Streptococcus pneumoniae **carriage study protocol - nasopharyngeal (NP) swab processing**

(da Gloria Carvalho *et al.* Revisiting pneumococcal carriage by use of broth enrichment and PCR techniques for enhanced detection of carriage and serotypes. J Clin Microbiol. 2010 May;48(5):1611-8. doi: 10.1128/JCM.02243-09. Epub 2010 Mar 10.)

1. Preparation of skim milk, tryptone, glucose, glycerol transport medium (STGG)

The formula of the Skim-milk tryptone glucose glycerol (STGG) transport medium is:

- Skim milk powder (from Difco or from grocery) 2 g
- Tryptone soya broth (TSB, from Oxoid) 3 g
- Glucose 0.5 g
- Glycerol 10 ml
- Distilled water 100 ml

a) Mix to dissolve all ingredients.
b) Dispense in 1.0 ml amounts in screw-capped 1.5-ml vials.
c) Loosen the screw-cap tops and autoclave for 10 minutes (at 15 pounds).
d) Tighten caps after autoclaving.
e) Store STGG frozen at -20°C or refrigerate until use. Use STGG medium within 6 months of preparation.

2. Inoculation of STGG with an NP swab

a) Thaw frozen tubes of STGG before use.
b) Label the tube with appropriate patient and specimen information.
c) Using a calcium alginate, Dacron or flocked swab, collect an NP swab from the patient.
d) Insert swab to the bottom of the STGG medium in thawed (room temperature) tube.
e) Raise the swab slightly and cut the wire portion (i.e., the shaft, or using a disinfected scissor) of the swab at the top level of the container. Allow the bottom portion of the swab (i.e., the tip) to drop into the tube. Discard the remaining shaft into disinfectant solution or a sharps container.
f) Tighten the screw-cap top securely.
g) Vortex on high speed for 10–20 seconds.
h) Freeze specimen immediately in upright position at -70°C, if possible.
i) Quality control test for sterility of the STGG medium has to be performed by plating a full loop of a homogenized vial from each lot onto trypticase soy agar with 5% sheep blood (BAP) and incubating the plate at 37°C for 24 h. Any growth should be considered contamination and the lot should be discarded.

3. Broth enrichment NP swab culture for enhanced pneumococcal growth

a. Thaw the NP-STGG specimens at room temperature (25°C) and vortex for approximately 10-20 s.
   - Re-freeze the specimen (*i.e.*, the STGG) as soon as possible; keep it cool (in an ice water bath if necessary) if the time is extended beyond a few minutes at room temperature.
   - Avoid multiple freeze-thaw cycles whenever possible. One way to decrease risk of freeze-thaw cycles within the freezer is to make sure the cryotubes are kept in the back of the freezer shelf and not the front or in the door.

b. Transfer 20**0** µl of the NP-STGG to 6 ml enrichment broth (5 ml of Todd Hewitt broth containing 0.5 % yeast extract (THY) to which 1 ml rabbit serum has been added).

c. Vortex and incubate for 6 hours at 37°C/CO_2 incubator or candle-jar.

d. Vortex and inoculate one loop (10 µl) of the THY enriched culture on BAP, streak in four quadrant fashion for colonies isolation and incubate for 18-24 hours at 37°C in CO2-incubator or candle-jar.

e. Transfer 1.0 ml of the THY enriched growth into screw-cap 1.5 ml vials (cryotube) and store at -20°C or -70°C for further DNA extraction followed by *lytA* gene real time PCR for *S. pneumoniae* and sequential multiplex PCR for pneumococcal serotyping (for lytA-positive specimens).

4. Pneumococcal isolate detection and identification

a. Carefully examine the BAP growth, for typical pneumococcal colonies, small, grayish, moist, watery surrounded by a greenish zone of alpha-hemolysis.

b. The suspected pneumococcal colonies should be passed to fresh BAP and incubated for 18-24 hours at 37°C in CO2-incubator or candle-jar. If enough growth proceed to optochin susceptibility and bile solubility tests. When more than one pneumococcal colony morphology is evident, all different morphologies should be tested.

c. **To perform the optochin susceptibility test:**
   - Streak the suspect alpha-hemolytic colony into BAP in confluent lines
   - Place 5 µg optochin disk with 6 mm diameter in the streaked area
   - Incubate in CO2-incubator or candle-jar at 35-37°C for 18-24 h
   - If susceptible to optochin (halo diameter >14 mm) it is identified as *S. pneumoniae*

Note: there are rare *S. pneumoniae* isolates optochin resistant with halo ≤14 mm that require bile solubility test in order to complete the identification tests.

d. **To perform the bile solubility:**

- Prepare a milky suspension (McFarland No.1) from an overnight culture in 1ml of 0.5% saline
- Divide the suspension in two tubes (test and control) of 0.5 ml
- Add 0.5 ml of 2% sodium desoxycholate (bile salts) to the test tube and 0.5 ml saline to the control tube
- Vortex and, incubate in CO2-incubator or candle-jar at 35-37°C for up to 2 h
- *S. pneumoniae* test tube will be completely transparent without any turbidity (please compare to the control tube), while any other alpha-hemolytic streptococci test tube will remain turbid after the 2 h incubation.

The test should not be performed on old cultures, as the active enzyme may be lost.

If identification confirmed the isolate as *S. pneumoniae*, a fresh culture (overnight/24h) should be stored at -70°C in 1.0 ml of STGG medium

5. Inoculate for Permanent Storage

a) 1 blood agar plate for -70°C storage
   - Examine this plate after overnight incubation.
   - If the culture is pure scrape all the growth using a sterile cotton tipped swab into a cryotube containing 1.0 ml of STGG medium.
   - Label and store as soon as possible at -70°C for future shipping to the reference laboratory for further typing and antimicrobial susceptibility testing.