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TITLE:

Metal-Limited Growth of *Neisseria Gonorrhoeae* for Characterization of Metal-Responsive Genes and Metal Acquisition from Host Ligands

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SUMMARY:

We describe here a method for growth of *Neisseria gonorrhoeae* in metal-restricted liquid medium to facilitate the expression of genes for metal uptake. We also outline downstream experiments to characterize the phenotype of gonococci grown in these conditions. These methods may be adapted to be suitable for characterization of metal-responsive genes in other bacteria.

ABSTRACT:

Trace metals such as iron and zinc are vital nutrients known to play key roles in prokaryotic processes including gene regulation, catalysis, and protein structure. Metal sequestration by hosts often leads to metal limitation for the bacterium. This limitation induces bacterial gene expression whose protein products allow bacteria to overcome their metal-limited environment. Characterization of such genes is challenging. Bacteria must be grown in meticulously prepared media that allows sufficient access to nutritional metals to permit bacterial growth while maintaining a metal profile conducive to achieving expression of the aforementioned genes. As such, a delicate balance must be established for the concentrations of these metals. Growing a nutritionally fastidious organism such as *Neisseria gonorrhoeae,* which has evolved to survive only in the human host, adds an additional level of complexity. Here, we describe the preparation of a defined metal-limited medium sufficient to allow gonococcal growth and the desired gene expression. This method allows the investigator to chelate iron and zinc from undesired sources while supplementing the media with defined sources of iron or zinc, whose preparation is also described. Finally, we outline three experiments that utilize this media to help characterize the protein products of metal-regulated gonococcal genes.

INTRODUCTION:

Neisseria gonorrhoeae causes the common sexually-transmitted infection gonorrhea. During infection, pathogenic *Neisseria* express a repertoire of metal-responsive genes that allow the bacteria to overcome metal restriction efforts by the human host[1-3]. Trace metals like iron and zinc play key roles in many cellular processes, such as binding to enzymes in catalytic sites, participation in redox reactions, and as structural factors in various proteins[4,5]. In metal-limited conditions, metal-responsive loci are derepressed and their resultant proteins can aid the acquisition of these nutrients. Characterization of these genes and proteins presents a unique technical challenge for the investigator. Metal ions must be withheld from bacteria in order to induce transcription of these genes from their native loci, but effective chelation of these ions from metal-laden media can be difficult to optimize. The different metal profiles of source water and inherent lot-to-lot variation[6] of powdered ingredients means that the amount of chelator required to remove a specific metal from a rich medium will vary between different locations, ingredient vendors, and even over time within a single laboratory as chemical inventory is replaced.

To circumvent this challenge, we describe the preparation of a defined medium that is treated with Chelex-100 resin during preparation to remove trace metals from the solution. This medium is sufficiently nutrient dense to allow for the growth of gonococcus, which is difficult to culture outside of the human host, and allows the investigator to introduce a specific metal profile by addition of their own defined sources and concentrations of metals. The method of controlled add-back of desired metals to depleted medium increases experimental consistency and allows for robust, replicable experiments regardless of factors such as water source and chemical lot numbers. Moreover, this media can be deployed as either a liquid or solid with only minor modifications, making it quite versatile.

In order to demonstrate the utility of the this medium, we outline a protocol for its use for gonococcal growth and describe three successful experiments to characterize metal-responsive *Neisseria* genes. First, we prepare gonococcal whole-cell lysates from metal-depleted or supplemented cultures and demonstrate variable levels of protein production from metal-responsive loci. We then outline a zinc-restricted growth assay in which gonococcal growth is controlled by supplementation of specific, useable zinc sources. Finally, we show binding assays that demonstrate whole gonococcal cells expressing metal-responsive surface receptors binding to their respective metal-containing ligands. Successful surface presentation of these receptors requires growth in metal-depleted medium.

The present protocol was optimized specifically for *Neisseria gonorrhoeae*, but numerous other bacterial pathogens employ metal acquisition strategies during infection[7], so this protocol may be adapted for the study of metal homeostasis in other bacteria. Optimizing this media and these experimental protocols for use in other bacteria will likely require slight modification of metal chelator concentrations and/or treatment time with Chelex-100, as other bacteria may have slightly different metal requirements than gonococcus. Iron and zinc are the primary metals of concern for the described investigations, but other metals (e.g., manganese) have been demonstrated as critical for bacteria, including *Neisseria*[8-12]. Furthermore, similar methods have been described for metal characterizations in eukaryotic cell culture work, which may also be

considered.[13]

PROTOCOL:

1. Preparation of Chelex-treated defined medium (CDM) stock solutions

1.1. Stock solution I

1.1.1. Combine NaCl (233.8 g), K_2SO_4 (40.0 g), NH_4Cl (8.8 g), K_2HPO_4 (13.9 g), and KH_2PO_4 (10.9 g) in deionized water to a final volume of 1 L.

1.1.2. Filter sterilize the solution and aliquot into 50 mL conical tubes.

1.1.3. Store at -20 °C.

1.2. Stock solution II

1.2.1. Combine thiamine HCl (0.2 g), thiamine pyrophosphate-Cl (0.05 g), calcium pantothenate (0.19 g), and biotin (0.3 g) in 50% (vol/vol) ethanol to a final volume of 1 L.

1.2.2. Aliquot into 50 mL conical tubes and store at -20 °C.

1.3. Stock solution III

1.3.1. Combine L-aspartate (4.0 g), L-glutamate (10.4 g), L-arginine (1.2 g), glycine (0.2 g), L-serine (0.4 g), L-leucine (0.72 g), L-isoleucine (0.24 g), L-valine (0.48 g), L-tyrosine (0.56 g), L-proline (0.4 g), L-tryptophan (0.64 g), L-threonine (0.4 g), L-phenylalanine (0.2 g), L-asparagine-H_2O (0.2 g), L-glutamine (0.4 g), L-histidine-HCl (0.2 g), L-methionine (0.12 g), L-alanine (0.8 g), L-lysine (0.4 g), and reduced glutathione (0.36 g) in 500 mL deionized water.

1.3.2. Dissolve L-cysteine (0.44 g) and L-cystine (0.28 g) in a minimal volume (~1 mL) of 1 M HCl and add to the above amino acid solution.

1.3.3. Adjust the pH of the solution with 10 N NaOH until all particulate is dissolved. The final pH will be 10.0–11.0.

1.3.4. Bring the final volume to 1 L with deionized water.

1.3.5. Filter sterilize the solution and aliquot into 250 mL volumes.

1.3.6. Store at -20 °C.

1.4. Stock solution IV

1.4.1. Dissolve glucose (200 g) in deionized water to a final volume of 1 L.

NOTE: The solution may have to be heated to dissolve the glucose.

1.4.2. Filter sterilize and aliquot the solution into 50 mL conical tubes.

1.4.3. Store at -20 °C.

1.5. Stock solution V

1.5.1. Combine hypoxanthine (5.0 g), uracil (5.0 g), and NaOH (4.0 g) in deionized water to a final volume of 1 L.

1.5.2. Filter sterilize and aliquot into 50 mL conical tubes.

1.5.3. Store at -20 °C.

1.6. Solution VI

1.6.1. Prepare a 1 M $CaCl_2$-$2H_2O$ (147 g/L) solution in deionized water. Filter sterilize and store at room temperature (RT).

1.7. Stock solution VII

1.7.1. Prepare a 1 M anhydrous $MgSO_4$ solution in deionized water. Filter sterilize and store at RT.

1.8. Stock solution VIII

1.8.1. Prepare a 1 M $NaHCO_3$ solution in deionized water. Filter sterilize and store at RT.

2. Preparation of 4x sterile concentrate and 1x CDM

NOTE: This procedure is to be performed in either acid treated sterile glassware or plastic to prevent leaching of metals into the solutions.

2.1. Combine stock solutions I (50 mL), II (20 mL), III (250 mL), IV (50 mL), and V (20 mL) with 20.0 g of HEPES and stir to mix.

2.2. Adjust pH to 7.4, then bring the final volume to 500 mL with deionized water.

2.3. Wash Chelex-100 resin in 1 L deionized water to remove preservatives prior to adding it to the 4x sterile concentrate. Do this by adding 50 g of resin to 1 L deionized water and stirring for at least 1 h. Remove the water by vacuum filtration and use this washed resin for step 2.4.

2.4. Add 50 g of resin and slowly stir for exactly 90 min.

2.5. Remove resin by filter sterilization and store the 4x sterile concentrate at 4 °C.

2.6. To prepare a 1x working concentration of CDM, first dilute the 4x concentrate with sterile deionized water, then add solution VI (125 µL per 500 mL of 1x solution), solution VII (535 µL per 500 mL), and solution VIII (10 mL per 500 mL).

NOTE: Although all stock solutions have already been sterilized, it is recommended to filter sterilize the 1x solution again after preparation.

3. Preparation of CDM plates

NOTE: The recipe below makes 1 L media for plates, but it is best to prepare these in smaller volumes. Everything scales down proportionally.

3.1. Washed agarose

3.1.1. Dissolve 50 g of agarose in 1 L deionized water, stirring for 1 h.

3.1.2. Transfer the solution to a centrifuge bottle and centrifuge at 1,200 x *g* for 15 min at RT. Carefully pour off the supernatant and discard.

3.1.3. Add sufficient deionized water to resuspend the agarose pellet, then transfer to a 1 L flask. Bring to a final volume of 1 L with deionized water and then stir and centrifuge as in step 3.1.2. Discard the supernatant.

3.1.4. Add sufficient 100% ethanol to resuspend the pellet, then transfer to a new 1 L flask. Bring to a final volume of 1 L with ethanol. Stir and centrifuge as in step 3.1.2.

3.1.5. Repeat step 3.1.4.

3.1.6. Add methanol to the agarose pellet to resuspend, then transfer to a 1 L flask. Bring to a final volume of 1 L with methanol. Stir and centrifuge as in step 3.1.2.

3.1.7. Repeat step 3.1.6.

3.1.8. Transfer washed agarose to a tray lined with aluminum foil and allow to dry in a fume hood. When dry, transfer to a metal-free container for long-term storage.

3.2. Add 10 g of washed agarose and 5 g of potato starch to 750 mL of deionized water.

3.3. Autoclave for 30 min at 121 °C, 100 kPa above atmospheric pressure.

3.4. Allow media to cool to ~65 °C, then add 250 mL of 4X CDM, 250 µL of solution VI, 1.07 mL of solution VII, and 20 mL of solution VIII.

3.5. If desired, add the metals of choice before pouring plates. The addition of chelators is not necessary to maintain metal-free conditions.

3.6. Pour into Petri dishes and allow to solidify.

4. Metal limited growth of *Neisseria gonorrhoeae*

NOTE: For most applications, it is not necessary to metal stress the bacteria prior to inoculation of CDM. The initial doubling step in CDM and the subsequent dilution is sufficient to deplete the gonococci of their internal iron and zinc stores. As such, the first two steps of the following procedure are conducted using agar plates made from GC medium base that have been supplemented with Kellogg's supplement I[14] and 12.5 µM $Fe(NO_3)_3$. If early metal stress is desired, we recommend preparing GC medium base plates without $Fe(NO_3)_3$ and with 5 µM TPEN (N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine) for zinc chelation or 10 µM deferoxamine for iron chelation. All incubation is conducted at 37 °C with 5% CO_2.

4.1. Two days prior to the experiment, on day -2, streak gonococci from freezer stocks onto GC medium plates and incubate for no more than 24 h.

4.2. On day -1, streak single colonies onto fresh GC medium plates. Try to do this 14–16 h prior to the growth experiment.

4.3. On the day of the experiment, add 5–10 mL 1x CDM to an acid washed 125 mL baffled sidearm flask (**Supplemental Figure 1**) and use this to blank a Klett colorimeter.

4.4. Use a sterile, cotton-tipped swab to inoculate CDM from healthy, single colonies. Aim for 20 Klett units.

NOTE: If a Klett colorimeter is not available, a spectrophotometer is suitable as well. There is no universal conversion formula for Klett units to OD, but a rough guide is available (https://support.hunterlab.com/hc/en-us/articles/214490283-Klett-Color-Scales).

4.5. Incubate with shaking at 250 rpm until approximately one mass doubling (40 Klett units). This should take between 1–2 h.

4.6. At this point, the cultures are back diluted by addition of a sufficient volume of CDM to reach half of the initial culture density (e.g., if 5 mL of culture has gone from 20 to 40 Klett units, 15 mL of CDM will bring it back down to 10 Klett units) and growth will continue as in step 4.5. The specific amount of back dilution, metal treatments, etc. depend on downstream applications. We give three examples below (sections 5, 6, or 7).

5. Western analysis of metal responsive gene products

5.1. Beginning at step 4.6, back dilute the cultures with three volumes of CDM (e.g., 15 mL of CDM if starting with 5 mL). At this point, add metal treatments if desired.

5.1.1. Iron-sensing genes can be derepressed with 12.5 µM $Fe(NO_3)_3$. Zinc-responsive genes may be derepressed with 10 µM $ZnSO_4$.

5.1.2. Additional iron or zinc stress is not necessary, as the media is already depleted, so responsive genes will already be expressed. If further stress is desired, we recommend no more than 1–2 µM of deferoxamine or TPEN, as excessive stress will prevent cultures from growing.

5.2. Grow cultures as in step 4.5 for 4 h and record the final cell density of the samples. Less metal-stressed cultures will grow to higher final densities.

5.3. Standardize cultures to a suitable density and prepare lysates.

5.3.1. Standardize whole-cell lysates to a density equivalent to 100 Klett units in 1 mL of culture. To accomplish this, divide 100 by the Klett unit density of your sample. The number you get is the volume, in mL, of culture that will be used to make the cell pellet.

5.4. Follow standard SDS-PAGE and Western blotting procedures[15] to probe for proteins of interest.

6. Metal-limited growth assays

NOTE: These assays describe premade growth premixes. The preparation of these mixes is described in section 8.

6.1. During the mass doubling in step 4.5, pretreat the wells of a 96 well microplate with 10x premixes. Additionally, designate three wells to serve as blanks. To these wells, add 10 µL of 10x premix and 90 µL of CDM.

6.2. Once the cultures in the sidearm flasks have doubled, add 100 µL of each culture to an unused well in the microplate and measure the optical density at 600 nm (OD_{600}). This may be done with cuvettes in a spectrophotometer, but with larger numbers of strains within an assay this can become cumbersome and is generally not advised unless your spectrophotometer can measure directly from the arm of the side arm flask (**Supplemental Figure 2**).

6.2.1. While measuring the OD, place the flasks back in the incubator to ensure the gonococci remain viable.

6.3. Calculate the correct amount of dilution required to bring the cultures to OD_{600} = 0.02. The

10x premixes have a negligible effect on OD and can be omitted from calculation.

6.4. Dilute cultures with CDM in small culture tubes and add sufficient volume to dilute the 10x premixes to 1x in the plate. If a plate warmer is available, keep the plate at 37 °C while working.

6.5. Incubate the plate for 8–12 h with shaking in a plate reader, taking OD_{600} measurements at desired intervals.

7. Detection of ligand binding by outer membrane metal transporters

7.1. Treat cultures as desired as in step 5.1, then incubate with shaking for 4 h.

7.2. Shortly before the 4 h mark, cut three pieces of filter paper and a piece of nitrocellulose to the approximate size needed to fit into a dot blot apparatus (**Supplemental Figure 3**). Presoak the nitrocellulose in deionized water, then assemble the apparatus with filter paper below the nitrocellulose.

7.3. At 4 h, record the cell densities and standardize to an appropriate final density. It is recommended to use ~10% of the density used for the preparation of the cell lysates in step 5. For example, if using 100 KU in 1 mL for the lysates, this means ~10 KU in 1 mL for these blots. The calculation is otherwise the same as described in 5.3.1, and cultures are added directly to the nitrocellulose in the calculated volumes rather than made into lysates.

7.4. Pipet cell cultures onto the nitrocellulose and allow sufficient time for the filter paper to absorb all the liquid.

7.5. Disassemble the apparatus, allow the blot to dry, and block the nitrocellulose membrane for 1 h in 5% bovine serum albumin or nonfat milk (w/v) in Tris-buffered saline.

7.6. Reassemble the dot blot apparatus, replacing the filter paper with paraffin film to create a leak-proof seal under the nitrocellulose.

7.7. Dilute the metal-binding ligand of interest to 0.2 µM in blocker and probe cells for 1 h.

7.8. Siphon off the liquid with a vacuum, wash the blot, then follow standard immunological procedures to develop the signal[16]. The wash steps may be done in or out of the apparatus.

8. Metal loading of transferrin, S100A7, and calprotectin, and preparation of 10x premixes

NOTE: As with CDM preparation, use acid washed glass or plastic for solution preparation.

8.1. Dissolve human transferrin at 10 mg/mL (125 µM) in initial buffer (100 mM Tris, 150 mM NaCl, 20 mM $NaHCO_3$, pH = 8.4). S100A7 and calprotectin are suspended in buffer consisting of 20 mM Tris, 100 mM NaCl, 10 mM 2-Mercaptoethanol, and 1 mM $CaCl_2$, pH = 8.0).

8.1.1. Add ferration solution (100 mM sodium citrate, 100 mM $NaHCO_3$, 5 mM $FeCl_3$-$6H_2O$, pH = 8.4) to the transferrin solution to achieve 30% iron saturation (e.g., 75 µL of ferration solution is suitable for 5 mL transferrin if made at 10 mg/mL).

8.1.2. Add $ZnSO_4$ to S100A7 or calprotectin at a 50% molar ratio to the protein to create 25% saturation (each protein molecule has two metal binding sites). Preparations of 100 µM S100A7 or calprotectin with 50 µM $ZnSO_4$ can be used.

8.1.3. For both cases, allow end-over-end mixing for at least 1 h for metal loading.

8.2. Prepare 4 L of dialysis buffer (40 mM Tris, 150 mM NaCl, 20 mM $NaHCO_3$, pH = 7.4). Split this into two separate 2 L volumes and place one at 4 °C.

8.3. Add the metal loaded proteins to a dialysis cassette using a syringe and dialyze against the first buffer volume for 4 h at RT.

8.4. Move the cassette to the second buffer volume and dialyze overnight at 4 °C. After these steps, any unbound metals should be removed.

NOTE: We advise a bicinchoninic acid assay to determine protein concentrations after dialysis.

8.5. Use a 10x transferrin premix for transferrin utilization as a sole iron source.

8.5.1. Prepare this premix by diluting 30% human Fe-transferrin and bovine apo-transferrin (prepared at 125 µM as described for human transferrin, without the iron loading step) to 75 µM and 30 µM, respectively, in PBS. A positive control premix replaces 30% Fe-transferrin with 75 µM $Fe(NO_3)_3$, and a negative control premix omits any added iron, retaining only the bovine apo-transferrin. In the growth assay, dilute 10 µL of these concentrates with 90 µL of culture. Final concentrations are 7.5 µM 30% human Fe-transferrin and 3 µM bovine apo-transferrin.

8.6. Use a modified version of the transferrin 10x premix for S100A7 or calprotectin utilization as a sole zinc source.

8.6.1. For a positive control premix, incorporate 50 µM $ZnSO_4$ into the transferrin premix. For a negative control, incorporate 50 µM TPEN and omit the zinc. Then, take 10 µL of each of these for every sample well needed, and move that volume to a new tube. Add to this half as much volume of sterile PBS. For example, if 10 wells will receive the positive control premix, take 100 µL of premix, move it to a new tube, and add 50 µL of PBS. Do the same for the negative control.

8.6.2. To make the S100A7 or calprotectin premix, take 10 µL of the negative control premix per well needed, move to a new tube, and add half that volume of the 25% Zn-S100A7 or calprotectin. In the growth assay, use 15 µL of premix and dilute with 85 µL of culture. Final concentrations for the transferrins remain the same as in step 8.5, with an added 5 µM of Zn, TPEN, or

S100A7/calprotectin.

REPRESENTATIVE RESULTS:

A specific defined medium in the absence of trace metals for the growth of *Neisseria gonorrhoeae* was developed and implemented for the characterization of metal-responsive genes and their gene products. In the optimized protocol, the metal profile of media is controlled by adding metals back at the discretion of the investigator, rather than by titrated chelation of a metal target, allowing for increased control and consistency from lab to lab and experiment to experiment. This media can be employed in both liquid and solid state, making it versatile across many experimental setups.

Differential protein production in variable metal concentrations can be seen in the included representative Western blots (**Figure 1**). The image shows the zinc-responsive outer-membrane transporters TdfJ and TdfH, which were upregulated in response to zinc chelation by TPEN. TdfJ was essentially undetectable when zinc was added back to the media, and TdfH was scarce. Moreover, the *tdfJ* promoter is known to be induced, rather than repressed, by iron. This is also visible in the blot. These blots utilized the iron-responsive lipoprotein TbpB[2] as a loading control. In conditions of iron add-back, TbpB production was reduced.

Metal-restricted growth assays demonstrate the utilization of specific, defined zinc sources by the gonococcus (**Figure 2**). **Figure 2A** shows *N. gonorrhoeae* growing in the presence of Zn-loaded calprotectin (CP), which requires the action of the zinc-responsive TdfH[17,18]. When no useable zinc source was available, either through no zinc add-back or by the absence of TdfH, growth was restricted. **Figure 2B** shows similar results in the presence of S100A7, which can serve as a sole zinc source when the outer membrane transporter TdfJ is produced[19]. In the presence of TPEN alone or when TdfJ was absent, growth was hampered, but addition of Zn-S100A7 recovered growth in a TdfJ-dependent manner. Finally, **Figure 2C** shows an experimental error. In this example, the dilution step of the gonococcal cultures was not sufficient to deplete the bacteria of internal zinc pools relative to the total culture volume in the microplate. As such, growth in the negative control exceeded the desired OD.

Specific binding of whole gonococcal cells to their respective ligands is demonstrated by dot blots from cultures prepared in metal-limiting and metal replete conditions (**Figure 3**). **Figure 3A,B** shows that gonococci grown in CDM were able to bind CP and S100A7 when producing TdfH and TdfJ, respectively, as a result of zinc scarcity. **Figure 3C** compares transferrin binding, which is accomplished by the cognate proteins made from the iron-sensing genes *tbpA* and *tbpB*[20], when gonococci are grown in CDM alone vs. GC medium broth treated with the iron chelator deferoxamine. This figure shows increased binding of transferrin by cultures grown in CDM, which is indicative of higher levels of protein expression due to the more iron-depleted nature of CDM compared to chelated GC broth.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative Western blot showing differential production of metal responsive proteins. (**A**) *Neisseria gonorrhoeae* wild type strain FA19 was grown in CDM supplemented with

$ZnSO_4$, $Fe(NO_3)_3$, or TPEN in the indicated concentrations. After treatment, cultures were grown for 4 h before whole-cell lysates of standardized density were produced and subjected to SDS-PAGE and Western blotting. Differential production levels for TdfJ, which is both repressed by zinc and induced by iron, can be clearly seen in response to zinc addition/depletion and iron addition. (**B**) Relative signal intensities for the Western blot were quantitated via densitometry. This figure is adapted from Maurakis et al[19].

Figure 2: Zinc-restricted growth of gonococci. Wild type strain FA19, or isogenic mutants of this strain that do not produce *tdfJ* or *tdfH,* were grown in (**A**) untreated CDM until the exponential phase was reached, (**B**) then back diluted to OD_{600} = 0.02 and (**C**) OD_{600} = 0.1. The samples in **A** were treated with premix containing calprotectin (top) or no added zinc (bottom) and grown for 8 h. The samples in **B** and **C** were supplemented with premix containing free zinc, no zinc (5 µM TPEN), or S100A7, and grown for 6 h. Growth in these conditions was only recovered upon supplementation of media with a useable zinc source, such as CP, S100A7, or free zinc in **A** and **B**, while **C** was not sufficiently diluted to deplete internal cellular zinc pools. **Figure 2A** is adapted from Jean et al.[17] **Figure 2B** is adapted from Maurakis et al[19].

Figure 3. Representative binding assays show host ligands binding to metal-stressed gonococci. (**A**) Wild type strain FA1090, or mutants of this strain that do not produce *tdfJ, tdfH,* or both, were grown in CDM without supplemental metals and were dotted onto nitrocellulose in standardized densities. Cells were probed with calprotectin, which is recognized by the zinc-responsive TdfH, and binding was assessed by detection with an anti-calprotectin antibody (top). Relative calprotectin binding was quantitated via densitometry, shown here on a log scale (bottom). (**B**) Binding experiments were performed as described in **A**, but instead using the FA19 wild type strain and *tdfJ* mutant and complemented strains in that background. Cells were probed with HRP-labeled S100A7, which is recognized by the zinc-responsive TdfJ. (**C**) Wild type strain FA19 was grown side-by-side in CDM or GC medium broth with 25 µM deferoxamine added to chelate free iron, dotted to nitrocellulose in standardized amounts, and probed with transferrin, which binds the iron-sensitive TbpA. These side-by-side tests show that CDM, unlike GC medium broth, required no additional chelation to achieve an iron-limited environment. **Figure 3A** is adapted from Jean et al. and the bottom from Maurakis et al[19].

DISCUSSION:

Growth media serves a variety of roles in microbiological research. Specialized media are used for selection, enrichment, and various other applications for many unique types of study. One such application is the induction of metal-responsive genes, which is typically accomplished by addition of a specific chelator that targets a particular metal ion. This method is limited, as the amount of chelation necessary for various trace metals is likely to be variable due to different water sources containing unique metal profiles, and two lots of the same media ingredient containing different metal concentrations[6]. To avoid this inherent shortcoming, we have described the preparation and use of a defined medium that is treated with Chelex-100 resin to remove all trace metals in bulk, allowing controlled addition of specified metals back into the medium as needed.

In the current protocol, the first important point of discussion is the source water. The protocol describes a Chelex treatment that is sufficient to remove metals from laboratory Type 2 (1.0 megaOhms-cm according to ISO 3696 specifications) water. Different water sources will likely require shorter or longer Chelex treatments. We have found that water of higher purity than Type 2, such as molecular biology grade water, will not support bacterial growth in this application. The choice of vessel for media preparation is just as important as the water source. We highly recommend clean plastic containers, as glassware may leach metal ions into the solution. If plasticware is not available, glassware must be acid washed to minimize contamination risk. The same acid washing is required for culture flasks when using CDM.

Growth of *Neisseria gonorrhoeae* in an in vitro setting can be quite challenging, as this organism has evolved to thrive specifically in human hosts[21]. While CDM is suitable to support growth for the duration of experiments, care must be taken during the inoculation and dilution steps to ensure that gonococci are not in atmospheric conditions longer than necessary. Due to the capnophilic nature of gonococci[22] and its predilection for temperatures found within human hosts, we do not recommend keeping cultures in atmospheric conditions for longer than ~15 min. If the initial mass doubling event described in step 4.5 of the method takes longer than 2 h, it is likely that gonococcal cultures have not been handled properly and the experiment should be aborted.

While the focus of this method is on methods for growth and characterization of *Neisseria gonorrhoeae* specifically, usage of this specific CDM is likely also suitable for study of other *Neisseria* species. Moreover, it can be easily applied to the characterization of other metal-sensing systems in other bacteria. For example, a similar metal-free media has been used to characterize metal uptake in *Staphylococcus aureus*[23] and *Escherichia coli*[24]. Utilization of the described recipe for other bacteria will likely involve small modifications or additions, depending on the specific nutritional needs of the bacteria in question. For example, supplement VIII is included in the recipe to aid with the gonococcus' need for supplemental CO_2. As described above, growth is performed at 37 °C with a 5% CO_2 atmosphere, but we have found that addition of supplemental bicarbonate in the media aids the initial stages of gonococcal growth in defined medium. For organisms without such a requirement, this ingredient may be omitted. Unfortunately, further examples of these modifications have to be empirically determined.

Despite the need to adapt the method somewhat for use with other bacteria, the basic framework should be appropriate for broad use. Characterization of metal-responsive genes and metal transporters is an ongoing endeavor in microbial study, with bacteria including *Neisseria meningitidis*[18,25-28], *S. aureus*[9], *Haemophilus influenzae*[29], *Salmonella enterica*[30], and *E. coli*[31] all receiving attention in this niche. Our own future utilization of this technique will aim to further the understanding of other gonococcal metal uptake systems beyond those already mentioned, which allow the gonococcus to acquire iron from host proteins such as lactoferrin[32,33], hemoglobin[34,35], and also from bacterial xenosiderophores[36], and to expand our studies into the effects of other metals such as manganese, which has been implicated in oxidative stress defense by *Neisseria*[12,37].

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to declare.

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Figure 1

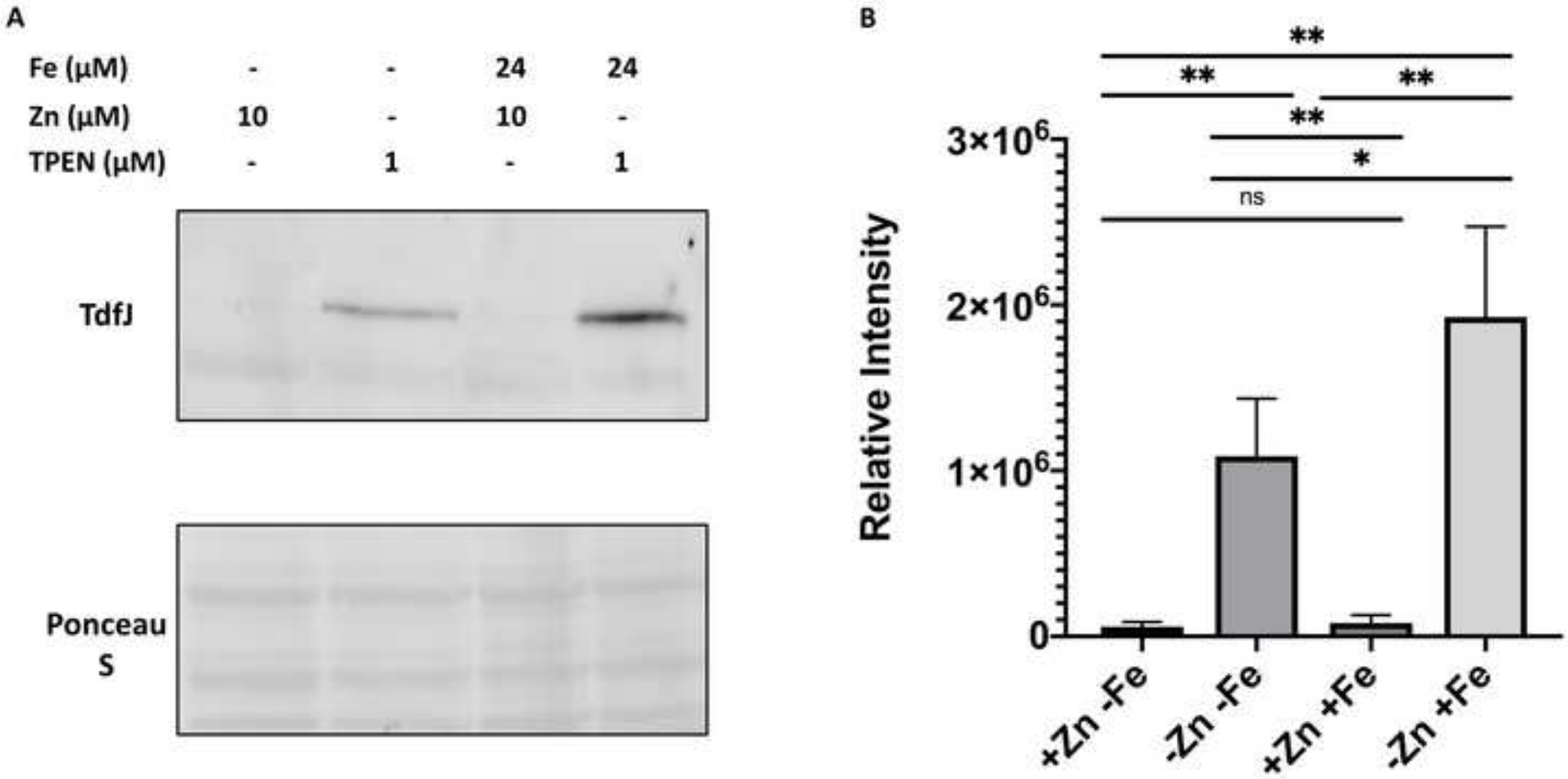


Figure 2

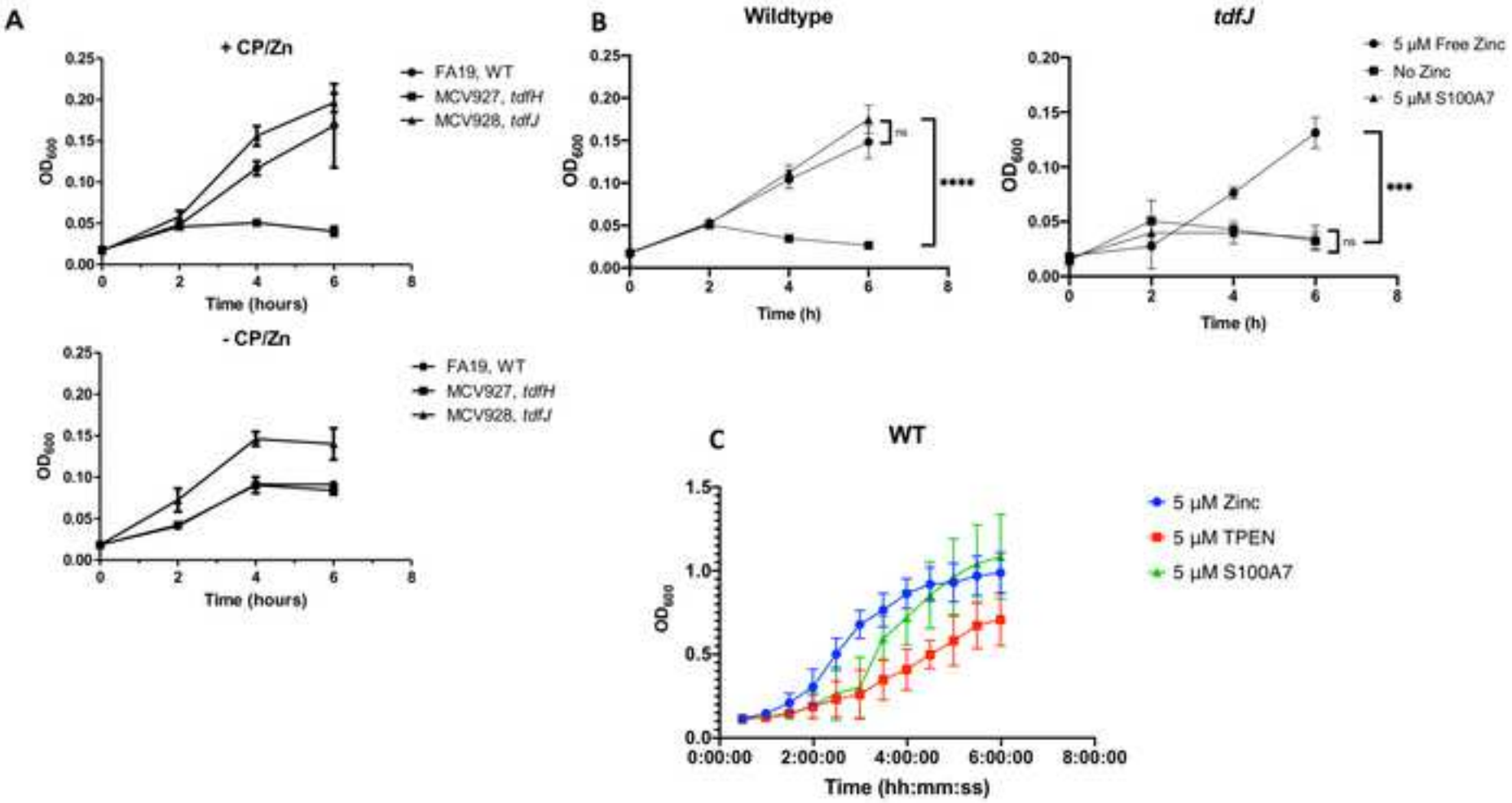
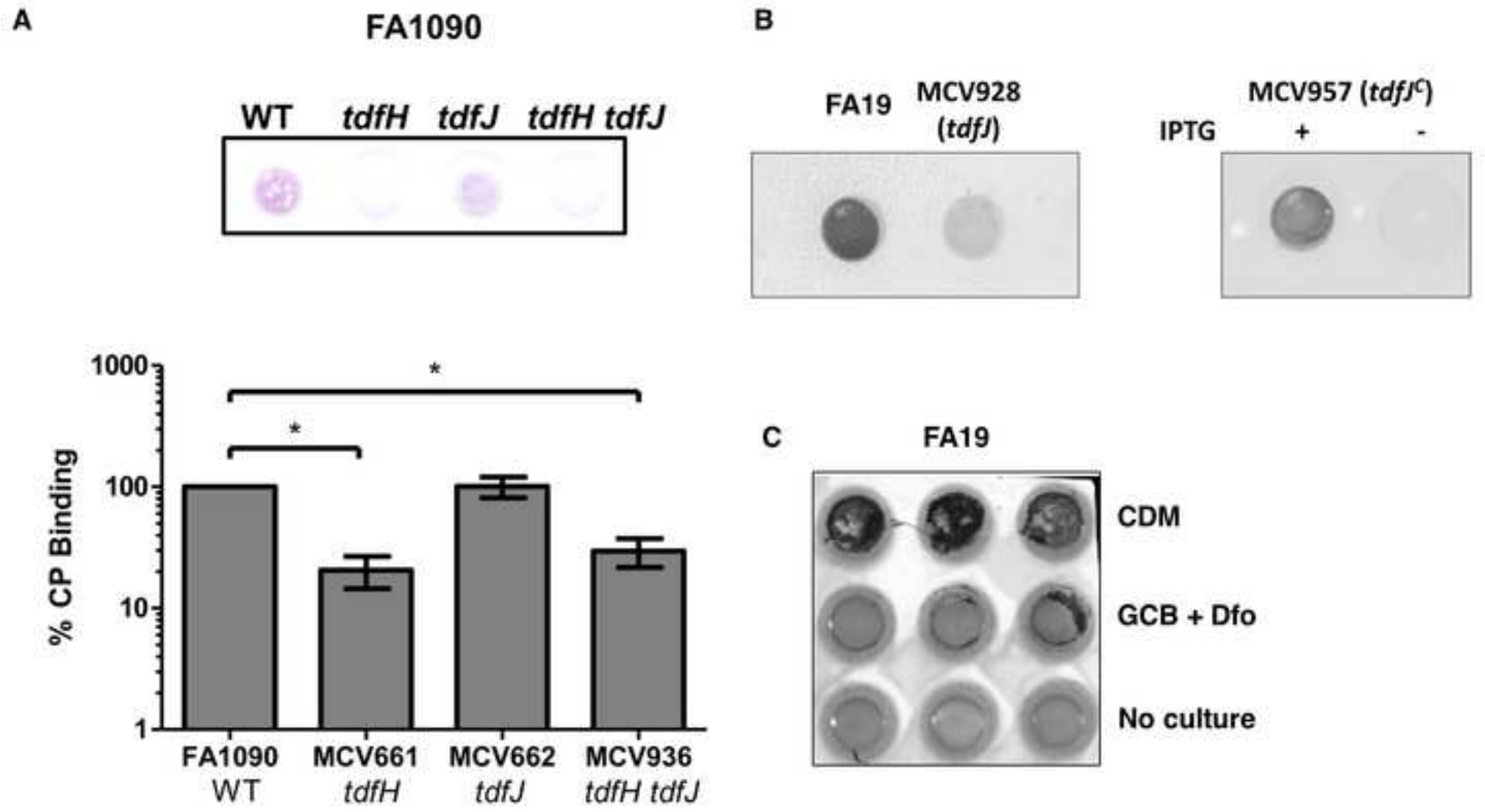


Figure 3



Name of Material/ Equipment	Company	Catalog Number
125 mL sidearm flasks	Bellco	2578-S0030
2-Mercaptoethanol	VWR	M131
3MM Paper	GE Health	3030-6461
Agarose	Biolone	BIO-41025
Ammonium chloride	Sigma-Aldrich	A9434
Biotin	Sigma-Aldrich	B4501
Blotting grade blocker	Bio-Rad	170-6404
Bovine serum albumin	Roche	3116964001
Bovine transferrin	Sigma-Aldrich	T1428
Calcium chloride dihydrate	Sigma-Aldrich	C5080
Calcium pantothenate	Sigma-Aldrich	C8731
Calprotectin	N/A	N/A
Chelex-100 Resin	Bio-Rad	142-2832
Cotton-tipped sterile swab	Puritan	25-806
Deferoxamine	Sigma-Aldrich	D9533
D-glucose	Sigma-Aldrich	G8270
Dialysis cassette	Thermo	66380
Dot blot apparatus	Schleicher & Schwell	10484138
Ethanol	Koptec	V1016
Ferric chloride	Sigma-Aldrich	F7134
Ferric nitrate nonahydrate	Sigma-Aldrich	F1143
GC medium base	Difco	228950
Glycine	Sigma-Aldrich	G8898
HEPES	Fisher	L-15694
Human transferrin	Sigma-Aldrich	T2030
Hypoxanthine	Sigma-Aldrich	H9377
Klett colorimeter	Manostat	37012-0000
L-alanine	Sigma-Aldrich	A7627
L-arginine	Sigma-Aldrich	A5006
L-asparagine monohydrate	Sigma-Aldrich	A8381

L-aspartate	Sigma-Aldrich	A9256
L-cysteine hydrochloride	Sigma-Aldrich	C1276
L-cystine	Sigma-Aldrich	C8755
L-glutamate	Sigma-Aldrich	G1251
L-glutamine	Sigma-Aldrich	G3126
L-histidine monohydrochloride	Sigma-Aldrich	H8125
L-isoleucine	Sigma-Aldrich	I2752
L-leucine	Sigma-Aldrich	L8000
L-lysine	Sigma-Aldrich	L5501
L-methionine	Sigma-Aldrich	M9625
L-phenylalanine	Sigma-Aldrich	P2126
L-proline	Sigma-Aldrich	P0380
L-serine	Sigma-Aldrich	S4500
L-threonine	Sigma-Aldrich	T8625
L-tryptophan	Sigma-Aldrich	T0254
L-tyrosine	Sigma-Aldrich	T3754
L-valine	Sigma-Aldrich	V0500
Magnesium sulfate	Sigma-Aldrich	M7506
Methanol	VWR	BDH1135-4LP
Nitrocellulose	GE Health	10600002
Potassium phosphate dibasic	Sigma-Aldrich	60356
Potassium phosphate monobasic	Sigma-Aldrich	P9791
Potassium sulfate	Sigma-Aldrich	P0772
Potato starch	Sigma-Aldrich	S4251
Reduced glutathione	Sigma-Aldrich	G4251
S100A7	N/A	N/A
Sodium bicarbonate	Sigma-Aldrich	S5761
Sodium chloride	VWR	470302
Sodium citrate	Fisher	S279
Sodium hydroxide	Acros Organics	383040010
Thiamine hydrochloride	Sigma-Aldrich	T4625

Thiamine pyrophosphate	Sigma-Aldrich	C8754
TPEN	Sigma-Aldrich	P4413
Tris	VWR	497
Uracil	Sigma-Aldrich	U0750
Zinc sulfte heptahydrate	Sigma-Aldrich	204986

Comments/Description
Must be custom ordered
Open in fume hood
Called "filter paper" in text
Powder
Powder
Powder
Nonfat dry milk
Powder
Powder
Powder
Powder
We are supplied with this by a collaborator
Wash with deionized water prior to use
Cotton is better than polyester for this application
Powder
Powder
Presoak in buffer prior to use
Lock down lid as tightly as possible before sample loading
Flammable liquid, store in flammables cabinet
Irritant, do not inhale
Irritant, do not inhale
Powder, already contains agar
Powder
Powder
Powder
Powder
Uses color transmission to assess culture density
Powder
Powder
Powder

Powder
Powder
Powder
Powder
Powder
Powder
Powder
Powder
Powder
Powder
Powder
Powder
Powder
Powder
Powder
Powder
Powder
Powder
Flammable liquid, store in flammables cabinet
Keep in protective sheath until use
Powder
Powder
Powder
Powder
Handle carefully. Can oxidize easily.
We are supplied with this by a collaborator
Powder
Powder
Powder

Highly hygroscopic
Powder

Also called cocarboxylase
Powder
Powder
Powder
Irritant, do not inhale

Responses to Reviewers' and Editorial Comments

Editorial Comments:

General:

1. The manuscript has been thoroughly screened for grammatical and typographical errors and appropriate corrections have been made.
2. The manuscript has been reformatted to adhere to the guidelines provided.
3. All instances of ® and ™ have been removed from the manuscript. The wording in the body of the text has been altered to limit commercial language as much as possible, and manufacturer names (ex. Bio-Rad) have been omitted from the text. They now appear only in the list of materials.

Protocol:

1. We believe the current protocol, as written, sufficiently answers the question of "how" each individual step is performed, and can be followed fully and without confusion. Where appropriate, references to established, common protocols are made.

Figures:

1. This manuscript contains figures that have been adapted from publications by Infection & Immunity (Jean et al) and PLOS Pathogens (Maurakis et al). Authors may reuse discrete portions of their own publications in Infection and Immunity, provided copyright credit is given to the American Society for Microbiology and the original publication, which has been done in this resubmission. PLOS Pathogens is an open access journal, and allows reuse and redistribution of its publications without restriction. A document containing links to these policies has been included in our resubmission.
2. The requested change in Figure 2C has been made.

References:

1. References were inserted into this manuscript using the EndNote format file provided on JoVE's website.

Table of Materials:

1. Information has been added to the table of materials to ensure all entries have associated information.

Reviewer #1:

Major Concerns:

1. The preparation of stock solutions VI-VIII has been separated into three entries within the protocol as a clarification.
2. "4X CDM" has been changed to "4X sterile concentrate" throughout the body of the text to clarify the protocol and create a distinction between the 4X sterile concentrate and the 1X CDM.
3. An explanation of back dilution has been added to address this concern.

4. A few sentences have been added to the text to discuss the study of other *Neisseria* species.
5. The wording of the text has been altered to clarify the importance of CO_2, along with the relative contributions of stock solution VIII in the media and atmospheric CO_2 present in the incubator. The text should now convey that both of these are necessary.

Minor Concerns:

1. The word “volume” has been added.
2. The original step 2.3.1 has been moved to step 2.3, and the previous step 2.3 is now 2.4, to clarify the order of these steps.
3. This typographical error has been fixed.
4. The word “step” has been added.

Reviewer #2:

Minor Concerns:

1. Densitometry analyses have been added to figures 1 and 3 to help illustrate the differences in signal intensity.
2. The figure legends have been modified to reflect the genetic background of the gonococcal strains being used in their respective experiments.

Reviewer #3:

1. This typographical error has been fixed.
2. The protocol has been adapted to include additional blanking steps.
3. Figure 2 has been uploaded again, this time at 300 dpi.

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Supplemental Figure 1

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Supplemental Figure 2

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Supplemental Figure 3

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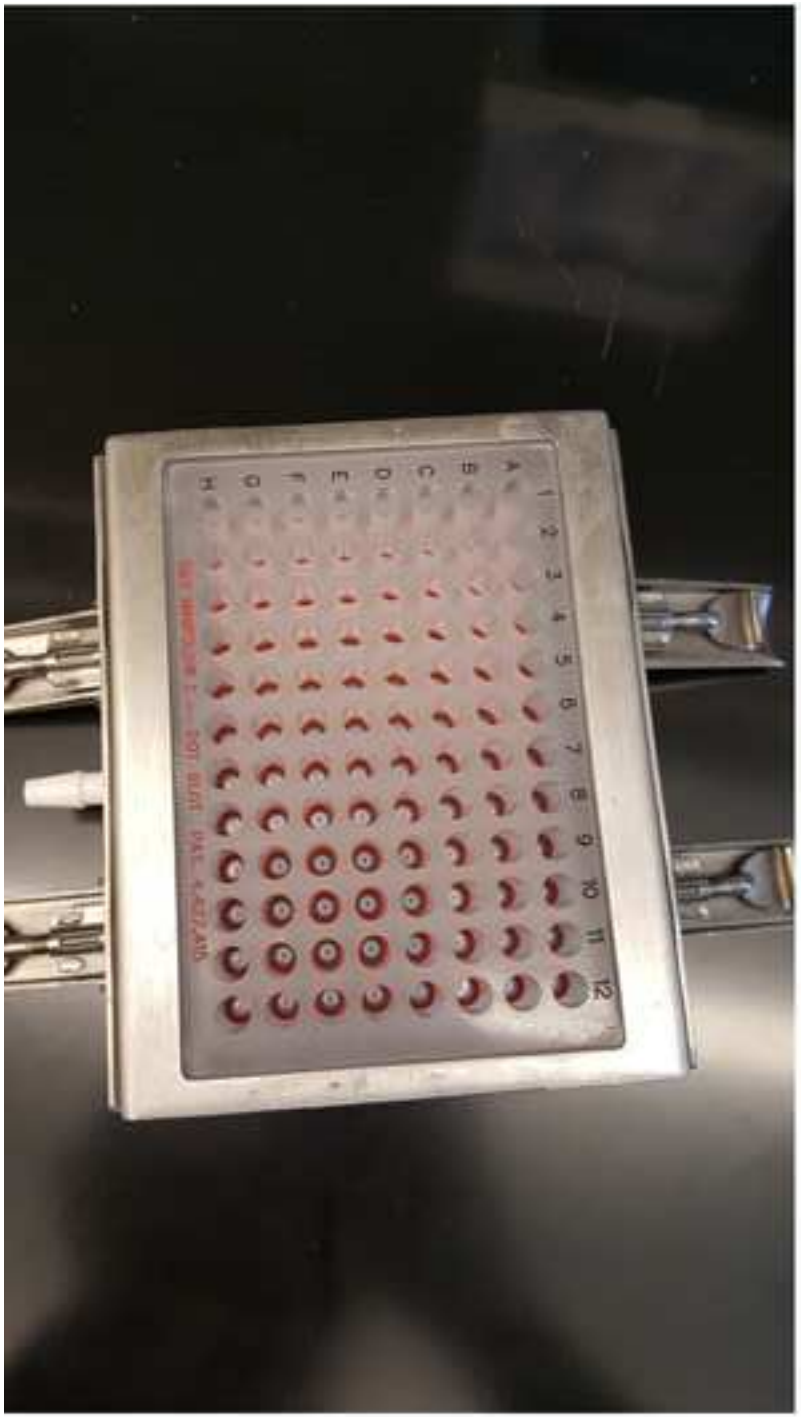

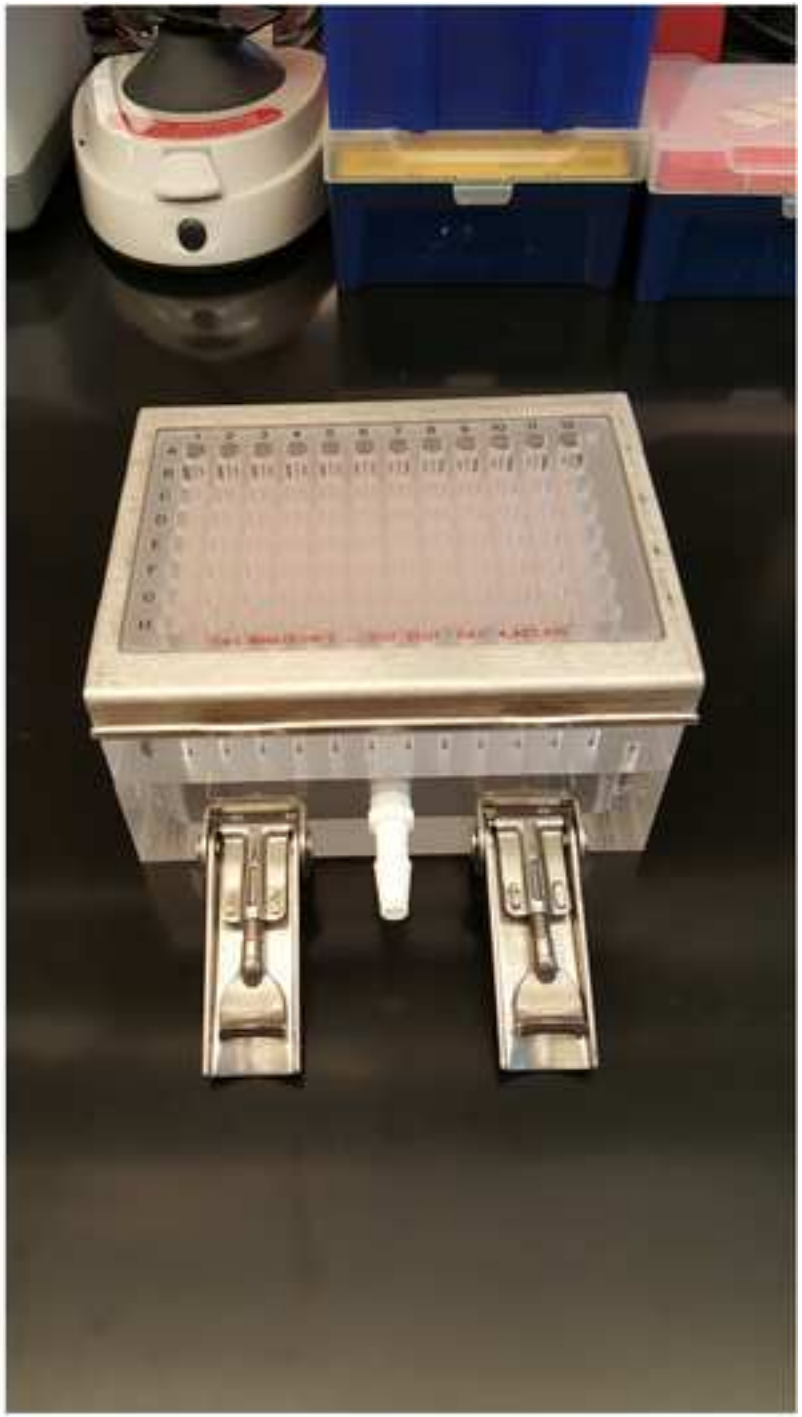

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