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SFGR Isolation from Clinical Diagnostic Material: Small volume inoculum

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ABSTRACT

Rickettsiae are small obligate intracellular gram-negative bacteria that are grown in a Biological Safety Level 3 (BSL-3) laboratory setting.This protocol uses antibiotic-free medium, and therefore cultures are susceptible to contamination by undesirable bacteria or fungi.This procedure provides guidance for the inoculation and growth of primary isolates derived from small volumes of clinical samples including blood and blood products in a small volume culture and includes topics of growth monitoring, changing media, freezing primary isolates, and troubleshooting in the event of contamination. This technique utilizes Vero E6 cells growth in small conical culture flasks.These flasks have a growth area of 10cm^2 and can accommodate up to 5 mL of media.All techniques are to be performed in a biological safety cabinet (BSC) in a BSL-3 laboratory.Proper training and extreme caution is of the utmost importance when working with live rickettsial cultures.

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KEYWORDS

Culture, Isolation, Clinical isolation, SFGR, Rickettsia

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GUIDELINES

1. All work is conducted in a BSL-3 laboratory.
2. Take care to ensure reagents are not contaminated or expired.
3. Use proper sterile technique when working in a BSL-3 laboratory.

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MATERIALS TEXT

Vortex mixer
Pipetteman
Inverted Light Microscope
High Speed Centrifuge capable of speeds up to 17,000 x g with fixed angle biosafe rotor
CO_2water jacketed incubator
Water bath
1X HBSS; Life Technologies Catalog # 14025092
5% Fetal Bovine Serum Minimum Essential Medium with supplements (Complete Media)
  Minimum Essential Medium (MEM), no glutamine; Life Technologies Catalog# 11090-081
  Non-Essential Amino Acids 10mM (NEAA) ; Life Technologies Catalog# 11140-050
  HEPES 1M; Life Technologies Catalog#15630-080
  L-Glutamine 200mM; Life Technologies Catalog# 25030-081
  Sodium Pyruvate 100mM; Life Technologies Catalog# 11360-070
  Fetal bovine serum (FBS), TET tested (low antibiotic); Atlanta Biologics Catalog# S10350
Sucrose Phosphate Glutamate (SPG) (1)
  Sucrose; Sigma Catalog # 84097
  Potassium phosphate monobasic; Sigma Catalog # P5655
  Potassium phosphate dibasic; Sigma Catalog # P3786
  L-glutamic acid; Sigma Catalog # G1251
  Potassium hydroxide (KOH), 10N; Sigma Catalog # P5958
Cell Culture Grade Sterile Water
70% Ethanol
5% Quaternary ammonium solution
Penicillin-Streptomycin (10,000 U/mL); Life Technologies Catalog # 15140-148
Fungizone; Life Technologies Catalog # 15290-026
4% Sheep Blood Agar Plate; Scireson Catalog # 9233
Cotrimoxazole, Ready Made Solution 100 mg/mL in DMSO; Sigma Catalog # A2487
Gentamicin (10mg/mL); Life Technologies Catalog # 15710-072
Lincomycin Hydrochloride; Sigma Catalog # L2774
Alcohol resistant laboratory marking pen
Sterile Glass Beads
5 mL disposable pipette tip, sterile and individually wrapped
10 mL disposable pipette tip, sterile and individually wrapped
PPE including: Disposable gloves, disposable rear closure gown, and Eye protection
10 cm^2 culture tube, Techno Plastic Products, Catalog # 91243

SAFETY WARNINGS

These procedures must be performed using BSL-3 facilities and practices. All manipulations must be performed in a BSC using sterile technique and practices. Follow universal precautions to protect yourself from blood-borne pathogens. Be aware that clinical samples may be contaminated with pathogens, including hepatitis B virus, hepatitis C virus, and human immunodeficiency virus (HIV). Don double gloves, eye protection and a designated disposable rear closure gown when working with live bacterial cultures. All liquid waste, used culture flasks, and disposable waste created in the BSL-3 from live bacterial cultures should be decontaminated prior to autoclaving by mixing or rinsing with a known inactivation agent such as a 5% quaternary

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ammonium solution. Contaminated items and gloves should be discarded into an autoclave pan kept in the BSC. All sharp items should be placed in a sharps container. The sharps container should be autoclaved when contents reach the indicated fill line. All rotors must to be bio-safe aerosol resistant and are only to be opened inside a BSC. Refer to CDC's Biosafety in Microbiological and Biomedical Laboratories (BMBL), https://www.cdc.gov/labs/BMBL.html.

BEFORE STARTING

1. Ensure 5% Complete Media is warmed to room temperature.
2. When freezing ensure SPG and freezing tubes are chilled.
3. Ensure all flasks, vials, and tubes are labeled with appropriate identifiers and dates.

Media Preparation

1 All reagents should be thawed, well mixed and not expired prior to use.Reagents should be stored at the appropriate temperatures recommended by the manufacturer.Prepared media expires 1 month after preparation date.

2 Clean the BSC with 5% quaternary ammonium solution followed by 70% ethanol. (NOTE: do not spray quaternary ammonium solution directly on the BSC surface as residual solution may cause rust.Instead spray on a lint free wipe and carefully decontaminate the surface, followed by removal with 70% ethanol.)

3 Wipe all reagent bottles down with 70% ethanol before placing in the BSC.

4 To prepare ~600 mL of Complete Media, pipet the following into a new 500 mL bottle of MEM:

4.1 6.15 mL of NEAA

4.2 6.15 mL of HEPES

4.3 6.15 mL of L-Glutamine

4.4 Note: L-Glutamine upon thaw from -20°C will be cloudy.Bring solution up to

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room temperature and vigorously mix until the reagent is **clear** with no particulate.Do not use if reagent is not properly mixed.

4.5 61.5 mL of Sodium Pyruvate

4.6 30 mL of Heat Inactivated TET Tested (low antibiotic) fetal bovine serum

5 Filter sterilize with a 0.22 µm Nalgene Filter Unit

6 Label with the media type, preparation date, and expiration date and store at 4°C until use.

Small Volume Inoculation: Fresh clinical blood or blood products can be used as an inoculum or blood or blood products frozen at a 1:1 ratio with SPG can be thawed and used as an inoculum.

7 Confirm 10cm^2 culture flasks are at least 99% confluent.

8 Clean the BSC with 5% quaternary ammonium solution followed by 70% ethanol. (NOTE: do not spray quaternary ammonium solution directly on the BSC surface as residual solution may cause rust.Instead spray on a lint free wipe and carefully decontaminate the surface, followed by removal with 70% ethanol.)

9 Carefully place the 10cm^2 culture flask of Vero E6 cells upright in a 50ml conical tube rack in the BSC and gently decontaminate with 70% ethanol.

10 Label with appropriate sample identifiers and date of inoculation.

11 Remove the spent media and replace with 3 mL of 5% Complete Media.

12 Obtain clinical blood or blood product and place in the BSC

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12.1 Either 50 µl of fresh clinical blood or blood product or

12.2 100 µl of previously frozen samples in a 1:1 ratio with SPG.

12.3 i.Quick thaw any frozen sample in a 36°C water bath, do not over heat.

13 Pipette the total volume of the inoculum directly into the 3 mL of media in a 10cm^2 culture flask.

14 Close the flask and gently mix the inoculum so it evenly spreads over the monolayer, careful not to wet the interior filtered lid.

15 Place the inoculated 10cm^2 culture flask in a 34°C 5% CO_2 water jacketed incubator.

Monitoring Isolate Growth

16

17 Incubate the inoculated 10cm^2 culture flask for 3 to 6 days, and sample the supernatant for DNA extraction.Do not disturb the monolayer.

18 Prior to sampling clean the BSC with 5% quaternary ammonium solution followed by 70% ethanol. (NOTE: do not spray quaternary ammonium solution directly on the BSC surface as residual solution may cause rust.Instead spray on a lint free wipe and carefully decontaminate the surface, followed by removal with 70% ethanol.)

19 To sample, carefully place the 10cm^2 culture flask upright in a 50ml conical tube rack in the

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BSC and gently decontaminate with 70% ethanol.

20 Prepare a 2 mL sterile tube labeled with appropriate sample identifiers, day of growth (day 0 is the day of inoculation), and date.

21 Using sterile technique, take a 200 µL supernatant sample and place into the labeled

22 mL sterile tube.Be sure to not disrupt the monolayer!

23 Seal the top of the 10cm^2 culture flask, decontaminate the exterior of the flask with 70% ethanol, and place back into the 34°C 5% CO_2 water jacketed incubator.

24 Decontaminate the exterior of the 2 mL sterile tube with 5% quaternary ammonium solution followed by 70% ethanol prior to removal from the BSC.

25 Freeze the supernatant sample at -80°C if it cannot be extracted immediately.

26 Repeat steps 2 to 8 once more within days 7 to 14 post inoculation.

27 Extract samples and run the PanR8 real-time PCR assay (2).If CT values increase from the first to second sample of≥ then 3.3 CT, indicating ≥ 1 fold increase in DNA copies between timepoints, the inoculated culture should be considered a viable isolate.

28 To visually monitor the inoculated culture, carefully remove the 10cm^2 culture flask from the incubator and place under the inverted light microscope.Care must be taken to not wet the interior of the lid.

29 Scan the monolayer for areas of cell lifting or the absence of cells caused by cell death with the 10x optical.

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30 If an area of cell lifting is identified, switch the optical to a 40x or 100x optical and scan for extracellular *Rickettsia*.If identified, take a DNA sample (if 2 have not been taken yet, and confirm growth via the PanR8 real-time PCR assay (2)Monitor 10cm^2 culture flask(s) for at least 14 days or until isolation confirmation via real-time PCR.

Media Changes

31 If > 2 DNA samples are taken over the 14 day culture incubation and minimal media coverage is not achieved, or the culture is incubated > 14 days, a media change is required.

32 Prior to culture manipulation, clean the BSC with 5% quaternary ammonium solution followed by 70% ethanol. (NOTE: do not spray quaternary ammonium solution directly on the BSC surface as residual solution may cause rust.Instead spray on a lint free wipe and carefully decontaminate the surface, followed by removal with 70% ethanol.)

33 Carefully place the 10cm^2 culture flask upright in a 50ml conical tube rack in the BSC and gently decontaminate with 70% ethanol.

34 Remove the spent media without disturbing the monolayer and discard into a waste bin containing 5% quaternary ammonium solution to decontaminate.

35 Add

36 mL of fresh 5% Complete Media to the 10cm^2 culture flask.Ensure media is added slowly so the monolayer is not disturbed.

37 Seal the top of the 10cm^2 culture flask, decontaminate the exterior of the flask with 70% ethanol, and place back into the 34°C 5% CO_2 water jacketed incubator.

Freezing Isolate Cultures (1)

38 Once the inoculated culture is confirmed to be a *Rickettsia* isolate, continue the incubation until 40 – 60% of the monolayer is lifted due to cell death.This will ensure a high concentration of viable bacteria to freeze.

39 Prior to culture manipulation, clean the BSC with 5% quaternary ammonium solution followed by 70% ethanol. (NOTE: do not spray quaternary ammonium solution directly on the BSC

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surface as residual solution may cause rust.Instead spray on a lint free wipe and carefully decontaminate the surface, followed by removal with 70% ethanol.)

40 Carefully place the 10cm^2 culture flask upright in a 50ml conical tube rack in the BSC and gently decontaminate with 70% ethanol.

41 Remove about half of the media, and place into a labeled sterile high-speed conical tube.Be sure to use a conical tube appropriate for high-speed centrifugation. (NOTE: never centrifuge a T10 culture tube at high speed.)

42 Add sterile glass beads to the 10cm^2 culture flask and seal the flask.

43 Shake or swirl the flask until all the monolayer is lifted.Do not shake so hard that bubbles and foam is formed.Do not wet the interior culture lid.

44 Place the 10cm^2 culture flask back in the rack so that the beads and media settle to the bottom.

45 Transfer cell suspension to the labeled sterile conical tube. Seal the tube. Decontaminate the tube with 5% quaternary ammonium solution followed by ethanol.

46 It is recommended to take ~10-50ul aliquot of the cell suspension for mycoplasma evaluation in a labeled 2 ml cryo-tube.Follow the steps 4-8 of Monitoring Isolate Growth procedure above.

47 Make an accurate balance for the centrifuge (by volume) if needed.

48 In the BSC, place high-speed conical tubes in a fixed angle biosafe rotor.Make sure centrifuge is balanced appropriately!

49 Decontaminate the rotor before removing from the BSC with 5% quaternary ammonium solution followed by 70% ethanol.

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50 Centrifuge at 17,000 x g for 30 min at 4˚C.

51 Remove rotor from the centrifuge and open in the BSC.

52 Remove samples from the rotor and place in a rack.

53 Decontaminate the rotor with 5% quaternary ammonium solution followed by 70% ethanol and remove from the BSC.

54 Remove the supernatant from the tube without disturbing the pellet and discard into a waste bin containing 5% quaternary ammonium solution to decontaminate.

55 Resuspend the cell pellet in 2 mL of ice cold SPG.

56 Aliquot 1 mL volumes of the cell suspension into 2 chilled sterile labeled 2 mL screw top cryotubes.Cryotubes should be labeled with the appropriate identifiers, isolate passage #, cell type, cell passage #, freezing media type, date, and initials.

57 Ensure tubes are sealed tight and decontaminate the tubes with quaternary ammonium solution followed by 70% ethanol. Place cold cell aliquots in a room temperature cell freezing container.

58 Place container in a -80°C freezer for at least 24 hours before long term storage in liquid nitrogen.

Troubleshooting Contamination

59 Isolates are grown in media without antibiotics.This makes them susceptible to contamination with unwanted and aggressive bacterial and fungal species.None of the reagents listed below will kill intracellular *Rickettsia*however, they may deter growth.It is best to act preemptively to stop contamination before it takes over a culture. If contamination is seen the culture media

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should be changed with media containing an antibiotic or antimycotic and incubated for 24 hours.It is important to observe contamination early so multiple doses are not needed.

See table below for reagents and concentrations.Serial dilution may be required to achieve correct final concentrations from the stock solutions.

References

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