol. of stock /500 ml of 4X concentrate	Conc. (mM) in 1X medium
Stock solution 1			50 ml	
NaCl	233.8			100.0
K ₂ SO ₄	40.0			5.7
NH ₄ Cl	8.8			4.1
K ₂ HPO ₄	13.9			2.0
KH ₂ PO ₄	10.9			2.0
Stock solution 2^a			20 ml	
Thiamine HCl	0.2			0.006
Thiamine pyrophosphate-Cl (=cocarboxylase)	0.05			0.001
Calcium pantothenate	0.19			0.004
Biotin	0.3			0.012
Stock solution 3	Per 1L	Per 4L	250 ml	
L-aspartic acid	4.0	16.0		3.76
L-glutamic acid	10.4	41.6		8.84
L-arginine	1.2	4.8		0.71
Glycine	0.2	0.8		0.33
L-serine	0.4	1.6		0.48
L-leucine	0.72	2.88		0.69
L-isoleucine	0.24	0.96		0.23
L-valine	0.48	1.92		0.51
L-tyrosine	0.56	2.24		0.39
L-cysteine HCl ^b	0.44	1.76		0.35
L-cystine ^b	0.28	1.12		0.15
L-proline	0.4	1.6		0.43
L-tryptophan	0.64	2.56		0.39
L-threonine	0.4	1.6		0.42
L-phenylalanine	0.2	0.8		0.15
L-asparagine·H ₂ O	0.2	0.8		0.17
L-glutamine	0.4	1.6		0.34
L-histidine HCl	0.2	0.8		0.13
L-methionine	0.12	0.48		0.10
L-alanine	0.8	3.2		1.12
L-lysine	0.4	1.6		0.29
Glutathione (reduced)	0.36	1.44		0.15
Stock solution 4			50 ml	
Glucose	200			27.8
Stock solution 5			20 ml	

Component	Conc. (g/L) of stock solution	Vol. of stock /500 ml of 4X concentrate	Conc. (mM) in 1X medium
Hypoxanthine	5.0		0.37
Uracil	5.0		0.45
NaOH	4.0		
N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid (HEPES)		20.0 gm	21.0
Stock solution 6			
1M CaCl ₂ ·2H ₂ O	147.0		0.25 (add 125 µl per 500 ml 1X solution)
Stock solution 7			
1M MgSO ₄ (anhydrous)	120.4		1.07 (add 535 µl per 500 ml 1X solution)
Stock solution 8			
1M NaHCO ₃	84.0		20.0 (add 10 ml per 500 ml 1X solution)

^aStock solution 2 is prepared in 50% (v/v) ethanol; all others are prepared in deionized H₂O.

^bL-cysteine and L-cystine are dissolved in a small amount of 1N HCl before addition to stock solution 3.