Published in final edited form as:

Curr Protoc Microbiol. ; 30: 9D.2.1–9D.2.13. doi:10.1002/9780471729259.mc09d02s30.

Laboratory Growth and Maintenance of *Streptococcus pyogenes* (The Group A Streptococcus, GAS)

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Abstract

Streptococcus pyogenes is a Gram-positive bacterium that strictly infects humans. It is the causative agent of a broad spectrum of diseases accounting for millions of infections and at least 517, 000 deaths each year worldwide (Carapetis et al., 2005). It is a nutritionally fastidious organism that ferments sugars to produce lactic acid and has strict requirements for growth. To aid in the study of this organism, this unit describes the growth and maintenance of *S. pyogenes*.

Keywords

Streptococcus pyogenes; the Group A Streptococcus; firmicute; facultative anaerobe; chemically defined medium

INTRODUCTION

This unit describes growth and maintenance of *Streptococcus pyogenes* or the Group A Streptococcus (GAS). These protocols should also be applicable to closely related species of Streptococci such as the Group C Streptococcus (e.g., GCS, *S. equi*) and Group G Streptococcus (e.g., GGS, *S. anginosus*) and potentially others. However, the protocols described here are specific for GAS.

S. pyogenes is a facultative anaerobe and is grown at 37°C in either ambient air or in 5–10% CO₂. Like all streptococci, GAS is both catalase and oxidase negative. GAS lacks the necessary enzymes for a functional TCA cycle and oxidative-cytochromes for electron transport; therefore, relies completely on fermentation of sugars for growth and energy production. It is a member of the lactic acid bacteria and is homofermentative for lactic acid production from glucose fermentation. Specific components of a rich growth medium for GAS include neo peptone extracts, glucose as carbon source, and a complex mixture of nutrients from beef heart infusion as first described by Todd & Hewitt (Todd and Hewitt, 1932). GAS is considered a multiple amino acid auxotroph requiring nearly all amino acids to be present in its growth media. A Chemically Defined Medium has been developed for GAS containing all of the necessary amino acids for GAS growth (van de Rijn, 1980).

This unit outlines protocols for culturing *S. pyogenes* (GAS) on solid medium (see Basic Protocol 1), various growth assays in liquid medium (see Basic Protocol 2,3,4), isolation from mixed cultures (see Basic Protocol 5), and making freezer stocks (see Basic Protocol 6). Methods for growth of *S. pyogenes* (GAS) in alternative environments such as chemically defined medium, low glucose and peptide rich medium, and human blood are also described. Support Protocols for differential identification of *S. pyogenes* (GAS) are also provided.

CAUTION: *Streptococcus pyogenes* (GAS) is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms as described in the Biosafety in Microbiological and Biomedical Laboratories (BMBL) manual from the Centers for Disease Control (CDC).

BASIC PROTOCOL 1

GROWTH OF *S. PYOGENES* (GAS) ON SOLID MEDIUM

Addition of 1.4% agar to growth media allows the generation of solid media plates. GAS grows best on complex “rich” medium such as Trypticase Soy Agar (TSA) supplemented with 5% Sheep Blood, where it typically produces large zones of β -hemolysis (the complete disruption of erythrocytes and the release of hemoglobin) (Fig. 1). Therefore, *S. pyogenes* is also called the Group A β -hemolytic Streptococcus (GABHS). GAS is also commonly grown on agar plates produced from Todd-Hewitt broth supplemented with 0.2% yeast extract (THY). Plates derived from Brain Heart Infusion (BHI) also support the growth of GAS. (See Appendix 2C for list of all media recipes found in CPMC.)

NOTE: All steps should be performed using sterile technique. GAS will remain viable on plates for only 5–7 days after streaking if stored at room temperature. GAS does not survive at 4°C.

Materials—TSA + 5% Sheep blood (Fisher #221239)

GAS culture from frozen stock (see Basic Protocol 6 for frozen stock)

Sterile wood sticks or inoculating loops

Pre-warm the TSA blood agar plates at 37°C to reduce condensation and help growth.

Using colonies from a plate of actively growing *S. pyogenes* (GAS)

1. Using a sterile disposable inoculating loop, sterilized re-usable loop, or sterile wood stick, pick one colony from the plate containing viable *S. pyogenes* colonies.
2. Streak along the edge of the plate.
3. Rotate the plate 90°, continue to streak, and repeat once more using the same loop.
4. Using a new inoculating loop, pass through the final streak and streak on clean zones towards the center of the plate to ensure isolated colonies (pure culture). Incubate overnight at 37°C, 5% CO₂ until colonies appear.

Optimal growth of GAS is seen in 5% CO₂; however, GAS will also grow in ambient air albeit a little slow.

Using a frozen stock of *S. pyogenes* (GAS)—To ensure a pure starting culture, it is recommended to start from a plate with colonies streaked out for isolation. However, a frozen stock can also be used as inoculum to start an overnight culture.

1. Remove the frozen stock from –80°C and place the tube on ice. Using a sterile inoculating loop or wooden stick, streak for isolated colonies on a blood agar plate (with antibiotic if needed) as described above.
2. Return the frozen stock to –80°C quickly to avoid freeze-thaw.
3. Incubate overnight at 37°C (or otherwise mentioned), 5% CO₂ until isolated colonies appear.

BASIC PROTOCOL 2

GROWTH OF *S. PYOGENES* (GAS) IN LIQUID MEDIUM

This protocol describes the inoculation of liquid medium from a single colony of *S. pyogenes*. To ensure best growth, pre-warming the medium to 37°C is recommended.

NOTE: This protocol describes the growth of *S. pyogenes* in rich THY medium. If the goal of the study is to limit nutrients such as carbohydrates or iron, Chemically Defined Medium (CDM) should be used (see Alternate Protocol for Growth 1). For growth in peptide rich conditions, C media (see Alternative Protocol for Growth 2) can be used.

NOTE: All steps should be performed using sterile technique to avoid contamination.

Materials—THY broth (see recipe) (Alpha Biosciences #T20-106)

Yeast Extract (Fisher #BP-9727-500)

Plate containing viable *S. pyogenes* (GAS)

Screw cap conical polypropylene tube 15 ml (Genesee #21-101) and 50 ml (Fisher #430290)

Sterile wood sticks or inoculating loops

Klett-Summerson colorimeter (Bel-Art Scienceware #370120000) or Spectrophotometer

Tubes/flasks for use with Klett-Summerson colorimeter

1. Using a sterile, disposable inoculating loop or wooden stick, pick one colony from plate containing viable *S. pyogenes* colonies.

Inoculation from a single, isolated colony will ensure that the broth culture is pure *S. pyogenes*. Inoculation of liquid medium from frozen culture cannot ensure the purity of the culture.

2. Suspend the colony in THY medium in a sterile 15 ml or 50 ml screw cap polypropylene conical tube depending on total volume required.

Tighten cap on tube to prevent release of CO₂ during growth. Try to use a volume close to maximum of tube to reduce the available air space. Since the tube is sealed, culture can be placed in non-CO₂ (ambient air) incubator. Shaking of the tube during growth is not necessary.

3. Incubate statically overnight at 37°C.
4. Since GAS cocci are non-motile and often aggregate (clump) during growth, they will be found settled to the bottom of the tube. Invert several times to resuspend bacteria prior to turbidity analysis or sample removal.
5. Check culture under a light microscope at 40× magnification for presence of non-motile chains of cocci (characteristic of GAS). Any motile cells will be contamination and culture should not be used.

BASIC PROTOCOL 3

GROWTH CURVES OF *S. PYOGENES* (GAS) USING KLETT TUBES

A Klett-Summerson colorimeter allows turbidity to be monitored directly without opening the tube or flask to remove culture. A Klett unit is an arbitrary measurement of absorbance.

In general for a *S. pyogenes* wild type strain, Klett units for a growth curve are as follows: mid log phase (Klett 60–80), late log phase (Klett 80–100), stationary phase (Klett 100–120). These values vary with the instrument used. If needed, OD can be used to monitor turbidity in a spectrophotometer at 600 nm by removing 1 ml aliquots for assay. It is important to perform a growth curve on the experimental strains before performing any assay, as there might be variation in Klett value with different strains.

1. Follow steps 1–5 from Basic Protocol 2.
2. Add 500 μ l (1:20) of an overnight culture to a 15 ml Klett tube containing 10 ml THY and appropriate antibiotic, if necessary. Mix well. For volumes more than 10 ml, use a 125 ml Klett flask with side tube for reading.
3. Incubate the tubes statically at 37°C.
4. For growth curves, measure the absorbance using the Klett-Summerson colorimeter every 30–45 min. Read more often if necessary.
5. Invert the tube once or twice before every reading.
6. If collecting the cells at a point in growth, put the Klett tube on ice to stop the growth when the cells reach the desired point in growth.
7. Transfer the culture to a conical tube and centrifuge at $8000 \times g$ for 10 min to pellet the cells. The pellet can be frozen at -20°C for further experiments (e.g., RNA isolation).

Some strains will not pellet easily and the pellet may not be stable due to capsule formation. Adding hyaluronidase (helps to break down the hyaluronic acid capsule of GAS) (85 μ g/ μ l) in overnight or during growth will help.

8. After use, fill Klett flasks or tubes with distilled water and autoclave to sterilize.
9. Dispose of the sterilized contents and rinse 2 $\times$ with distilled water.

Use acid resistant (Neoprene coated) gloves while handling concentrated H_2SO_4 (VWR # 32920-042).

10. Fill the Klett flask with concentrated H_2SO_4 containing 0.5 g NOCHROMIX (Godax labs #19-010) and let sit for at least 12 h. Soak the tubes in a large glass beaker filled with the NOCHROMIX solution and let it sit overnight.
11. Rinse thoroughly 6–8 times with distilled water.
12. Dry the flasks completely and autoclave for sterilization.

Growth of GAS can be influenced even with traces of detergent in the tube/flask, so acid washing is preferred when possible for reusable glass containers. Glass washing with detergent can be used but must be followed by multiple hand washes in distilled water to remove any remaining detergent.

BASIC PROTOCOL 4

GROWTH CURVES OF *S. PYOGENES* (GAS) IN PLATE READER

When multiple growth conditions and/or longer kinetic growth experiments are required, growth assays can be performed in multi-well plates using a plate reader capable of temperature control and shaking. 48- or 96-well plates can lead to significant clumping upon

saturated growth due to their smaller well size, thus 24-well plates (Corning # 3524) are optimal for most GAS strains (some strains might be difficult to grow in multi-well plates).

NOTE: The protocol described here is for use of a 24-well plate in a BMG Fluostar Optima plate reader for growth using Chemically Defined Medium (CDM). However, it can be modified for any medium or reader. All steps should be carried out using sterile technique to avoid contamination.

1. Follow steps 1–5 from Basic Protocol 2.
2. Prepare a stock of 25 ml CDM (see preparation in recipe).
3. Add 1 ml of prepared CDM to each well of a 24-well plate.
4. Pellet an overnight culture grown in 15 ml polypropylene conical tube at $8000 \times g$ for 10 min at 4°C .
5. Wash the cells with saline to remove excess media.
6. Resuspend the pellet in saline to bring the OD_{600} to 0.5.

Alternately, scrape the cells from the plate containing viable *S. pyogenes* colonies and resuspend in saline to bring the OD_{600} to 0.5.

7. Inoculate each well with 20 μl (1:50) of the culture from step 10b, leaving one well blank. Seal the plate well with a transparent optical tape (Bio-Rad #2239444).

Prewarm the plate reader to 37°C before starting the growth assay. Sealing the plate with optical tape mimics growth in a sealed tube. Although not sterile, use of a dedicated stock of optical sealing tape did not show any contamination of plates.

8. The conditions used for typical growth of most GAS strains are:
 - Temperature: 37°C
 - Number of cycles: 40 (depends on the length of the growth curve)
 - Cycle time: 1800 s
 - Number of flashes per well: 20
 - Wavelength: 600 nm
 - Shaking frequency: 400 rpm
 - Shaking mode: double orbital (figure eight)
 - Additional shaking time before each cycle: 10 sec

The shaking prior to each read helps disperse any GAS clumping that might lead to improper turbidity determination.

9. Growth curves can be generated using the software associated with the plate reader (Omega) at the end of the growth cycle.

BASIC PROTOCOL 5

ISOLATION OF *S. PYOGENES* (GAS) FROM A MIXED CULTURE

Clinical samples are rarely pure cultures, so it is necessary to follow appropriate protocols commonly used in clinical laboratories to identify and isolate *S. pyogenes*. As stated above, GAS produce zones of β -hemolysis on TSA blood agar plates and are Gram-positive cocci (see Gram stain in Support Protocol 1) that grow in chains of varying lengths (long in

laboratory media, short in clinical material). Furthermore, GAS lack both oxidase and catalase (see Support Protocols 2 and 3) activity. Mixed cultures should be cultured for isolated colonies on TSA blood agar (see Basic Protocol 1), and then assayed with oxidase and catalase tests to establish if they are negative. Finally, potential GAS strains can be subjected to a Rapid Strep Test (Diagnostic Test Group #DTG-STP50) or PCR analysis to confirm.

SUPPORT PROTOCOLS FOR DIFFERENTIAL IDENTIFICATION OF *S. PYOGENES* (GAS) (adapted from [microbelibrary.org](http://www.microbelibrary.org))

Support Protocol 1: Gram Stain (<http://www.microbelibrary.org/component/resource/gram-stain/2886-gram-stain-protocols>)

The Gram stain is used for phenotypic characterization of bacteria. The staining procedure differentiates the bacteria according to their cell wall structure. Gram-positive cells have a thick peptidoglycan layer and stain blue to purple. Gram-negative cells have a thin peptidoglycan layer and stain red to pink.

Materials—Slides

Crystal violet

Gram's iodine

Ethanol

Safranin

1. Prepare slide for staining: Add samples to the slide. Air dry, and heat fix.
2. Place slide in staining tray, flood with crystal violet. Stain for 30 sec.
3. Gently rinse with water and drain.
4. Flood the slide with gram's iodine. Let it stand for 30 sec.
5. Rinse with water and drain.
6. Hold the slide at an angle, dripping the 95% v/v ethanol over the sample until run-off becomes colorless.
7. Quickly rinse the slide with water.
8. Flood slide with safranin. Allow stain to remain for 30 sec.
9. Rinse with water and drain.
10. Blot slide within a pad of blotting paper. Do not rub. The slide should be completely dry before microscopic examination.
11. Use 1000× magnification to observe individual bacterial cells.
12. Observe for the color.

Gram-positive cells are purple, and Gram-negative cells are pink/red.

Support Protocol 2: Catalase Assay (<http://www.microbelibrary.org/index.php/library/laboratory-test/3226-catalase-test-protocol>)

The catalase test facilitates the detection of the enzyme catalase in bacteria. It is essential for differentiating catalase-positive from catalase-negative. GAS is catalase negative. The

catalase test is also valuable in differentiating aerobic and obligate anaerobic bacteria, as anaerobes are generally known to lack the enzyme.

Materials—Slides

10% Hydrogen Peroxide

1. Using a sterile inoculating loop transfer culture from an isolated colony to the slide (Do not add water).
Be careful to not pick agar, it may result in a false positive reaction, particularly if grown on blood agar media.
2. Place a drop of hydrogen peroxide onto the slide containing the colony. Do not mix.
3. Observe for immediate bubble formation.

The appearance of bubbles indicates a positive reaction.

Support Protocol 3: Oxidase Assay (<http://www.microbelibrary.org/index.php/library/laboratory-test/3229-oxidase-test-protocol>)

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase. Oxidase positive means the bacterium contains cytochrome c oxidase and can use oxygen for energy production with an electron transfer chain. GAS is oxidase negative.

Materials—Sterile cotton tip applicators

Oxidase reagent (Dalynn Biologicals #R095)

1. Snap open the oxidase reagent in the middle, but do not remove cap.
2. Use the cotton tip applicator to sample one isolated colony from a fresh (18–24 h) culture of bacteria.
3. Squeeze one or two drops of oxidase reagent onto the cotton tip applicator.
4. Observe for color changes.

Microorganisms are considered oxidase positive (+) when the color change to dark purple occurs within 30 sec.

Microorganisms are oxidase negative (–) if no color change occurs or it takes longer than 1min.

BASIC PROTOCOL 6

MAKING A *S. PYOGENES* (GAS) FREEZER STOCK

This protocol describes how to generate and maintain a stock of *S. pyogenes* at –80°C for long-term storage.

Materials—80% sterile glycerol solution

Liquid culture of viable and pure *S. pyogenes* in media (e.g., THY)

2 ml cryogenic vials (Sarstedt # 72-694-006)

–80°C freezer

1. Add 400 μ l of 80% glycerol to a 2 ml cryogenic vial.
2. Add 1.2 ml of *S. pyogenes* culture to the 2 ml vial (20% glycerol final).
3. Mix the culture and glycerol well by inversion or vortex.
4. Immediately place in -80°C freezer for storage.

S. pyogenes (GAS) can also be frozen directly from colonies on agar plates. Scrape up using a sterile loop and resuspend in sterile 20% glycerol in THY broth.

ALTERNATE PROTOCOL 1

GROWTH OF *S. PYOGENES* (GAS) IN CHEMICALLY DEFINED MEDIUM (CDM)

Chemically Defined Medium contains the exact components required for growth of *S. pyogenes* (van de Rijn and Kessler, 1980; Wood et al., 2005). This media should be used to assess the role of specific nutrients for growth, such as carbohydrates or metals. CDM can be made from scratch (see recipe) or is available as a custom-order powder (MP Biomedicals #1009-617) to allow consistency from batch to batch.

Prepare (see recipe) and filter-sterilize CDM prior to addition of sterilized final ingredients. We have found preparing the medium as 2 $\times$ enhances the growth.

CDM should be prepared fresh, filter sterilized, and stored at 4°C covered with foil. The prepared medium should not be stored for more than 2 weeks. CDM can be supplemented with any carbohydrate source of choice.

1. Prewarm the medium at 37°C .
2. Pellet the overnight culture at $8000 \times g$ for 10 min at 4°C .
3. Wash the cells with saline to remove media.
4. Resuspend the pellet in saline to bring the OD_{600} to 0.5.
5. Inoculate the Klett tube containing 10 ml of sterile CDM with 1:20 of overnight culture.
6. Monitor the growth using a Klett-Summerson colorimeter.
7. For growth assays using a plate reader see Basic Protocol 4.

ALTERNATE PROTOCOL 2

GROWTH OF *S. PYOGENES* (GAS) IN LOW GLUCOSE C MEDIUM

C medium (Kang et al., 2010) is rich in peptides and poor in carbohydrates. Unlike THY medium, C medium contains only trace amounts of glucose unless added separately. C medium is thought to mimic environments encountered by GAS during infection of soft tissues and other *in vivo* human niches limiting in carbohydrates.

1. Prepare the medium by dissolving 0.5% (w/v) protease peptone no. 3 (Difco), 1.5% (w/v) yeast extract and 17 mM NaCl final concentration to completion in distilled water. Adjust the pH to 7.5.
2. Autoclave the solution.
3. Add filter sterilized 0.4 mM MgSO_4 and 10 mM of K_2HPO_4 (final concentration) to the prepared solution.
4. Follow Basic Protocol 2, 3 or 4.

ALTERNATE PROTOCOL 3

GROWTH OF *S. PYOGENES* (GAS) IN HUMAN BLOOD

The Lancefield Bactericidal Assay (Lancefield, 1957) is used to determine the ability of GAS strains to survive and disseminate in whole human blood, a major virulence attribute for this human pathogen. It tests whether GAS is able to evade phagocytic killing and utilize human blood as a growth medium.

1. Start the overnight culture as in Basic Protocol 2.
2. Add 1:20 dilution of the overnight culture to THY with appropriate antibiotic.
3. Grow to mid log phase (Klett 30–40 or $OD_{600} = 0.1 - 0.15$).
4. Vortex the cells for 10 min at room temperature.

This helps to break up the chains typically found for GAS growth in broth culture to better assess the colony forming units (cfu).

5. Perform 10-fold serial dilutions of the cells to 10^{-4} in saline.
6. Add 50 μ l of 10^{-4} dilution (100 – 200 CFU) in 500 μ l whole human blood and incubate in 37°C with rotation for 3 h.
7. After the incubation vortex the cells for 10 min as before.
8. Plate 100 μ l of serial dilutions to 10^{-1} . Multiplication factor can be calculated by dividing the CFU obtained after incubation by initial CFU inoculated ($cfu_{final}/cfu_{initial}$).

Collection of human blood requires approval from the Institutional Review Board (IRB) at your institution. Human blood should be freshly drawn and used immediately. Since donors may be immune to your serotype of GAS, only strains that show a multiplication factor of 50–100 fold or more should be used for blood growth assays.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

THY broth

As per manufacturer's (Alpha Biosciences) instructions, add 30 g of Todd-Hewitt (TH) broth (peptone 20 g, beef heart infusion 3.1 g, sodium carbonate 2.5 g, dextrose 2 g, sodium chloride 2 g, disodium phosphate 0.4 g) in 0.8 L distilled water. Add 2g of Yeast Extract and dissolve to completion. Make up the volume to 1 L. Autoclave for 20 min and let it cool at room temperature.

THY agar

As per manufacturer's (Alpha Biosciences) instructions, add 30 g of THY broth in 0.8 L distilled water. Add 2g of Yeast Extract and 14g of Agar (Fisher # BP1423). Dissolve to completion and make up the volume to 1 L. Autoclave for 20 min and let it cool in 55°C water bath for 30 min. Add the antibiotic, mix well and pour 25 ml of agar in each petri dish. Let the agar set overnight at room temperature and then store it at 4°C.

C media

Dissolve 0.5% (w/v) Protease peptone 3 (Difco), 1.5% (w/v) yeast extract and 17 mM NaCl to completion in distilled water. Adjust the pH to 7.5 and autoclave for 20 min and allow it

to cool at room temperature. Add filter sterilized 0.4 mM MgSO₄ and 10 mM of K₂HPO₄ (final concentration) to the prepared solution.

CDM

This medium (see recipe below) has been adapted and modified by Buttaro and colleagues (Wood et al., 2005) based on the original recipe developed by Van de Rijn & Kessler (van de Rijn and Kessler, 1980)

Recipe for Chemically Defined Medium (CDM)—Prepare 17g/L of powdered CDM Medium (containing the ingredients listed) for GAS (MP Biomedicals #111009617) in dH₂O

Component	Units/Liter
Adenine Sulfate, 2H ₂ O	0.0200 G
DL-Alanine	0.1000 G
L-Arginine, FB	0.1000 G
L-Asparagine, Anhydrous	0.1000 G
L-Aspartic Acid	0.1000 G
Biotin	0.0002 G
Calcium Chloride, Anhydrous	0.0005 G
D-Calcium Pantothenate	0.0020 G
Cyanocobalamin	0.0001 G
L-Cystine, 3HCl	0.0652 G
Folic Acid	0.0008 G
L-Glutamic Acid	0.1000 G
L-Glutamine	0.2000 G
Glycine	0.1000 G
Guanine, HCl, H ₂ O	0.0200 G
L-Histidine, FB	0.1000 G
Hydroxy L-Proline	0.1000 G
L-Isoleucine	0.1000 G
L-Leucine	0.1000 G
L-Lysine, HCl	0.1249 G
Magnesium Sulfate, Anhydrous	0.3419 G
L-Methionine	0.1000 G
Niacinamide	0.0010 G
β-NAD, 3H ₂ O	0.0025 G
Para-Aminobenzoic Acid	0.0002 G
L-Phenylalanine	0.1000 G
Potassium Phosphate, Dibasic, Anhydrous	0.2000 G
Potassium Phosphate, Monobasic, Anhydrous	1.0000 G
L-Proline	0.1000 G
Pyridoxal, HCl	0.0010 G
Pyridoxamine, 2HCl	0.0010 G
Riboflavin	0.0020 G

Component	Units/Liter
L-Serine	0.1000 G
Sodium Acetate, Anhydrous	2.7126 G
Sodium Phosphate, Dibasic, Anhydrous	7.3500 G
Sodium Phosphate, Monobasic, H ₂ O, ACS	3.1950 G
Thiamine, HCl	0.0010 G
L-Threonine	0.2000 G
L-Tryptophan	0.1000 G
L-Tyrosine, 2Na, 2H ₂ O	0.1442 G
Uracil	0.0200 G
L-Valine	0.1000 G
Filter sterilize the prepared medium	

Add to Prepared Sterilized Medium:

Carbohydrate	X (Glucose 0.25%); others (1%)
Ferric Nitrate, 9H ₂ O 0.7 mg/ml (good for a month)	1.424 ml/L (2.4 µM)
Ferrous Sulfate, 7H ₂ O 1mg/ml (good for a month)	5 ml/L (17.9 µM)
Manganese Sulfate, H ₂ O 1.7 mg/ml	2 ml/L (29.5 µM)
NaHCO ₃ (125mg/ml) (make fresh)	20 ml/L
L-Cysteine (35.4 mg/ml) (make fresh)	20 ml/L

COMMENTARY

Background Information

A hallmark of a successful pathogen is its ability to rapidly adapt to changing environments encountered during infection to obtain required nutrients, evade the host immune response and infect different tissues. GAS is a Gram-positive human pathogen responsible for a broad spectrum of diseases ranging from benign, self-limiting infections (pharyngitis or ‘strep throat’, impetigo) to life-threatening invasive disorders (necrotizing fasciitis, streptococcal toxic shock syndrome) to immune sequelae (acute rheumatic fever) (Kreikemeyer et al., 2003). Approximately 500,000 deaths are reported worldwide each year due to severe GAS diseases, thus making it a leading cause of mortality and morbidity (Musser et al., 1993). In addition, millions of milder GAS infection cases are reported each year (Olsen RJ, 2009). Given its considerable importance to human health and substantial financial burden, GAS pathogenesis has long been an area of active investigation. Despite these efforts, significant questions regarding molecular mechanisms involved in GAS pathogenesis remain unanswered. Importantly, no licensed vaccine for GAS is available, emphasizing the need for continued efforts for better understanding the underlying mechanisms of GAS pathogenesis (Olsen RJ, 2009).

Environmental signals typically link to co-ordinate change in gene expression, resulting in production of a defined set or regulon of virulence factors appropriate for the given situation. Unlike many other pathogenic bacteria, GAS does not appear to use alternative sigma factors to regulate virulence gene expression (Opdyke et al., 2001). Instead, it depends on transcriptional regulators, whose activities are responsive to specific conditions influencing growth. It is well established that the capacity of GAS to adapt and persist at so many different tissue sites is a key factor in its success as a pathogen.

Critical Parameters and Troubleshooting

Providing an optimal growth environment is really important for the successful laboratory cultivation of *Streptococcus pyogenes*. GAS is a fastidious organism and requires a minimum concentration of nutrients such as sugars and peptides for best growth. The attached Chemically Defined Medium (CDM) recipe represents the minimum set of basic nutrients required for growth. GAS is defined as metabolically anaerobic, but aerotolerant, and grows best when incubated static or stationary (no shaking). On plates, GAS is viable for 5–7 days at room temperature and does not survive well at 4°C. Highly encapsulated GAS strains are difficult to pellet and may require addition of hyaluronidase (85 µg/µl) to degrade the hyaluronic acid capsule.

Anticipated Results

Under appropriate growth conditions, GAS has a doubling time of 40 min to 1 hour in rich medium. Colony formation should occur after overnight incubation at 37°C with 5% CO₂ or 36 to 48 h when grown at 30°C with 5% CO₂; however, the growth rates may be affected by the presence of antibiotics or other selective agents. Increased lag phase is typically observed when cultured in CDM or C media and is often a strain dependent phenomenon. All GAS strains form zones of β-hemolysis on blood agar plates, mostly after overnight incubation at 37°C with 5% CO₂ but some strains such as M4 have delayed hemolytic activity.

Time Considerations

The amount of time required to saturate the medium with *Streptococcus pyogenes* varies depending on the inoculum and the strain being used. Starting from a single colony, GAS will grow to saturation in about 12–16 h, whereas when saturated liquid culture is used as inoculum it will take about 10–12 h to reach saturation in THY. The time will vary with the medium used, resulting in slow growth in the medium with limited nutrients. In most GAS strains, zones of β-hemolysis are formed after overnight incubation.

Acknowledgments

We thank Zehava Eichenbaum, Yoann Le Breton, and members of the McIver lab for critical review of this manuscript. This work was supported in part by funding to K.S.M. through the National Institutes of Health (NIH), National Institute for Allergy and Infectious Diseases (NIAID) for R01 AI47928 and through the American Heart Association for 10GRNT39000017.

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Figure 1.

Types of hemolysis observed upon growth on sheep blood agar: Alpha (α) hemolysis (top) exhibits incomplete clearance with a zone of greenish discoloration around the colony (e.g. *Streptococcus pneumoniae*). Gamma (γ) hemolysis (center) shows no clearing around the colony (e.g. *Enterococcus faecalis*). Beta (β) hemolysis (bottom) produces a clear zone of hemolysis immediately around the colony (e.g. *Streptococcus pyogenes*). Image published with permission of Lippencott, Williams, and Wilkins Press (<http://lww.com>).