1. Definitions

    1.1 SOP: Standard Operating Procedure

    1.2 CRF: Case Report Form

2. Purpose / Background

    2.1. The purpose of this SOP is to provide guidance for the processing, testing and storage of all PERCH study specimens.

3. Scope / Applicability

    3.1. This SOP applies to all laboratory personnel involved in the processing of laboratory specimens.

4. Prerequisites / Supplies Needed

    4.1. See test specific SOPs for supplies needed.

5. Roles / Responsibilities

    [Site specific, as needed]

6. Procedural Steps

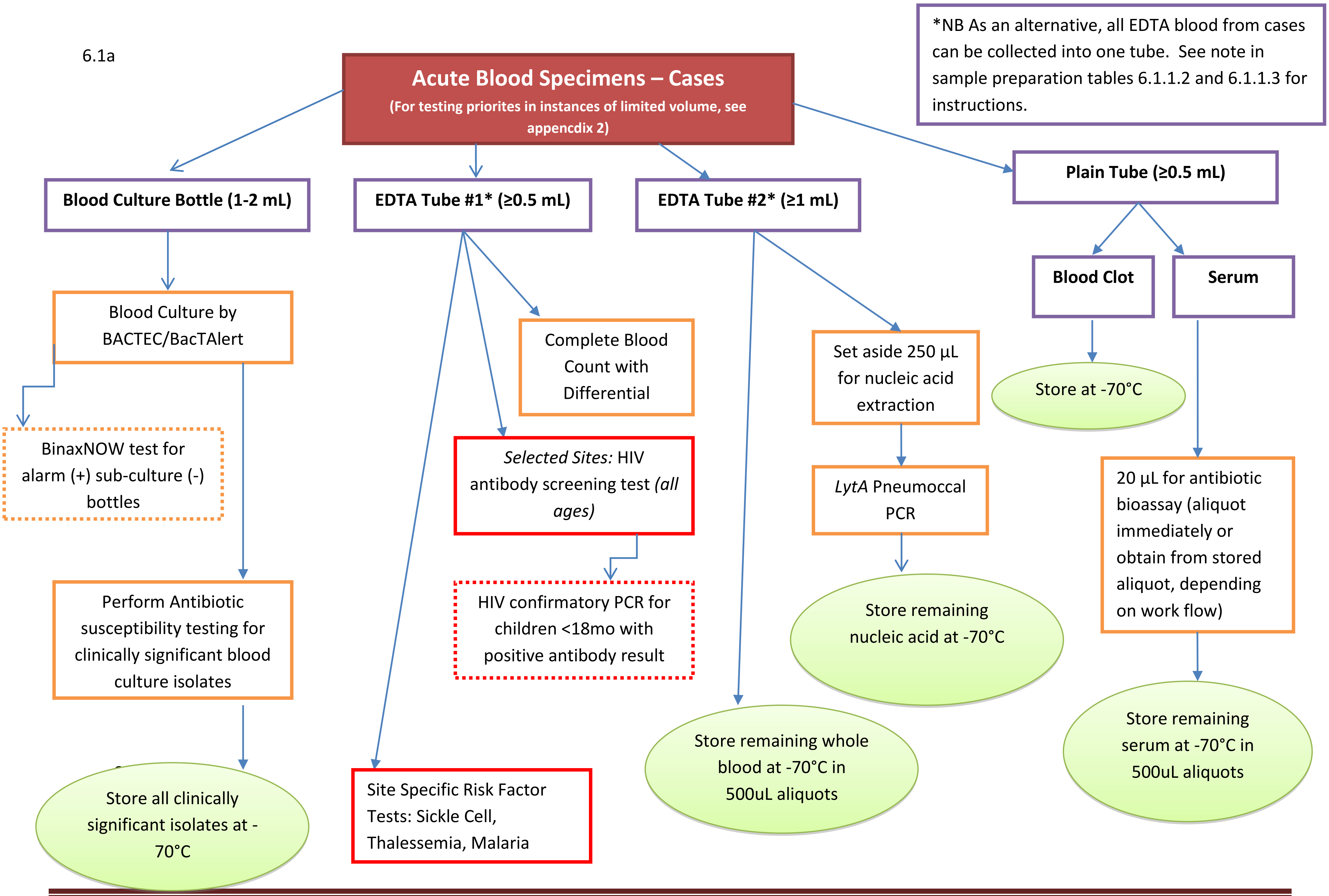
    6.1 Accepting/Rejecting Specimens:

    Staff who receive specimens in the laboratory are responsible, to the best of their ability, for ensuring that specimens have been stored and transported under the conditions specified in Appendix 1, Specimen Transport and Storage Conditions. Specimens will only be rejected for processing for the following reasons:

    (a) specimen is unlabeled
    (b) specimen ID does not match the requisition form
    (c) blood is hemolyzed, or anticoagulated specimens contain clots
    (d) specimen container is leaking.

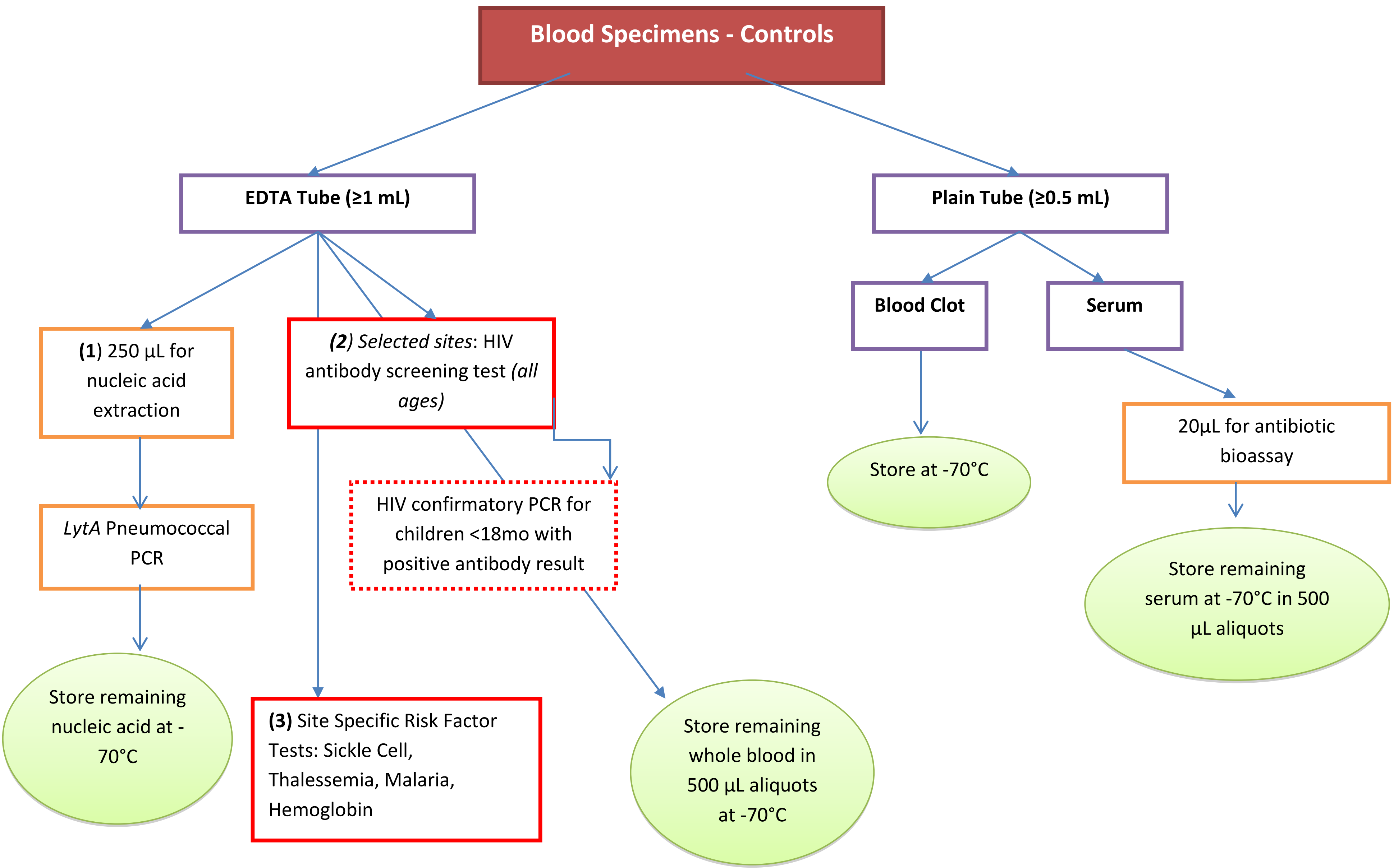
    6.2 Process all study specimens according to the flow charts and specimen preparation tables below. Any departure from the flow charts listed below (e.g. instances of insufficient volume) should be documented as part of the laboratory's quality management process. For specific processing instructions, please refer to the relevant specimen processing SOP.

6.1a



*NB As an alternative, all EDTA blood from cases can be collected into one tube. See note in sample preparation tables 6.1.1.2 and 6.1.1.3 for instructions.
Acute Blood Specimens – Cases
(For testing priorites in instances of limited volume, see appencdix 2)
Blood Culture Bottle (1-2 mL)
EDTA Tube #1* (≥0.5 mL)
EDTA Tube #2* (≥1 mL)
Plain Tube (≥0.5 mL)
Blood Clot
Serum
Blood Culture by BACTEC/BacTAlert
BinaxNOW test for alarm (+) sub-culture (-) bottles
Perform Antibiotic susceptibility testing for clinically significant blood culture isolates
Store all clinically significant isolates at -70°C
Complete Blood Count with Differential
Selected Sites: HIV antibody screening test (all ages)
HIV confirmatory PCR for children <18mo with positive antibody result
Site Specific Risk Factor Tests: Sickle Cell, Thalessemia, Malaria
Set aside 250 µL for nucleic acid extraction
LytA Pneumoccal PCR
Store remaining nucleic acid at -70°C
Store remaining whole blood at -70°C in 500uL aliquots
Store at -70°C
20 µL for antibiotic bioassay (aliquot immediately or obtain from stored aliquot, depending on work flow)
Store remaining serum at -70°C in 500uL aliquots

6.1b



Blood Specimens - Controls
EDTA Tube (≥1 mL)
Plain Tube (≥0.5 mL)
(1) 250 µL for nucleic acid extraction
(2) Selected sites: HIV antibody screening test (all ages)
Blood Clot
Serum
Store at -70°C
20µL for antibiotic bioassay
HIV confirmatory PCR for children <18mo with positive antibody result
LytA Pneumococcal PCR
Store remaining serum at -70°C in 500 µL aliquots
Store remaining nucleic acid at -70°C
(3) Site Specific Risk Factor Tests: Sickle Cell, Thalessemia, Malaria, Hemoglobin
Store remaining whole blood in 500 µL aliquots at -70°C

6.1.1. Specimen Preparation: Acute Blood

6.1.1.1 Whole Blood in Plain Tube (cases and controls**)**

<table>
<tr><td>Specimen type:</td><td colspan="2">Whole blood in plain tube (≥0.5 ml).</td></tr>
<tr><td>Storage:</td><td colspan="2">≤3 days at 2-8°C until specimen separation</td></tr>
<tr><td>Materials and equipment:</td><td colspan="2">Sterile 2 ml Sarstedt tubes or equivalent
Sterile transfer pipettes
Centrifuge</td></tr>
<tr><td rowspan="4">Procedure:</td><td>Step</td><td>Action</td></tr>
<tr><td>1</td><td>Centrifuge the plain blood tube at 3000 rpm for 10 min to separate blood cells</td></tr>
<tr><td>2</td><td>Transfer serum in 500 µl aliquots into Sarstedt tubes labeled with participant details. Store at -70°C.</td></tr>
<tr><td>3</td><td>Transfer blood clot from plain tube into a Sarstedt tubes labeled with participant details. Store at -70°C.</td></tr>
</table>

NB: 20 µl of serum is used for the antibiotic bioassay. This can either be aliquoted separately immediately after specimen separation or can be obtained from one of the stored 500 µl aliquots depending on work flows.

6.1.1.2 Whole Blood in EDTA Tube #1*

Specimen type:	Whole blood in EDTA tube (≥0.5 ml).	
Storage:	≤3 days at 2-8°C until specimen separation	
Materials:	Sterile 2 ml Sarstedt tubes or equivalent Sterile transfer pipettes	
Procedure:	*Step*	*Action*
	1	Process according to local assay requirements for complete blood count, and for relevant sites: HIV antibody testing, sickle cell testing, thalessemia screen and malaria testing.

*NB As an alternative, all EDTA blood from cases can be collected into one tube. In this situation, process as for Whole Blood in EDTA (controls), i.e. the 250 µl aliquot for *Lyt*A PCR testing is first obtained, then an aliquot for complete blood count, HIV antibody testing, sickle cell testing, thalessemia screen and malaria testing. The remainder is then stored in 500 µl aliquots at -70°C.

6.1.1.3 Whole Blood in EDTA Tube #2*

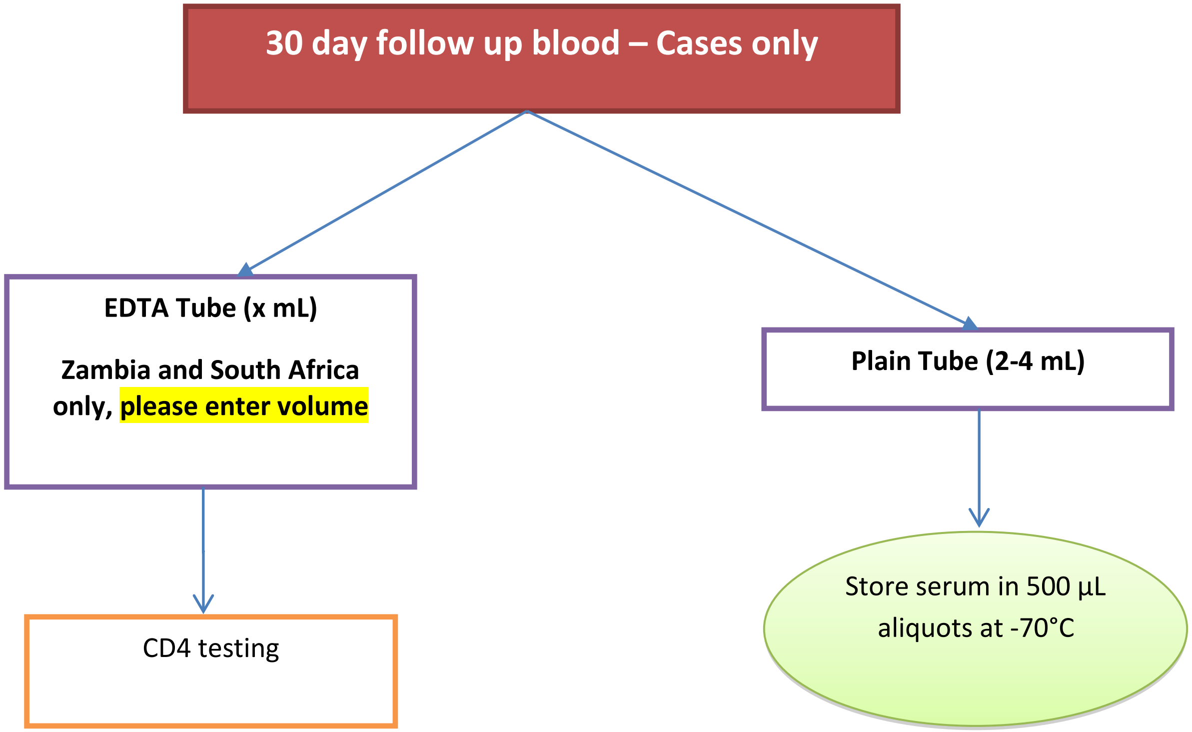
Specimen type:	Whole blood in EDTA tube (≥1 ml, for PCR).	
Storage:	≤3 days at 2-8°C until specimen separation	
Materials:	Sterile 2 ml Sarstedt tubes or equivalent Sterile transfer pipettes	
Procedure:	*Step*	*Action*
	1	Transfer 250 µl of whole blood into a Sarstedt tube labeled with participant details. This will be used for *LytA* PCR testing. This aliquot should undergo total nucleic acid extraction as soon as possible. If not extracted within 72 h, store at -70°C.
	2	Transfer remaining whole blood in 500 µl aliquots into Sarstedt tubes labeled with participant details. Store at -70°C.

*NB As an alternative, all EDTA blood from cases can be collected into one tube. In this situation, process as for Whole Blood in EDTA (controls), i.e. the 250 µl aliquot for *Lyt*A PCR testing is first obtained, then an aliquot for complete blood count, HIV antibody testing, sickle cell testing, thalessemia screen and malaria testing. The remainder is then stored in 500 µl aliquots at -70°C.

6.1.1.4 Whole Blood in EDTA (controls)

<table>
<tr><td>Specimen type:</td><td colspan="2">Whole blood in EDTA tube (≥1 ml, for PCR).</td></tr>
<tr><td>Storage:</td><td colspan="2">≤3 days at 2-8°C until specimen separation</td></tr>
<tr><td>Materials:</td><td colspan="2">Sterile 2 ml Sarstedt tubes or equivalent
Sterile transfer pipettes</td></tr>
<tr><td rowspan="4">Procedure:</td><td>Step</td><td>Action</td></tr>
<tr><td>1</td><td>Transfer 250 µl of whole blood into a Sarstedt tube labeled with participant details. This will be used for LytA PCR testing. This aliquot should undergo total nucleic acid extraction as soon as possible. If not extracted within 72 h, store at -70°C.</td></tr>
<tr><td>2</td><td>Process according to local assay requirements for complete blood count, and for relevant sites: HIV antibody testing, sickle cell testing, thalessemia screen and malaria testing.</td></tr>
<tr><td>3</td><td>Transfer remaining whole blood in 500 µl aliquots into Sarstedt tubes labeled with participant details. Store at -70°C.</td></tr>
</table>

6.2


30 day follow up blood – Cases only
EDTA Tube (x mL)
Zambia and South Africa only, please enter volume
Plain Tube (2-4 mL)
CD4 testing
Store serum in 500 µL aliquots at -70°C

6.2.1 Specimen Preparation: 30 day follow up blood

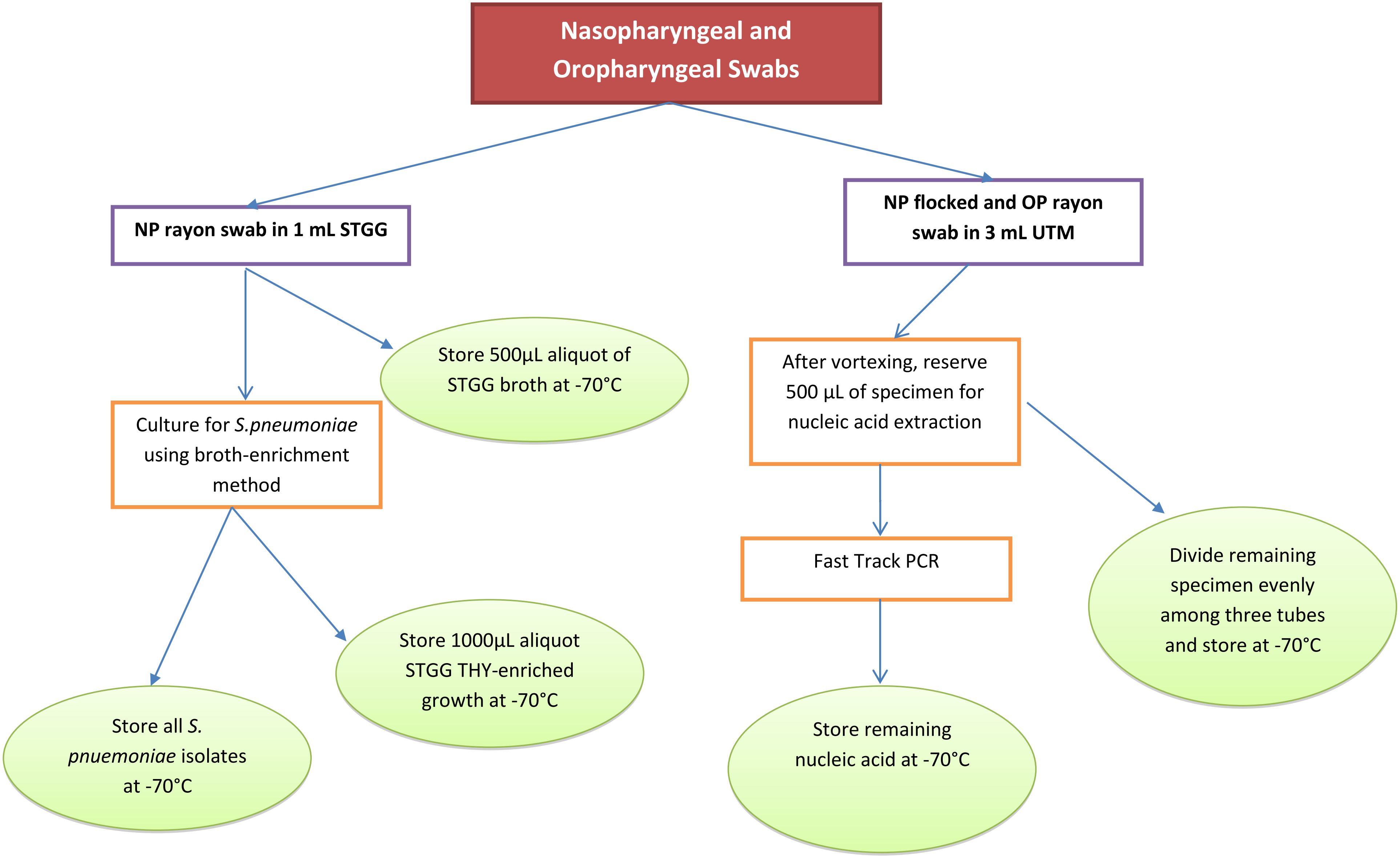
6.2.1.1 30-Day Follow-Up Whole Blood in Plain Tube (all cases)

Specimen type:	Whole blood in plain tube 2-4 ml).	
Storage:	≤3 days at 2-8°C until specimen separation	
Materials and equipment:	Sterile 2 ml Sarstedt tubes or equivalent Sterile transfer pipettes Centrifuge	
Procedure:	*Step*	*Action*
	1	Centrifuge the plain blood tube at 3000 rpm for 10 min to separate blood cells
	2	Transfer serum in 500 µL aliquots into Sarstedt tubes labeled with participant details. Store at -70°C.

6.2.1.2 30-Day Follow-Up Whole Blood in EDTA (cases from Zambia and South Africa only)

Specimen type:	Whole blood in EDTA tube	
Storage:	≤3 days at 2-8°C until specimen separation	
Materials:	Sterile 2 ml Sarstedt tubes or equivalent Sterile transfer pipettes	
Procedure:	*Step*	*Action*
	1	Process according to local assay requirements for CD4 testing

6.3


Nasopharyngeal and Oropharyngeal Swabs
NP rayon swab in 1 mL STGG
NP flocked and OP rayon swab in 3 mL UTM
Store 500µL aliquot of STGG broth at -70°C
Culture for *S.pneumoniae* using broth-enrichment method
After vortexing, reserve 500 µL of specimen for nucleic acid extraction
Fast Track PCR
Divide remaining specimen evenly among three tubes and store at -70°C
Store 1000µL aliquot STGG THY-enriched growth at -70°C
Store all *S. pnuemoniae* isolates at -70°C
Store remaining nucleic acid at -70°C

6.3.1 Specimen Preparation: NP/OP Swabs

6.3.1.1 Nasopharyngeal/Oropharyngeal Swabs in Viral transport Medium

<table>
<tr><td>Specimen type:</td><td colspan="2">Nasopharyngeal swab and oropharyngeal swab in 3 ml universal transport medium (UTM).</td></tr>
<tr><td>Storage:</td><td colspan="2">≤24 h at 2-8°C (≤2 h at room temperature) until processing</td></tr>
<tr><td>Materials:</td><td colspan="2">Sterile 2 ml Sarstedt tubes or equivalent
Sterile transfer pipettes</td></tr>
<tr><td rowspan="5">Procedure:</td><td>Step</td><td>Action</td></tr>
<tr><td>1</td><td>Vortex for 20-30 seconds with swabs still in the fluid</td></tr>
<tr><td>2</td><td>Remove swabs (fluid can be expressed from the swabs by pressing on the inside of the vial before removal). Swabs can then be discarded.</td></tr>
<tr><td>3</td><td>Transfer 500 µl fluid into a new Sarstedt tube labeled with participant details. This will be used for extraction and multiplex PCR. Store at -70°C (or 2-8°C (max 24-48 h)) until further processing.</td></tr>
<tr><td>4</td><td>Transfer remaining fluid into each of 3 Sarstedt tubes labeled with participant details. Store at -70°C.</td></tr>
</table>

6.3.1.2 Nasopharyngeal Swabs in STGG

Specimen type:	Nasopharyngeal swab in 1 ml STGG	
Storage:	<8 h at 2-8°C until freezing at -70°C	
Materials:	Sterile 2 ml Sarstedt tubes or equivalent Sterile transfer pipettes	
Procedure:	*Step*	*Action*
	1	Process immediately, making sure to store or store at -70°C for delayed processing. After processing, store one 500µL aliquot of STGG at -70°C in a Starstedt tube.
	2	Following the STGG broth enrichment step, Transfer 1.0 ml of the THY enriched growth into screw-cap 1.5 ml vials (cryotube) and store at -70°C.

6.4

Note: In instances of limited volume, prioritize mycobacterial testing, followed by PCR testing.

Initial Induced Sputum Specimen

Make Gram stain smear and inoculate media culture.

Store gram-stained slide in a box in chronological order

If ≤ 500 µL of specimen remains at this point, send all for mycobacterial testing and stop. Otherwise, proceed as below.

If there is enough volume, store two 500 µL aliquots of specimen at -70°C

The remainder of the specimen, (at least 500 µL) should be reserved for mycobacterial testing and may be transferred in the original container (ensure that this includes some of the purulent portion of the specimen).

Set aside 500 µL of specimen for Nucleic Acid Extraction

Fast Track PCR

Store remaining specimen at -70°C

Store remaining nucleic acid at -70°C

Perform Antibiotic susceptibility testing for clinically significant culture isolates

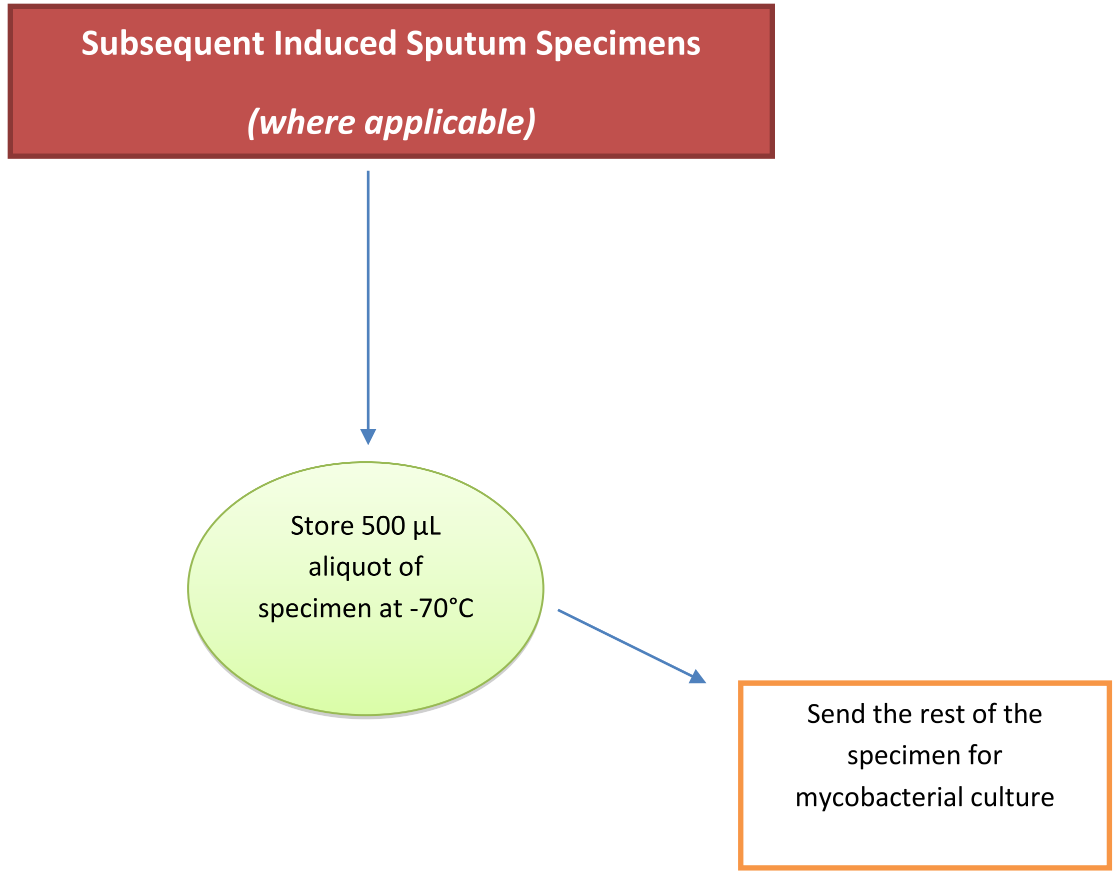
Store all clinically significant isolates at -70°C

6.4.1 Specimen Preparation: Initial Induced Sputum Specimen

6.4.1.1 Initial Induced Sputum Specimen

Specimen type:	Induced sputum in a sterile container.	
Storage:	≤24 h at 2-8°C (≤2 h at room temperature) until processing	
Materials:	Sterile 2 ml Sarstedt tubes or equivalent Sterile transfer pipettes	
Procedure:	*Step*	*Action*
	1	Make Gram stain smear and inoculate media for culture.
	2	If ≤ 500 µL of specimen remains at this point, send all for mycobacterial testing and stop. Otherwise, proceed as below.
	3	Transfer 500 µl of sputum into a Sarstedt tube labeled with participant details. Ensure some of the purulent portion is included if it is a purulent specimen.This will be used for nucleic acid extraction and multiplex PCR. Store at -70°C (or 2-8°C (max 24-48 h)) until further processing.
	4	Transfer at least 500 µl of remaining original sputum into each of two Sarstedt tubes labeled with participant details. Store at -70°C.
	5	The remainder of the original specimen (at least 500 µl) is reserved for mycobacterial testing and may be transferred in the original container. Ensure some of the purulent portion goes for mycobacterial culture if it is a purulent specimen.

6.5.

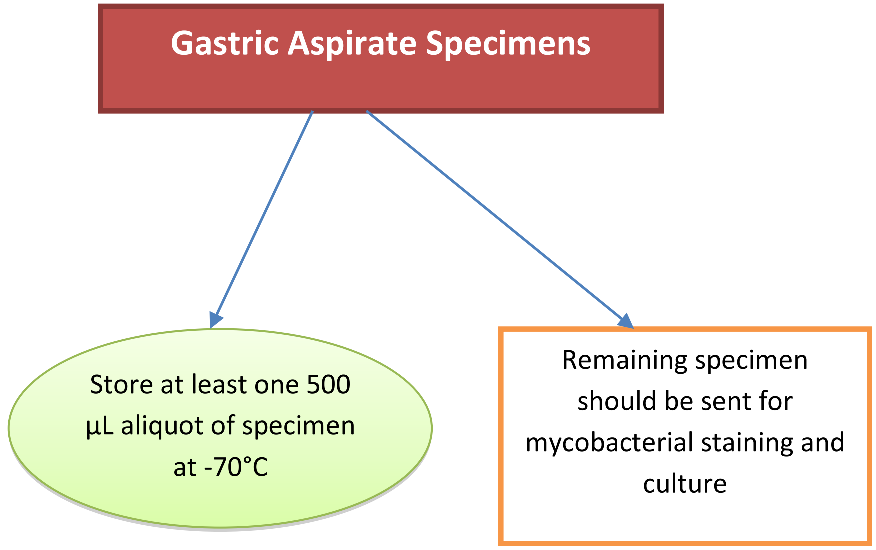

Subsequent Induced Sputum Specimens
(where applicable)
Store 500 µL aliquot of specimen at -70°C
Send the rest of the specimen for mycobacterial culture

6.5.1 Specimen Preparation: Subsequent Induced Sputum Specimen, when collected

6.5.1.1 Subsequent Induced Sputum Specimen

Specimen type:	Induced sputum in a sterile container.	
Storage:	≤24 h at 2-8°C (≤2 h at room temperature) until processing	
Materials:	Sterile 2 ml Sarstedt tubes or equivalent Sterile transfer pipettes	
Procedure:	*Step*	*Action*
	1	Transfer 500 µl of sputum into a Sarstedt tube labeled with participant details. Store at -70°C.
	2	Send remaining specimen for mycobacterial testing.

6.6

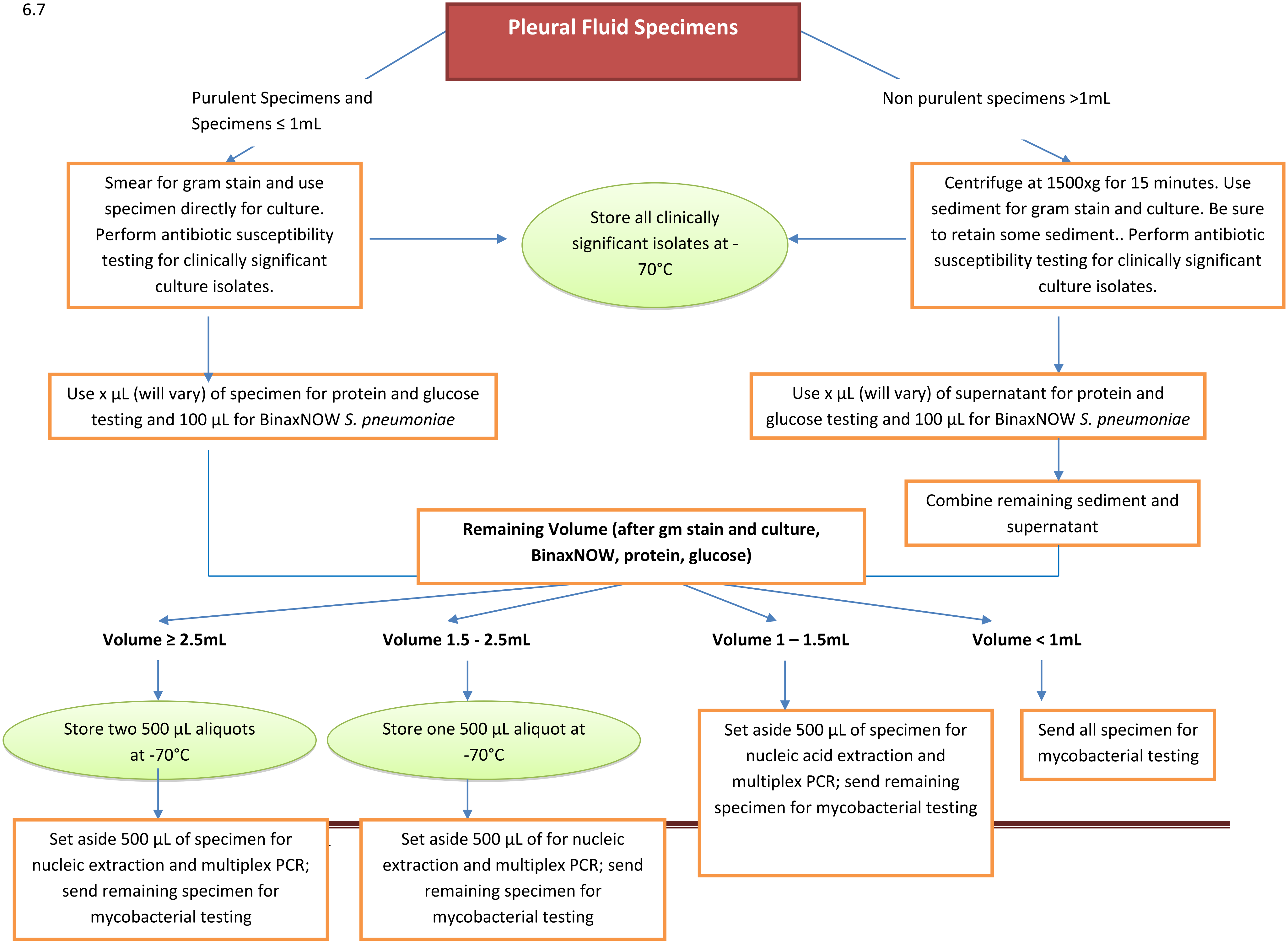

Gastric Aspirate Specimens
Store at least one 500 µL aliquot of specimen at -70°C
Remaining specimen should be sent for mycobacterial staining and culture

6.6.1 Specimen Preparation: Gastric Aspirate

6.6.1.1 Gastric Aspirate

<table>
<tr><td>Specimen type:</td><td colspan="2">Gastric aspirate in a sterile container</td></tr>
<tr><td>Storage:</td><td colspan="2">≤24 h at 2-8°C (≤2 h at room temperature) until processing</td></tr>
<tr><td>Materials and equipment:</td><td colspan="2">Sterile 2 ml Sarstedt tubes or equivalent
Sterile transfer pipettes</td></tr>
<tr><td rowspan="3">Procedure:</td><td>Step</td><td>Action</td></tr>
<tr><td>1</td><td>Transfer 500 µl of gastric aspirate into a Sarstedt tube labeled with participant details. Store at -70°C.</td></tr>
<tr><td>2</td><td>Send remaining specimen for mycobacterial testing</td></tr>
</table>

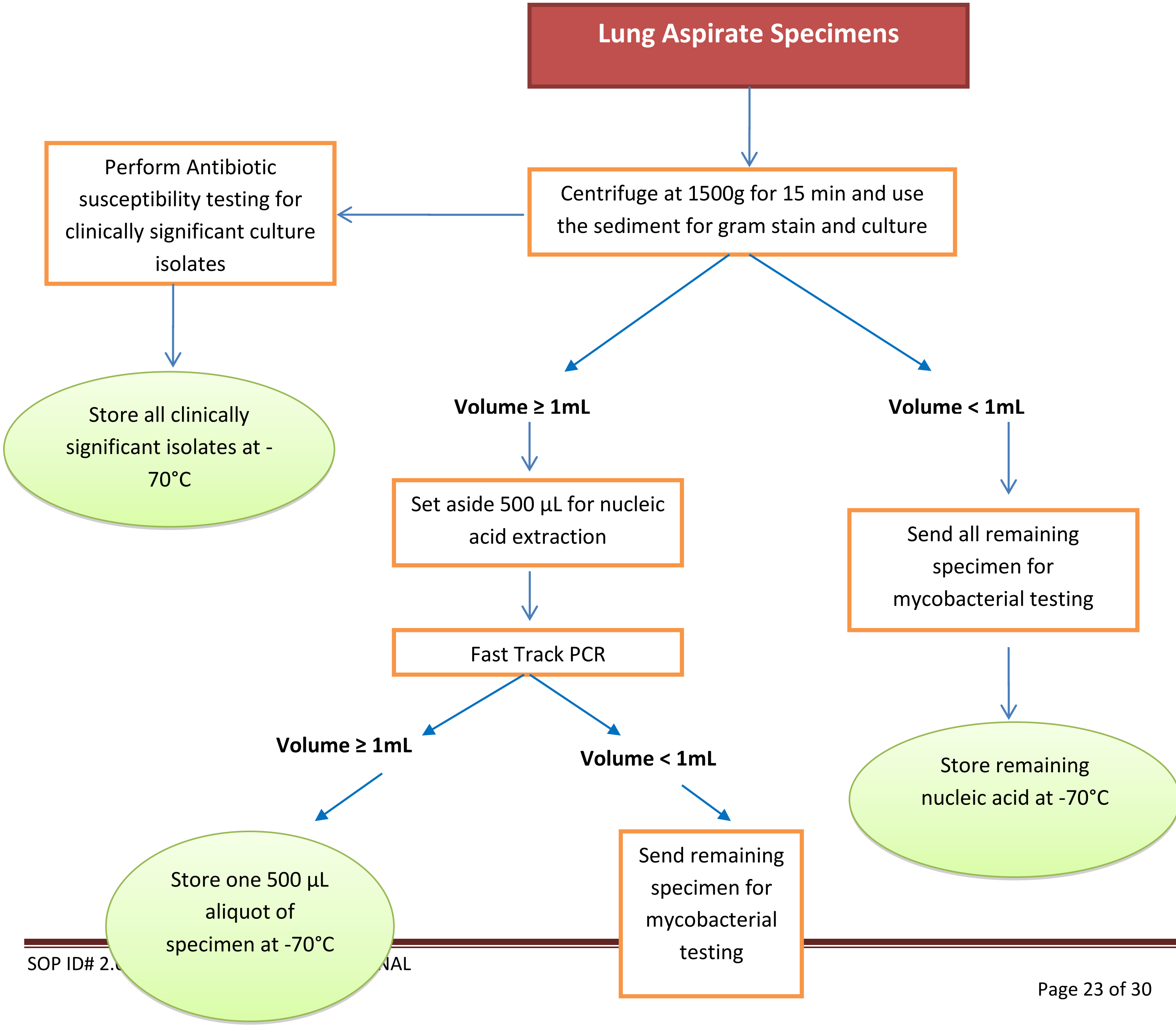
6.7


Pleural Fluid Specimens
Purulent Specimens and Specimens ≤ 1mL
Non purulent specimens >1mL
Smear for gram stain and use specimen directly for culture. Perform antibiotic susceptibility testing for clinically significant culture isolates.
Store all clinically significant isolates at -70°C
Centrifuge at 1500xg for 15 minutes. Use sediment for gram stain and culture. Be sure to retain some sediment.. Perform antibiotic susceptibility testing for clinically significant culture isolates.
Use x µL (will vary) of specimen for protein and glucose testing and 100 µL for BinaxNOW S. pneumoniae
Use x µL (will vary) of supernatant for protein and glucose testing and 100 µL for BinaxNOW S. pneumoniae
Combine remaining sediment and supernatant
Remaining Volume (after gm stain and culture, BinaxNOW, protein, glucose)
Volume ≥ 2.5mL
Volume 1.5 - 2.5mL
Volume 1 – 1.5mL
Volume < 1mL
Store two 500 µL aliquots at -70°C
Store one 500 µL aliquot at -70°C
Set aside 500 µL of specimen for nucleic acid extraction and multiplex PCR; send remaining specimen for mycobacterial testing
Send all specimen for mycobacterial testing
Set aside 500 µL of specimen for nucleic extraction and multiplex PCR; send remaining specimen for mycobacterial testing
Set aside 500 µL of for nucleic extraction and multiplex PCR; send remaining specimen for mycobacterial testing

6.7.1 Specimen Preparation: Pleural Fluid

Specimen type:	Pleural fluid in a sterile container	
Storage:	≤24 h at 2-8°C (≤2 h at room temperature) until processing	
Materials and equipment:	Sterile 2 ml Sarstedt tubes or equivalent Sterile transfer pipettes Centrifuge	
Procedure:	*Step*	*Action*
	1	If specimen is purulent or ≤1 mL, make a smear for a Gram stain and use the specimen directly for culture.
	2	If specimen is non-purulent and >1 mL, centrifuge at 1500xg for 15 min. Use the sediment for the Gram stain and culture.
	3	Use sufficient volume of specimen (or supernatant for centrifuged specimens) for protein and glucose testing (volume will depend on local assay requirements), and 100 µL for BinaxNOW *S. pneumoniae* testing
	4	For centrifuged specimens, combine remaining sediment and supernatant.
	5	The remaining specimen should be aliquoted according to the following schema: • If volume ≥2.5 mL: transfer 500 µl of specimen into a Sarstedt tube for nucleic acid extraction and multiplex PCR; store two additional 500 µL aliquots at -70°C; send remaining specimen for mycobacterial testing. • If volume 1.5-2.5 mL: transfer 500 µl of specimen into a Sarstedt tube for nucleic acid extraction and multiplex PCR; store one additional 500 µL aliquot at -70°C; send remaining specimen for mycobacterial testing. • If volume 1-1.5 mL: transfer 500 µl of specimen into a Sarstedt tube for nucleic acid extraction and multiplex PCR; send remaining specimen for mycobacterial testing. • If volume <1mL: send all for mycobacterial testing.

6.8

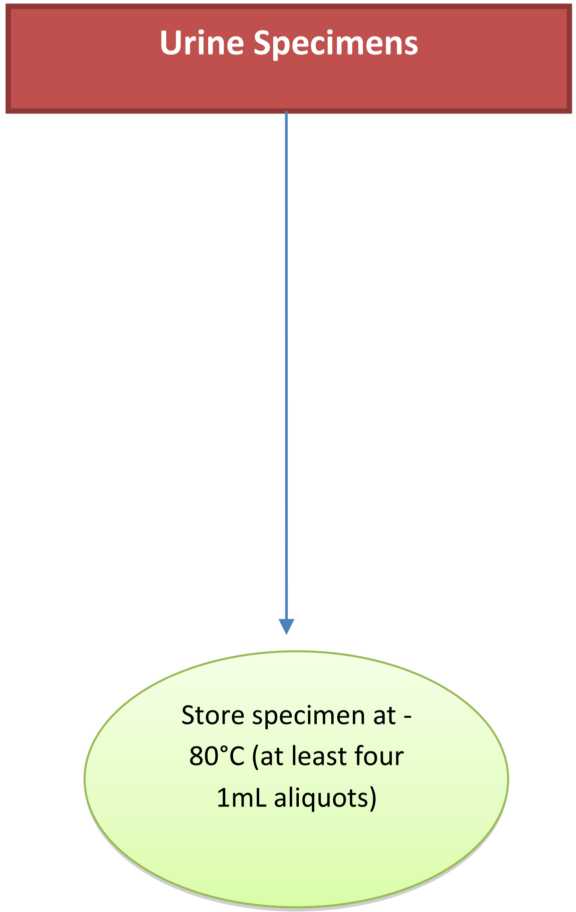


Lung Aspirate Specimens
Centrifuge at 1500g for 15 min and use the sediment for gram stain and culture
Perform Antibiotic susceptibility testing for clinically significant culture isolates
Store all clinically significant isolates at -70°C
Volume ≥ 1mL
Volume < 1mL
Set aside 500 µL for nucleic acid extraction
Send all remaining specimen for mycobacterial testing
Fast Track PCR
Store remaining nucleic acid at -70°C
Volume ≥ 1mL
Volume < 1mL
Store one 500 µL aliquot of specimen at -70°C
Send remaining specimen for mycobacterial testing

6.8.1 Specimen Preparation: Lung Aspirate

<table>
<tr><td>Specimen type:</td><td colspan="2">Lung aspirate in a sterile container</td></tr>
<tr><td>Storage:</td><td colspan="2">≤24 h at 2-8°C (≤2 h at room temperature) until processing</td></tr>
<tr><td>Materials and equipment:</td><td colspan="2">Sterile 2 ml Sarstedt tubes or equivalent
Sterile transfer pipettes</td></tr>
<tr><td rowspan="4">Procedure:</td><td>Step</td><td>Action</td></tr>
<tr><td>1</td><td>Centrifuge at 1500 g for 15 min and use the sediment for the Gram stain and culture</td></tr>
<tr><td>2</td><td>If specimen volume is ≥1 ml, transfer 500 µl into a Sarstedt tube labeled with participant details. This will be used for extraction and multiplex PCR. Store at -70°C (or 2-8°C (max 24-48 h)) until further processing. Send remaining specimen for mycobacterial testing.</td></tr>
<tr><td>2</td><td>If specimen volume is <1 ml send all remaining specimen for mycobacterial testing.</td></tr>
</table>

6.9


Urine Specimens
Store specimen at -80°C (at least four 1mL aliquots)

6.9.1 Specimen Preparation: Urine

Specimen type:	Urine in a sterile container	
Storage:	≤24 h at 2-8°C (≤2 h at room temperature) until processing	
Materials and equipment:	Sterile 2 ml Sarstedt tubes or equivalent Sterile transfer pipettes	
Procedure:	*Step*	*Action*
	1	Transfer urine into a Sarstedt tubes labeled with participant details (at least four 1mL aliquots). Store at -70°C.

NB: testing urine for antibiotic activity will be delayed for at least a few months into the study until we have assessed the success of urine collection.

6.10 Stored samples for Cases and Controls

	Specimen	Sample	Number	Volume
Cases	Acute Blood*	Blood culture isolates	As appropriate	n/a
		Whole blood aliquots	1-2	500 µL
		Extracted NA	As appropriate	All remaining
		Clot from plain tube	1	n/a
		Serum	1-2	500 uL
	Convalescent Blood*	Serum	1-4	500 uL
	NP STGG swab	S. pneumoniae isolates	As appropriate	n/a
		STGG broth	1	500 uL
		STGG THY enriched growth	1	1mL
	NP/OP VTM swab	Extracted NA	As appropriate	All remaining
		VTM	3	All remaining, split between 3 vials
	1st Induced Sputum	Gram Stain Slide	1	n/a
		Culture isolates	As appropriate	n/a
		Extracted NA	As appropriate	All remaining
		Specimen aliquots	0-2	500 uL
	2nd Induced Sputum	Specimen aliquots	0-3	500 uL
	Gastric Aspirate	Specimen aliquots	1-2	500 uL
	Pleural Fluid	Culture isolates	As appropriate	n/a
		Extracted NA		All remaining
		Specimen aliquots	0-2	500 uL
	Lung Aspirate	Culture isolates	As appropriate	n/a
		Extracted NA	As appropriate	All remaining
		Specimen aliquots	0-2	500 uL
	Urine	Specimen aliquots - option 1	4	1 mL
		Specimen aliquots - option 2	2	2 mL
Controls	Blood*	Extracted NA	As appropriate	All remaining
		Clot from plain tube	1	n/a
		Serum	1-2	500 uL
	NP STGG swab	S. pneumoniae isolates	0-1	n/a
		STGG broth	1	500 uL
	NP/OP VTM swab	Extracted NA	As appropriate	All remaining
		VTM	3	All remaining, split between 3 vials
	Urine	Specimen aliquots	4	1 mL

***Note: remaining blood/serum should NEVER be discarded, even if final aliquot is less than specified volume.**

7. Record Management

CRF 19 (Specimen requisition/reception) should be completed for all PERCH specimens received by the laboratory. Internal logs or other quality management tools should document any reasons why testing for a specimen is not completed according to the algorithms in this SOP. The relevant CRFs 20-27 should be completed for all study results.

8. Quality Assurance / Quality Control

8.1 The specimen reception SOP should be followed for all specimens received in the laboratory, and any departures from the SOP should be documented.

8.2 A laboratory supervisor must review all laboratory results CRFs

9. References

PERCH LAB SOP 2.0	Testing Algorithm
PERCH LAB SOP 2.1A	Processing of Blood Cultures BACTEC
PERCH LAB SOP 2.1B	Processing of Blood Cultures BacT_ALERT
PERCH LAB SOP 2.2	Antibiotic Susceptibility Testing (AST)
PERCH LAB SOP 2.3	Antibiotic Bioassay
PERCH LAB SOP 2.4	*lytA* pneumococcal PCR
PERCH LAB SOP 2.5	Processing of NP STGG Swab
PERCH LAB SOP 2.6	Processing of Induced Sputum
PERCH LAB SOP 2.7	Processing of Pleural Fluid
PERCH LAB SOP 2.8	Processing of Lung Aspirate
PERCH LAB SOP 2.9.1	Fast Track 33 Manual
PERCH LAB SOP 2.9.2	Fast Track 33 Quantification Manual
PERCH LAB SOP 2.9.3	Fast Track EQA Manual
PERCH LAB SOP 2.10	Nucleic Acid Extraction

APPENDIX 1 Specimen Transport and Storage Conditions

Specimen	Transport/storage conditions*	Until
Blood culture	≤24 h, room temperature or according to manufacturer's instructions	Placement in blood culture machine
Whole blood (EDTA and plain tubes)	<3 days, 2-8°C	Specimen separation
Urine	≤24 h, 2-8°C (≤2 h, room temperature)	Freezing (-70°C)
NP/OP swabs in Viral Transport Medium	≤24 h, 2-8°C (≤2 h, room temperature)	Freezing (-70°C)
NP swab in STGG	<8 h, 2-8°C	Freezing (-70°C)
Induced Sputum	≤96 h for mycobacterial culture (store at 2-8°C in interim); 24 h, 2-8°C (≤2 h, room temperature) for all other testing	Inoculation onto culture media and other primary laboratory processing
Lung Aspirate	≤24 h, 2-8°C (≤2 h, room temperature)	Inoculation onto culture media and other primary laboratory processing
Gastric Aspirate	≤96 h, 2-8°C (≤15 min, room temperature)	Mycobacterial culture
Pleural Fluid	≤24 h, 2-8°C (≤2 h, room temperature)	Inoculation onto culture media and other primary laboratory processing
Lung Tissue	≤24 h, 2-8°C (≤2 h, room temperature)	Inoculation onto culture media and other primary laboratory processing

Appendix 2 – Case Acute Blood Testing Priorities in Instances of Limited Volume

1) Blood cultures
   CBC
   malaria slides (for endemic sites)
   HIV serology (for high prevalence sites)

2) Purple top tube for PCR, etc., (up to 1 ml max.)

3) If there is sufficient volume, any remaining blood should be placed in the red top tube

SOP Updates:

9September11:

- *Flow chart 6.3, table 6.3.1.2, and table 6.10* corrected to reflect storage of 1ml STGG THY enriched growth aliquot as well as 500ul STGG aliquot from specimen collection container.
- *Flow chart 6.1a, tables 6.1.1.2 and 6.1.1.3* corrected to align numbering EDTA tubes with CRF 06
- *Appendix 1* corrected to allow for refrigerated storage of induced sputum and gastric aspirates for up to 96 hours prior to mycobacterial culture.

►Standard Operating Procedure◄	
Section: Laboratory	Version: FINAL Initials: ______
Title: 2.1A Processing of Blood Cultures_BACTEC	Revision Date: 20 April 2011

1. Definitions.
   1.1. CRF – Case Report Form

2. Purpose / Background
   2.1. The blood of healthy individuals is usually sterile. Blood cultures are a standard laboratory tool for detecting invasive bacterial/fungal disease. The BACTEC is an automated blood culture system which incubates and continuously monitors blood cultures. A positive result is signaled immediately upon detection of carbon dioxide production in the blood culture bottle.

   2.2. The quality of blood collection greatly affects the sensitivity and reliability of any blood culture system. Critical factors are:
   Timing of blood collection. Before administration of antibiotics.
   Volume of blood collected. Sensitivity increases with volume; bacteremias can be missed by relying upon small blood volumes, which is a particular issue in children.
   Skin disinfection. Contamination of blood cultures with bacteria that commonly reside on the skin can cause false positive results for bacteremia, may prevent identification of a true pathogen, and complicate the interpretation of our laboratory results. Thorough disinfection of the venipuncture site can significantly reduce the number of contaminated blood cultures.

   2.2. The purpose of this SOP is to give guidance on isolation of organisms found in blood specimens.

3. Scope / Applicability
   3.1. This SOP is applicable to all trained laboratory technicians/technologists/scientists working in the microbiology laboratory.

4. Roles / Responsibilities
   [Site specific]
5. Specimen
   5.1. Blood received in the laboratory in BACTEC Paeds PLUS blood culture bottles (see appendix 1, collection of blood for blood culture)

6. Prerequisites / Supplies Needed
   6.1. Equipment
   6.1.1 CO_2 Incubators or Candle jar
   6.1.2 Aerobic incubator
   6.1.3 BD BACTEC machine

6.2. Media

6.2.1 5% Sheep Blood agar
6.2.2 Chocolate agar
6.2.3 MacConkey agar

6.3. Materials

6.3.1 Alcohol
6.3.2 Antimicrobial susceptibility discs.
6.3.3 Biochemical reagents
6.3.4 Paediatric blood culture bottles BACTEC Paeds Plus
6.3.5 Cotton wool/gauze
6.3.6 Gram stain reagents
6.3.7 Syringes and needles
6.3.8 Sterile gloves
6.3.9 Tourniquet
6.3.10 Microscope Slides
6.3.11 Sharps container

7. Safety/Risk Assessment

7.1 Wear Personal Protective Equipment at ALL times when processing blood culture samples. Process all blood cultures in a biosafety cabinet.

7.2 Waste disposal: Discard all sharps in sharp boxes. Autoclave all culture plates and other clinical wastes before taking them for incineration. Reusable material must be autoclaved before washing

8. Procedural Steps

8.1. **Pre-processing specimen handling**

Blood culture bottles should be placed in the BACTEC blood culture instrument as soon as possible after arrival in the laboratory. Store at air-conditioned room temperature if unable to process immediately. *[This will need to be modified based on specific manufacturers' instructions, as some systems allow incubation in a standard incubator before placement on automated instrument]*

8.2. **Initial processing of blood culture bottles**

Each blood culture bottle should be weighed and the value subtracted from the uninoculated weight of the bottle. The result is the weight of the inoculated blood and is recorded on the CRF.

[specific to each automated system]

8.3. **Protocol for positive blood cultures (BD Bactec 9050)**

Day 1

8.3.1. Unload positive blood culture bottle(s) according to BD Bactec 9050 instrument operation protocol (See instrument operation protocol below).

8.3.2. In the Biohazard cabinet, sterilise the rubber top of the bottle with 70% alcohol. Using a BD vent needle inoculate the following:

5% Blood Agar (aerobic)
Chocolate Agar
MacConkey Agar
Slide for Gram stain

Spread plates and incubate aerobic plates at 35-37°C with 5% CO_2

8.3.3. Leave Gram to air dry then stain and examine.

8.3.4. Once the Gram stain reaction and morphology is known, notify the clinician.

Day 2

8.3.5. Examine plates and identify isolates according to standard microbiological methods.

8.3.6. Perform antibiotic susceptibility testing (refer to AST SOP 2.2).

8.3.7. Reincubate plates for a further 24 hours.

Day 3

8.3.8. Re-examine plates. Identify and perform antibiotic susceptibility testing on any further isolates that may have grown.

8.3.9. Report confirmed sensitivities and final identification if available.

Notes:

- All positive blood cultures are initially regarded as significant.
- All clinically significant isolates should be frozen at -80°C.

8.4. **Reporting Isolates of Doubtful Significance**

8.4.1. **Coagulase Negative Staphylococcus**

Only perform antibiotic susceptibilities or freeze coagulase negative staphylococci when:

- it has been isolated from another normally sterile site e.g. CSF, or
- when isolated from more than blood culture from different times and/or sites.
  All other isolates are regarded as contaminants.

8.4.2 **Micrococcus and Propionibacterium**

These bacteria are regarded as contaminants. Do not perform antibiotic susceptibility testing.

8.4.3 **Corynebacterium spp.**

Corynebacterium bacteria should only be identified and antibiotic susceptibility testing performed if:

- isolated from more than one set of blood cultures from different times and/or sites, or
- the same corynebacterium has been isolated from another, normally sterile, site e.g. CSF.
  All other isolates are regarded as contaminants.

8.4.4 **Bacillus spp.**

- Identification and freezing is only necessary if *Bacillus cereus* or *B. anthracis* is suspected, (**NB. if *B. anthracis* is suspected any work-up should be performed in a BSL-3 laboratory) or**
- If the same *Bacillus* species has been isolated from another, normally sterile, site eg. CSF
  All other isolates are regarded as contaminants.

8.4.5 **Alpha –hemolytic streptococci**

Only perform antibiotic susceptibilities on alpha-hemolytic streptococci when:

- *S. pneumoniae* or *Enterococcus* species have first been ruled out, and
- it has been isolated from another normally sterile site, or
- when isolated from more than one set of blood cultures from different times and/or sites, or endocarditis is suspected
  All other isolates are regarded as contaminants.

8.5. Protocol for "False Positive" blood cultures

If a bottle has been flagged positive by the BacT/ALERT instrument and the Gram stain is negative, sub-culture as outlined for a positive culture above and keep the blood culture bottle at room temperature. If the 24-hour plates do not show any growth, test with the NOW Streptococcus pneumoniae Test® (Binax). This rapid immunochromatographic assay is designed to detect *S. pneumoniae* antigens in the urine of patients with pneumonia and cerebrospinal fluid from patients with meningitis, but the test can be modified to detect pneumococcal antigens in blood culture medium even when no organisms can be seen on Gram stain or culture.

Procedure

8.5.1 Place blood culture bottles in a biosafety cabinet to avoid accidental aerosolization of potentially infectious materials. Use standard precautions for safe handling of blood.

8.5.2 Allow blood cultures to equilibrate to room temperature (15-30ºC) and swirl gently to resuspend antigens before beginning tests.

8.5.3 Wipe gloves with 70% alcohol, then carefully disinfect the top (rubber septum) of each bottle with 70% alcohol.

8.5.4 Unwrap one testing device for each specimen to be tested and lay flat in biosafety cabinet without touching the reaction area of the testing device. Label it with the identification number of the blood culture bottle to be tested.

8.5.5 Remove one Binax swab per sample from the kit, and use the foil package from the testing device as a tray for the swab. Do not use other swabs for this test.

8.5.6 Using a BD Vent needle, aseptically remove approximately 0.5 mLs of blood culture media from each positive bottle.

8.5.7 Drop culture media from the needle onto a Binax swab until the swab head is completely soaked, but not lying in a puddle of excess media. If the swab head drips when picked up, remove the excess liquid by pressing against inside edge of the foil package.

8.5.8 Insert the swab into the bottom hole (swab well) on the inner right panel of the testing device. Firmly push upwards so that the swab tip is fully visible in the top hole. Do not remove the swab.

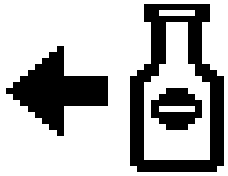
8.5.9 Hold the Reagent A vial vertically (straight up and down) 1-2 cm above the device. Slowly let 3 drops of Reagent A fall into the bottom hole.

8.5.10 Immediately remove the adhesive liner from the right edge of the test device, and close and seal the device. Repeat all steps for each apparent false positive bottle.

8.5.11 Read the result in the window 15 minutes after closing the device. Results read after 15 minutes may not be accurate; strongly positive samples may produce a visible sample line in less than 15 minutes.

8.5.12 One or two lines should appear in the window on the testing device. A single pink-to-purple colored Control Line in the top half of the window means that the test was performed correctly, but no pneumococcus antigens were detected. The appearance of two pink-to purple colored lines, the Control Line and a Sample Line, indicated a positive result even if the sample line is very faint. If no lines appear, or only the bottom Sample Line appears, the test results are invalid. If this happens, the test should be repeated using three samples: the pre-packaged positive and negative control swabs, and the blood culture again.

8.6. **Protocol for negative blood cultures (BD Bactec 9050)**



A minus sign will be flagged on the screen/panel of the BD Bactec 9050 machine indicating that the bottle/s do not contain any growing microbes and should be removed.
This should be set for 5 days of incubation.
(Instrument operation protocol for removing negative blood culture bottles is shown below).

9. Record Management

Access, location, and retention of records pertaining to the SOP – site specific

10. Quality Assurance / Quality Control

10.1 Initial training and competency assessment of all appropriate staff in this SOP.
10.2 Ongoing monitoring of blood culture bottle weights
10.3 Ongoing monitoring of blood culture contamination rates
10.4 Ongoing monitoring of reports

11. References

11. 1 Canterbury Health Laboratories. Bacteriology blood culture manual. 2008

11. 2 Thailand Ministry of Public Health – U.S. Centers for Disease Control and Prevention. Population-based surveillance for microbial agents of pneumonia and sepsis with detection of *Streptococcus pneumoniae*. Standard operating procedures for clinical and laboratory staff. 2008

11.3 World Health Organization. Manual for the Laboratory Identification and Antimicrobial Susceptibility Testing of Bacterial Pathogens of Public Health Importance in the Developing World. Geneva:WHO, 2003

12. Appendices

Appendix 1 - Collection of blood for blood culture (adapted from WHO manual):

Infection can be transmitted from patient to staff and from staff to patient during the blood-taking procedure. Viral agents pose the greatest hazard and in some instances are potentially lethal. Of particular importance are the hepatitis viruses and the human immunodeficiency virus (HIV; the virus causing acquired immunodeficiency syndrome [AIDS]). To decrease the risk of transmission of these viral agents, the following recommendations should be practiced:

a) Wear latex or vinyl gloves impermeable to liquids.
b) Change gloves between patients.
c) Inoculate blood into blood-culture media immediately to prevent the blood from clotting in the syringe. Syringes and needles should be disposed of in a puncture-resistant, autoclavable container. No attempt should be made to recap the needle. A new syringe and needle must be used for each patient.
d) Wipe the surface of the blood-culture bottle and the gloves with a disinfectant.
e) Label the bottle.
f) For the transport to the microbiology laboratory, place the blood-culture medium in a container that can be securely sealed.
g) Specimen containers should be individually and conspicuously labeled. Any containers with blood on the outside should be wiped thoroughly. Such containers should be transported in individual, sealed plastic envelopes.
h) Remove gloves and discard in an autoclavable container.
i) Wash hands with soap and water immediately after removing gloves.
j) Transport the specimen to the microbiology laboratory or, if that facility is closed, store the specimen in an approved location.
k) In the event of a needle-stick injury or other skin puncture or wound, wash the wound thoroughly with soap and water, encouraging bleeding. Report any contamination of the hands or body with blood, or any puncture wound, or any cut to the supervisor and the health service for treatment, as appropriate.

Venipuncture
a) Gather everything needed to complete the blood collection process: gloves, syringe, needle, tourniquet, gauze squares, cotton balls, adhesive bandage, puncture resistant container, culture medium and antiseptic; iodine tincture (100 ml of 70% isopropyl alcohol to 1 g of iodine) or povidone-iodine is preferred, but 70% alcohol is an acceptable alternative. The size of the needle will depend on the collection site and the size of the vein. A 23-gauge needle that is 20 – 25 mm in length or a butterfly needle is generally used for children. Collecting a large amount of blood from a child can be difficult: 1 – 3 ml is usually sufficient, but volume of blood is directly related to culture yield. Blood cultures from young children should be diluted to 1 – 2 ml of blood in 20 ml of broth (1:10 to 1:20). Blood cultures from adults should be diluted to 5 – 10 ml of blood in 50 ml of broth (1:5 to 1:10).
b) Select an arm and apply a tourniquet to restrict the flow of venous blood. The most prominent vein is usually chosen for venipuncture.

c) Vigorously wipe the skin with the 70% alcohol, and swab with the iodine tincture or povidone-iodine. Rub over the selected area. Allow to dry. If the vein is palpated again, repeat the skin disinfection.
d) After the disinfectant has dried, insert the needle into the vein with the bevel of the needle face-up. Once the vein is entered, withdraw the blood by pulling back the barrel of the syringe in a slow, steady manner. Air must not be pumped into a vein. After the desired amount of blood is obtained, release the tourniquet and place a sterile cotton ball over the insertion site while holding the needle in place. Withdraw the needle and have the patient hold the cotton ball firmly in place until the wound has stopped bleeding. Inoculate the culture medium. Put the adhesive bandage on the wound.
e) Use vacutainer tubes for blood collection, if they are available. Specimens should be put into a blood-culture bottle immediately and placed in an incubator as soon as possible; if incubation is not feasible, the blood culture bottle can be kept at room temperature (20° – 25°C) for up to 8 hours. Ideally, the blood samples should be processed in a bacteriology laboratory as soon as possible after collection (i.e., within 2 hours).

Appendix 2 – Recording blood culture contamination rates

Definition:

Blood culture contamination rate = No. blood cultures positive for contaminating organisms/ Total No. of blood cultures collected

Contaminating organisms
The following are considered to be blood culture contaminants unless regarded as significant:

Coagulase-negative staphylococci
Micrococcus Species
Propionibacterium species
Corynebacterium species
Bacillus species
Apha-hemolytic streptococci

Appendix 3: BLOOD CULTURE MACHINE Instructions

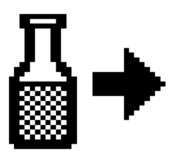
BACTEC 9050

Instrument Operation.

- The instrument has 3 primary operating functions:

(1). Entering Cultures.

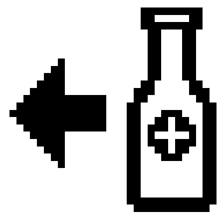
- Press home the rotor instrument key
- Open the door of the instrument
- Press the soft key under the load bottle icon
- Scan the bottle bar code
- Enter the bottle into the position identified
- Confirm the completion of the load operation



- Repeat for each bottle that needs to be loaded

(2). Removing Positives.

- Positive light of instrument will be illuminated and the instrument's audible alarm will sound.
- Press home the rotor instrument key
- Open the door of the instrument
- Press the remove positive soft key
- The display identifies the bottle position
- Scan the bottle barcode
- Repeat to remove all positives
- All positive bottles need to be gram stained and subcultured onto the appropriate media



(3). <u>Removing Negatives.</u>

- At the conclusion of the defined protocol, bottle are classified as final negatives
- Press home, the rotor instrument key
- Open the door of the instrument
- Press the remove negative soft key
- The display identifies the bottle positions
- Scan the bottle barcode
- Repeat for all negative bottles

1. Definitions
   1.1. CRF- Case Report Form

2. Purpose / Background
   2.1. The blood of healthy individuals is usually sterile. Blood cultures are a standard laboratory tool for detecting invasive bacterial/fungal disease. The BacT/ALERT is an automated blood culture system which incubates and continuously monitors blood cultures. A positive result is signaled immediately upon detection of carbon dioxide production in the blood culture bottle.

   2.2. The quality of blood collection greatly affects the sensitivity and reliability of any blood culture system. Critical factors are:
   Timing of blood collection. Before administration of antibiotics.
   Volume of blood collected. Sensitivity increases with volume; bacteremias can be missed by relying upon small blood volumes, which is a particular issue in children.
   Skin disinfection. Contamination of blood cultures with bacteria that commonly reside on the skin can cause false positive results for bacteremia, may prevent identification of a true pathogen, and complicate the interpretation of our laboratory results. Thorough disinfection of the venipuncture site can significantly reduce the number of contaminated blood cultures.

   2.2. The purpose of this SOP is to give guidance on isolation of organisms found in blood specimens.

3. Scope / Applicability
   3.1. This SOP is applicable to all trained laboratory technicians/technologists/scientists working in the microbiology laboratory.

4. Roles / Responsibilities
   [Site specific]

5. Specimen
   5.1. Blood received in the laboratory in BacT/ALERT Pediatric FAN blood culture bottles (see appendix 1, collection of blood for blood culture)

6. Prerequisites / Supplies Needed
   6.1. Equipment
   6.1.1 CO_2 Incubators or Candle jar
   6.1.2 Aerobic incubator
   6.1.3 BD BacT/Alert machine

6.2 Media

6.2.1 5% Sheep Blood agar
6.2.2 Chocolate agar
6.2.3 MacConkey agar

6.3 Materials

6.3.1 Alcohol
6.3.2 Antimicrobial susceptibility discs.
6.3.3 Biochemical reagents
6.3.4 Paediatric blood culture bottles BacT/ALERT Pediatric FAN
6.3.5 Cotton wool/gauze
6.3.6 Gram stain reagents
6.3.7 Syringes and needles
6.3.8 Sterile gloves
6.3.9 Tourniquet
6.3.10 Microscope Slides
6.3.11 Sharps container

7. Safety/Risk Assessment

7.1 Wear Personal Protective Equipment at ALL times when processing blood culture samples. Process all blood cultures in a biosafety cabinet.

7.2 Waste disposal: Discard all sharps in sharp boxes. Autoclave all culture plates and other clinical wastes before taking them for incineration. Reusable material must be autoclaved before washing.

8. Procedural Steps

8.1. **Pre-processing specimen handling**

Blood culture bottles should be placed in the BacT/ALERT blood culture instrument as soon as possible after arrival in the laboratory. Store at air-conditioned room temperature if unable to process immediately. *[This will need to be modified based on specific manufacturers' instructions, as some systems allow incubation in a standard incubator before placement on automated instrument]*

8.2. **Initial processing of blood culture bottles**

Each blood culture bottle should be weighed and the value subtracted from the un-inoculated weight of the bottle. The result is the weight of the inoculated blood and is recorded on the CRF.

[specific to each automated system]

8.3. **Protocol for positive blood cultures (**BacT/ALERT**)**

Day 1

8.3.1. Unload positive blood culture bottle(s) according to BacT/ALERT instrument operation protocol (See instrument operation protocol below).

8.3.2. In the Biohazard cabinet, sterilise the rubber top of the bottle with 70% alcohol. Using a vent needle inoculate the following:

5% Blood Agar (aerobic)
Chocolate Agar
MacConkey Agar
Slide for Gram stain

Spread plates and incubate aerobic plates at 35-37°C with 5% CO_2

8.3.3. Leave Gram to air dry then stain and examine.

8.3.4. Once the Gram stain reaction and morphology is known, notify the clinician.

Day 2

8.3.5. Examine plates and identify isolates according to standard microbiological methods.

8.3.6. Perform antibiotic susceptibility testing (refer to Antibiotic Susceptibility Testing SOP).

8.3.7. Reincubate plates for a further 24 hours.

Day 3

8.3.8. Re-examine plates. Identify and perform antibiotic susceptibility testing on any further isolates that may have grown.

8.3.9. Report confirmed sensitivities and final identification if available.

Notes:

- All positive blood cultures are initially regarded as significant.
- All clinically significant isolates should be frozen at -80°C.

8.4. **Reporting Isolates of Doubtful Significance**

8.4.1. **Coagulase Negative Staphylococcus**

Only perform antibiotic susceptibilities or freeze coagulase negative staphylococci when:

- it has been isolated from another normally sterile site e.g. CSF, or
- when isolated from more than blood culture from different times and/or sites.
  All other isolates are regarded as contaminants.

8.4.2 **Micrococcus and Propionibacterium**

These bacteria are regarded as contaminants. Do not perform antibiotic susceptibility testing.

8.4.3 **Corynebacterium spp.**

Corynebacterium bacteria should only be identified and antibiotic susceptibility testing performed if:

- isolated from more than one set of blood cultures from different times and/or sites, or
- the same corynebacterium has been isolated from another, normally sterile, site e.g. CSF.
  All other isolates are regarded as contaminants.

8.4.4 **Bacillus spp.**

- Identification and freezing is only necessary if *Bacillus cereus* or *B. anthracis* is suspected, (**NB. if *B. anthracis* is suspected any work-up should be performed in a BSL-3 laboratory) or**
- If the same *Bacillus* species has been isolated from another, normally sterile, site eg. CSF
  All other isolates are regarded as contaminants.

8.4.5 **Alpha –hemolytic streptococci**

Only perform antibiotic susceptibilities on alpha-hemolytic streptococci when:

- *S. pneumoniae* or *Enterococcus* species have first been ruled out, and
- it has been isolated from another normally sterile site, or
- when isolated from more than one set of blood cultures from different times and/or sites, or
- endocarditis is suspected
  All other isolates are regarded as contaminants.

8.5. Protocol for "False Positive" blood cultures

If a bottle has been flagged positive by the BacT/ALERT instrument and the Gram stain is negative, sub-culture as outlined for a positive culture above and keep the blood culture bottle at room temperature. If the 24-hour plates do not show any growth, test with the NOW Streptococcus pneumoniae Test® (Binax). This rapid immunochromatographic assay is designed to detect *S. pneumoniae* antigens in the urine of patients with pneumonia and cerebrospinal fluid from patients with meningitis, but the test can be modified to detect pneumococcal antigens in blood culture medium even when no organisms can be seen on Gram stain or culture.

Procedure

8.5.1 Place blood culture bottles in a biosafety cabinet to avoid accidental aerosolization of potentially infectious materials. Use standard precautions for safe handling of blood.

8.5.2 Allow blood cultures to equilibrate to room temperature (15-30ºC) and swirl gently to resuspend antigens before beginning tests.

8.5.3 Wipe gloves with 70% alcohol, then carefully disinfect the top (rubber septum) of each bottle with 70% alcohol.

8.5.4 Unwrap one testing device for each specimen to be tested and lay flat in biosafety cabinet without touching the reaction area of the testing device. Label it with the identification number of the blood culture bottle to be tested.

8.5.5 Remove one Binax swab per sample from the kit, and use the foil package from the testing device as a tray for the swab. Do not use other swabs for this test.

8.5.6 Using a BD Vent needle, aseptically remove approximately 0.5 mLs of blood culture media from each positive bottle.

8.5.7 Drop culture media from the needle onto a Binax swab until the swab head is completely soaked, but not lying in a puddle of excess media. If the swab head drips when picked up, remove the excess liquid by pressing against inside edge of the foil package.

8.5.8 Insert the swab into the bottom hole (swab well) on the inner right panel of the testing device. Firmly push upwards so that the swab tip is fully visible in the top hole. Do not remove the swab.

8.5.9 Hold the Reagent A vial vertically (straight up and down) 1-2 cm above the device. Slowly let 3 drops of Reagent A fall into the bottom hole.

8.5.10 Immediately remove the adhesive liner from the right edge of the test device, and close and seal the device. Repeat all steps for each apparent false positive bottle.

8.5.11 Read the result in the window 15 minutes after closing the device. Results read after 15 minutes may not be accurate; strongly positive samples may produce a visible sample line in less than 15 minutes.

8.5.12 One or two lines should appear in the window on the testing device. A single pink-to-purple colored Control Line in the top half of the window means that the test was performed correctly, but no pneumococcus antigens were detected. The appearance of two pink-to purple colored lines, the Control Line and a Sample Line, indicated a positive result even if the sample line is very faint. If no lines appear, or only the bottom Sample Line appears, the test results are invalid. If this happens, the test should be repeated using three samples: the pre-packaged positive and negative control swabs, and the blood culture again.

Protocol for negative blood cultures (BacT/ALERT**)**
[needs BacT/ALERT-specific information. Remove negative bottles after 5 days]

9. Record Management

Access, location, and retention of records pertaining to the SOP – site specific

10. Quality Assurance / Quality Control

10.1 Initial training and competency assessment of all appropriate staff in this SOP.
10.2 Ongoing monitoring of blood culture bottle weights
10.3 Ongoing monitoring of blood culture contamination rates
10.4 Ongoing monitoring of reports

11. References

11. 1 Canterbury Health Laboratories. Bacteriology blood culture manual. 2008

11. 2 Thailand Ministry of Public Health – U.S. Centers for Disease Control and Prevention. Population-based surveillance for microbial agents of pneumonia and sepsis with detection of *Streptococcus pneumoniae*. Standard operating procedures for clinical and laboratory staff. 2008

11.3 World Health Organization. Manual for the Laboratory Identification and Antimicrobial Susceptibility Testing of Bacterial Pathogens of Public Health Importance in the Developing World. Geneva:WHO, 2003

12. Appendices

Appendix 1 - Collection of blood for blood culture (adapted from WHO manual):

[this may not be needed if sites have their own version]

Infection can be transmitted from patient to staff and from staff to patient during the blood-taking procedure. Viral agents pose the greatest hazard and in some instances are potentially lethal. Of particular importance are the hepatitis viruses and the human immunodeficiency virus (HIV; the virus causing acquired immunodeficiency syndrome [AIDS]). To decrease the risk of transmission of these viral agents, the following recommendations should be practiced:

a) Wear latex or vinyl gloves impermeable to liquids.
b) Change gloves between patients.
c) Inoculate blood into blood-culture media immediately to prevent the blood from clotting in the syringe. Syringes and needles should be disposed of in a puncture-resistant, autoclavable container. No attempt should be made to recap the needle. A new syringe and needle must be used for each patient.
d) Wipe the surface of the blood-culture bottle and the gloves with a disinfectant.
e) Label the bottle.
f) For the transport to the microbiology laboratory, place the blood-culture medium in a container that can be securely sealed.
g) Specimen containers should be individually and conspicuously labeled. Any containers with blood on the outside should be wiped thoroughly. Such containers should be transported in individual, sealed plastic envelopes.
h) Remove gloves and discard in an autoclavable container.
i) Wash hands with soap and water immediately after removing gloves.
j) Transport the specimen to the microbiology laboratory or, if that facility is closed, store the specimen in an approved location.
k) In the event of a needle-stick injury or other skin puncture or wound, wash the wound thoroughly with soap and water, encouraging bleeding. Report any contamination of the hands or body with blood, or any puncture wound, or any cut to the supervisor and the health service for treatment, as appropriate.

Venipuncture
a) Gather everything needed to complete the blood collection process: gloves, syringe, needle, tourniquet, gauze squares, cotton balls, adhesive bandage, puncture resistant container, culture medium and antiseptic; iodine tincture (100 ml of 70% isopropyl alcohol to 1 g of iodine) or povidone-iodine is preferred, but 70% alcohol is an acceptable alternative. The size of the
needle will depend on the collection site and the size of the vein. A 23-gauge needle that is 20 – 25 mm in length or a butterfly needle is generally used for children. Collecting a large amount of blood from a child can be difficult: 1 – 3 ml is usually sufficient, but volume of blood is directly related to culture yield. Blood cultures from young children should be diluted to 1 – 2 ml of blood in 20 ml of broth (1:10 to 1:20). Blood cultures from adults should be diluted to 5 – 10 ml

of blood in 50 ml of broth (1:5 to 1:10).
b) Select an arm and apply a tourniquet to restrict the flow of venous blood. The most prominent vein is usually chosen for venipuncture.
c) Vigorously wipe the skin with the 70% alcohol, and swab with the iodine tincture or povidone-iodine. Rub over the selected area. Allow to dry. If the vein is palpated again, repeat the skin disinfection.
d) After the disinfectant has dried, insert the needle into the vein with the bevel of the needle face-up. Once the vein is entered, withdraw the blood by pulling back the barrel of the syringe in a slow, steady manner. Air must not be pumped into a vein. After the desired amount of blood is obtained, release the tourniquet and place a sterile cotton ball over the insertion site while holding the needle in place. Withdraw the needle and have the patient hold the cotton ball firmly in place until the wound has stopped bleeding. Inoculate the culture medium. Put the adhesive bandage on the wound.
e) Use vacutainer tubes for blood collection, if they are available. Specimens should be put into a blood-culture bottle immediately and placed in an incubator as soon as possible; if incubation is not feasible, the blood culture bottle can be kept at room temperature (20° – 25°C) for up to 8 hours. Ideally, the blood samples should be processed in a bacteriology laboratory as soon as possible after collection (i.e., within 2 hours).

Appendix 2 – Recording blood culture contamination rates

Definition:

Blood culture contamination rate = No. blood cultures positive for contaminating organisms/ Total No. of blood cultures collected

Contaminating organisms
The following are considered to be blood culture contaminants unless regarded as significant:

Coagulase-negative staphylococci
Micrococcus Species
Propionibacterium species
Corynebacterium species
Bacillus species
Apha-hemolytic streptococci

1. Definitions
   1.1. CLSI – Clinical Laboratory Standards Institute
   1.2. HTM – Haemophilus Test Medium
   1.3. MH – Mueller Hinton
   1.4. MHB – Mueller Hinton agar with 5% sheep blood
   1.5. MIC – Minimum Inhibitory Concentration
   1.6. TSB - Trypticase Soy Broth
   1.7. ATCC – American Type Culture Collection

2. Purpose / Background
   2.1. Antimicrobial susceptibility tests are performed in order to determine whether a pathogen is likely to be susceptible or resistant to specific antibiotic treatment. Criteria for in vitro testing of isolates have been developed to provide the best guidance for clinical management, and must be used in conjunction with other variables such as drug absorption, penetration of the drug into the appropriate body compartment, and recognized in vivo limitations of some antibiotic-microbe combinations. Correctly identifying an isolate is therefore key to accurate interpretation of antibiotic susceptibility tests.

   Antibiotic susceptibility testing in the PERCH project will follow criteria provided by the Clinical Laboratory Standards Institute (CLSI) and will be based around disk diffusion methodology whenever possible. **These criteria are outlined in CLSI document M100-S21. This document and subsequent updates should always be the primary resource for interpretative criteria.**

   2.2. The purpose of this SOP is to give guidance in performing the antibiotic susceptibility testing.

3. Scope / Applicability
   3.1. This SOP is applicable to all trained laboratory technicians/technologists/scientists working in the *[name]* microbiology laboratory.

4. Roles / Responsibilities

4.1 *[site specific]*

5. Specimen
    5.1. Pure 24-hour isolate on a culture plate.

6. Prerequisites / Supplies Needed
    6.1 Incubators
    6.2 Vernier calipers or ruler
    6.3 Antimicrobial disks (store frozen with a desiccant)
    6.4 Mueller Hinton Agar (MH agar) (e.g. BD #221275 or equivalent)
    6.5 Mueller Hinton Agar with 5% sheep blood (MHB) (e.g. BD #221176 or equivalent)
    6.6 Haemophilus Test Media (HTM) (e.g. BD #221992 or equivalent)
    6.7 Trypticase Soy Broth (TSB) or Mueller Hinton broth (MH broth) (3 mL)
    6.8 Turbidometer or 0.5 McFarland turbidity Standard
    6.9 Sterile saline
    6.10 Sterile swabs
    6.11Forceps
    6.12 Disc dispenser

7. Safety/Risk Assessment
    7.1 Standard precautions, including personal protective equipment.

8. Procedural Steps
    8.1. **Principle**

The disk diffusion method of susceptibility testing has been standardized primarily for testing of rapidly growing bacteria. To perform the test, filter paper disks containing a specific amount of antimicrobial agent are applied to the surface of an agar medium that has been inoculated with a known amount of the test organism. The drug in the disk diffuses through the agar. As the distance from the disk increases, the concentration of the antimicrobial agent decreases creating a gradient of drug concentrations in the agar medium. At the same time as the drug diffuses through the agar, the bacteria try to multiply and grow across the agar. In areas where the concentration of drug is inhibitory, no growth occurs, forming a zone of inhibition around each disk.

Criteria currently recommended for interpreting zone diameters and MIC results for commonly used antimicrobial agents are published by CLSI. Results are reported categorically as Susceptible (S), Intermediate (I), or Resistant (R).

Susceptible. An infection due to the strain may be appropriately treated with the dosage of the antibiotic recommended for that type of infection.

Intermediate. Zones falling into this range may be considered equivocal. The antibiotic may be used but response will depend on doses used, the site of infection and other factors.

Resistant. Not inhibited by the usually achievable systemic concentration of the agent, or have specific microbial resistance mechanisms, e.g. β-lactamases.

8.2. **Procedure**

8.2.1 Allow disks to come to room temperature before opening the container.

8.2.2 Using the turbidometer or McFarland turbidity standard, prepare a suspension of the test organism in sterile saline equivalent to a 0.5 McFarland standard using isolated colonies. If there is not enough growth, inoculate the organism into TSB or MH broth, and incubate at 35°C for 2-4 hours or until it reaches the turbidity of a 0.5 McFarland standard. Use the suspension within 15 mins of preparing it.

8.2.3 Using a sterile cotton swab, inoculate the organism onto an appropriate agar plate, streaking in 3 directions over the entire agar surface. For organisms that grow rapidly use MH agar. For *Haemophilus* species use HTM and for *S. pneumoniae* use MHB. For other organisms that do not grow on MH, use MHB. Wait 5-15 mins for the suspension to adsorb into the agar, but no longer.

8.2.4 Using forceps or a disk dispenser, apply the appropriate antimicrobial disks onto the agar. Place the disks with an equal distance apart from each other and put no more than 6 disks on a 100mm diameter plate.

8.2.5. Incubate plates as follows:

Organism	Incubator conditions	Duration of incubation
Haemophilus species	35±2°C in 5% CO_2	16-18 hours
S. pneumoniae, other streptococci, meningococcus	35±2°C in 5% CO_2	20-24 hours
S. aureus for oxacillin and *Enterococcus* for vancomycin	35±2°C in ambient air	24 hours
Acinetobacter, Stenotrophomonas, Burkholderia	35±2°C in ambient air	20-24 hours
Others	35±2°C in ambient air	16-18 hours

8.3. Interpretation

After incubation, measure the diameters of the zone of complete inhibition (as judged by the unaided eye) with calipers or ruler.

8.3.1 For MH and HTM agar (except for *Staphylococcus* spp. with oxacillin, vancomycin OR Enterococcus spp. with vancomycin):

8.3.1.1 Measure from the back of the plate.

8.3.1.2 Hold the petri plate a few inches above a black, nonreflecting background illuminated with reflected light.

8.3.1.3 The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye. Ignore faint growth of tiny colonies that can be detected only with a magnifying lens at the edge of the zone of inhibited growth.

8.3.1.4. Strains of *Proteus* spp. may swarm into areas of inhibited growth around certain antimicrobial agents. With Proteus spp., ignore the thin veil of swarming growth in an otherwise obvious zone of growth inhibition.

8.3.1.5 With trimethoprim and the sulfonamides, antagonists in the medium may allow some slight growth; therefore, disregard slight growth (20% or less of the lawn of growth) and measure the more obvious margin to determine the zone diameter.

8.3.2 For *Staphylococcus* spp. with oxacillin, vancomycin OR Enterococcus spp. With vancomycin):

8.3.2.1. Measure from the back of the plate.

8.3.2.2 Use transmitted light (plate held up to light source).

8.3.2.3. The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye. Ignore faint growth of tiny colonies that can be detected only with a magnifying lens at the edge of the zone of inhibited growth.

8.3.2.4 Any discernable growth within the zone of inhibition is indicative of resistance.

8.3.3. For MHB agar:

8.3.3.1. Measure from the upper surface of the plate.

8.3.3.2 Use transmitted light (plate held up to light source).

8.3.3.3 The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye. Ignore faint growth of tiny colonies that can be detected only with a magnifying lens at the edge of the zone of inhibited growth.

8.3.3.4 With trimethoprim and the sulfonamides, antagonists in the medium may allow some slight growth; therefore, disregard slight growth (20% or less of the lawn of growth) and measure the more obvious margin to determine the zone diameter.

Refer to CLSI Document M100-S20 for the zone size interpretations. Report susceptible, resistant and intermediate as appropriate. The antibiotics to report for each bacteria are listed in the tables in the appendix.

8.4. Etest® for MIC testing of *S. pneumoniae*

Disk diffusion testing with oxacillin screens for reduced susceptibility of *S. pneumoniae* to penicillin, but does not differentiate whether non-susceptible strains have complete or intermediate resistance. Therefore, all *S. pneumoniae* isolates with reduced susceptibility with the oxacillin screen should also have an MIC determined to penicillin using the Etest® (BioMerieux, low dose strips).

There are now other epsilometer tests available in addition to the Etest® produced by AB Biodisk. These may be acceptable as long as they have documented similar performance to the Etest®.

The procedure for Etest® determination of penicillin resistance in *S. pneumoniae* isolates is as follows:

8.4.1 Suspend viable colonies from a freshly grown overnight blood agar plate in a MH broth tube, adjusting the suspension to the equivalent of a 0.5 MacFarland turbidity standard.

8.4.2 Dip a sterile cotton swab into the bacterial suspension, removing excess fluid against the side of the tube, and inoculate the entire surface of the MHB agar plate evenly with the same swab, rotating the plate 60 degrees after each inoculation to ensure confluent growth.

8.4.3 Allow the plate to dry completely (5-15 minutes), but <u>no</u> longer. While the plate is drying, remove the Etest strips from the -20°C freezer and allow the strips that will be used to warm to room temperature. Unused strips must always be stored at -20°C.

8.4.4 Place the Etest® strip onto the inoculated agar plate with sterile forceps. Avoid making air bubbles below the strip. Make sure that the printed MIC values face upward. Once applied, do not move the antimicrobial strip.

8.4.5 Incubate the plates in an inverted position (upside down) at 35°C in a 5% CO_2 incubator for 20-24 hours, following the manufacturer's instructions.

8.4.6 MICs are read by examining the intersection of the ellipse-formed zone of inhibition with the value printed on the Etest® strip, read using oblique light, with a magnifying glass if necessary. The intersection should be determined from the point of inhibition of all growth, including hazes and isolated colonies. Follow the manufacturer's instructions in the package insert for interpreting and reporting antimicrobial susceptibility results. If the intersection of the printed MIC value and the zone of inhibition lies between two markings, read the next highest standard value.

8.4.7 Interpret the antimicrobial susceptibility of the isolate, checking to be sure that the results for the positive control reference strain *S. pneumoniae* ATCC 49619 lie within the acceptable control range.

8.5. **Screening for Extended Spectrum β-Lactamase (ESBL) Production**

ESBL-producing Enterobacteriaceae (especially *E. coli, Klebsiella pneumoniae, K. oxytoca,* and *Proteus mirabilis*) have become increasingly widespread. ESBL-producing isolates are resistant to extended-spectrum cephalosporins, such as cefotaxime and ceftazidime, and aztreonam, as well as to penicillins and narrow spectrum cephalosporins.

E. coli, K. pneumoniae, K. oxytoca, and *Proteus mirabilis* considered significant enough for susceptibility testing should be screened for ESBL by using a cefotaxime (30µg) disk or ceftazidime (30µg) disk. Zone sizes ≤27mm for cefotaxime or ≤22mm for ceftazidime may indicate ESBL production and should be confirmed an additional phenotypic test.

The confirmatory procedure (outlined in CLSI Document M100-S21 Table 2A-S1) involves testing the strain against ceftazime, ceftazidime-clavulanic acid, cefotaxime, cefotaxime-clavulanic acid using standard disk diffusion recommendations. After 16-18 hours incubation at 35±2°C in ambient air, a ≥5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid compared to its zone when tested alone indicates an ESBL

9. Record Management

Access, location, and retention of records pertaining to the SOP – site specific

10. Quality Assurance / Quality Control

10.1 Test the following organisms each time a new batch of MH agar is prepared and once weekly. Subculture the organisms to sheep blood agar the day before setting up the QC.

- *S. aureus* ATCC 25923

- *E. coli* ATCC 25922
- *P. aeruginosa* ATCC 27853
- In addition, test *E. faecalis* ATCC 29212 each time a new batch of MH is prepared.

Perform weekly Quality Control on HTM agar with *Haemophilus influenzae* ATCC 49247. Test for growth of *Haemophilus influenzae* ATCC 10211 on each new batch HTM.

See CLSI Document M100-S21 Table 3 for acceptable QC results.

For troubleshooting out-of range QC results, see CLSI Document M100-S21 Table 3D.

11. References

11.1. Clinical and Laboratory Standards Institute (CLSI) Document - Performance Standards for Antimicrobial Susceptibility Testing M100-S21, 2011

11.2. Clinical and Laboratory Standards Institute (CLSI) Document - Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Proposed Guideline. M45-P, 2006

APPENDIX Antibiotic Susceptibility Panels for PERCH

The following antibiotics will be tested for isolates recovered from PERCH clinical specimens.

Acinetobacter

DRUGS	**Disc concentration**	**S** ≥	**I**	**R** ≤
Gentamicin (CN)	10 µg	15	13-14	12
Ciprofloxacin (CIP)	5 µg	21	16-20	15
Imipenem (IPM)	10 µg	16	14-15	13

Alpha-Hemolytic Streptococci

DRUGS	**Disc concentration**	**S** ≥	**I**	**R** ≤
Penicillin (P)[a]		MIC ≤0.12	MIC 0.25-2	MIC ≥4
Cefotaxime (CTX)	30 µg	28	26-27	25
Vancomycin (VA)	30 µg	17		

[a] Disk diffusion is unreliable for penicillin. For significant isolates (e.g. suspected endocarditis) the MIC should be determined by Etest.

Beta-Hemolytic Streptococci.

DRUGS	Disc concentration	S ≥	I	R ≤
Penicillin (P)	10 Units	24		
Erythromycin (ERY)	15 µg	21	16-20	15
Clindamycin (DA)	2 µg	19	16-18	15
Tetracycline (TET)	30 µg	23	19-22	18

Note:

- Strains that appear resistant to erythromycin but susceptible to clindamycin need to be checked for the presence of inducible resistance to clindamycin.
- Note: susceptibility to other penicillins and cephalosporins can be inferred from the penicillin result.

Burkholderia cepacia

DRUGS	Disc concentration	S ≥	I	R ≤
Cotrimoxazole (SXT)	1.25/23.75 µg	16	11-15	10
Ceftazidime (CAZ)	30 µg	21	18-20	17

Enterococci

DRUGS	Disc concentration	S ≥	I	R ≤
Ampicillin (AMP)	10 µg	17		16
Vancomycin (VA)	30 µg	17	15-16	14

Enterobacteriaceae

DRUGS	**Disc concentration**	**S** ≥	**I**	**R** ≤
Ampicillin (AMP)	10 µg	17	14-16	13
Amoxicillin/clavulanic acid (AMC)	20/10 µg	18	14-17	13
Gentamicin (CN)	10 µg	15	13-14	12
Cotrimoxazole (SXT)	1.25/23.75 µg	16	11-15	10
Cefotaxime (CTX)	30 µg	26	23-25	22
Chloramphenicol (CH)	30 µg	18	13-17	12
Ciprofloxacin (CIP)	5 µg	21	16-20	15
Ciprofloxacin (CIP)[a]	5 µg	31	21-30	20
Ceftazidime (CAZ)	30 µg	21	18-20	17
Ceftriaxone (CRO)	30 µg	23	20-22	19
Imipenem (IPM)	10 µg	23	20-22	19
Cefoxitin (FOX)	30 µg	18	15-17	14

[a] For reporting against S. typhi and extraintestinal Salmonella spp. only

Notes:

- *Salmonella* species should be interpreted as resistant to Gentamicin irrespective of their susceptibility testing, as it is inactive *in vivo*.
- *E. coli, K. pneumoniae, K. oxytoca,* and *Proteus mirabilis* which are resistant to ceftazidime, ceftriaxone, or cefotaxime need to be tested for the presence of an Extended Spectrum Beta-Lactamase (ESBL) before the results of susceptibly to any β-lactam drugs can be used. If an ESBL is confirmed, the isolate will be reported as resistant to all penicillins and cephalosporins regardless of the initial disc diffusion result for each drug.
- *Klebsiella* species and the Enterobacteriaceae which typically possess inducible cephalosporinases (eg. *Enterobacter, Citrobacter, Serratia, Morganella and Providencia* species) are always resistant to ampicillin *in vivo*.
- The Enterobacteriaceae which typically possess inducible cephalosporinases (eg. *Enterobacter, Citrobacter freundi, Serratia marcescens, Morganella morganii and Providencia* species) are likely to develop resistance to cephalosporins *in vivo* even if it appears susceptible *in vitro*.
- Cefoxitin is not a drug that is used in patient care but it can be a marker for an *AmpC* cephalosporinase.

***Haemophilus influenzae*:**

DRUGS	Disc concentration	S ≥	I	R ≤
Ampicillin (AMP)	10 µg	22	19-21	18
Amoxicillin/clavulanic acid (AMC)	20/10 µg	20		19
Ciprofloxacin (CIP)	5 µg	21		
Chloramphenicol (CH)	30 µg	29	26-28	25
Tetracycline (TET)	30 µg	29	26-28	25
Cefotaxime (CTX)	30 µg	26		
Cotrimoxazole (SXT)	1.25/23.75 µg	16	11-15	10

Note:

- β-lactamase test should be done for *Haemophilus influenzae*. If the isolate is β-lactamase positive then report as resistant to Ampicillin.

Moraxella catarrhalis

There are no CLSI disk diffusion methods for *M. catarrhalis*. Test for β-lactamase production and interpret as resistant to ampicillin if β-lactamase positive

***Neisseria meningitidis*:**

DRUGS	Disc concentration	S ≥	I	R ≤
Penicillin (P)[a]		MIC ≤0.06	MIC 0.12-0.25	MIC ≥0.5
Chloramphenicol (Ch)	30 µg	26	20-25	19
Ciprofloxacin (Cip)	5 µg	35	33-34	32
Cefotaxime (CTX)	30 µg	34		

Note:

- [a]Disk diffusion tests with penicillin are unreliable for *N. meningitidis*. MIC tests (e.g. Etest) should be used for this organism

***Pasteurella multocida*:**

DRUGS	Disc concentration	S ≥	I	R ≤
Ampicillin (AMP)	10 µg	27		
Penicillin (P)	10 Units	25		
Ceftriaxone (CRO)	30 µg	34		
Tetracycline (TET)	30 µg	23		

Pseudomonas aeruginosa

DRUGS	Disc concentration	S ≥	I	R ≤
Gentamicin (Cn)	10 µg	15	13-14	12
Ciprofloxacin (Cip)	5 µg	21	16-20	15
Ceftazidime (CAZ)	30 µg	18	15-17	14
Imipenem (IPM)	10 µg	19	16-18	15

***Staphylococcus* species:**

DRUGS	Disc concentration	S ≥	I	R ≤
Gentamicin (CN)	10 µg	15	13-14	12
Chloramphenicol (CH)	30 µg	18	13-17	12
Ciprofloxacin (CIP)[a]	5 µg	21	16-20	15
Erythromycin (ERY)	15 µg	23	14-22	13
Penicillin (P)	10 Units	29		28
Cotrimoxazole (SXT)	1.25/23.75 µg	16	11-15	10
Cefoxitin (FOX)[a]	30 µg	22 (*S. aureus* and *S. lugdunensis*) 25 (coagulase-negative staphylococci except *S. lugdunensis*)		21 (*S. aureus* and *S. lugdunensis*) 24 (coagulase-negative staphylococci except *S. lugdunensis*)
Oxacillin (OX)[b]	1 µg	13 (*S. aureus*)	11-12 (*S. aureus*)	10 (*S. aureus*)
Tetracycline (TET)	30 µg	19	15-18	14
Clindamycin (DA)	2 µg	21	15-20	14

Notes:

[b]Either oxacillin or cefoxitin can be used to test for susceptibility to penicillinase-stable penicillins

***Streptococcus pneumoniae*:**

DRUGS	**Disc concentration**	**S ≥**	**I**	**R ≤**
Chloramphenicol (CH)	30 µg	21		20
Cotrimoxazole (SXT)	1.25/23.75 µg	19	16-18	15
Tetracycline (TET)	30 µg	23	19-22	18
Oxacillin (OX)	1 µg	20		
Erythromycin (ERY)	15 µg	21	16-20	15

Note:

- If susceptible to the oxacillin screening test, the isolate may be interpreted as susceptible to penicillin, cefotaxime and ceftriaxone. If resistant or intermediate to oxacillin, the MIC by E-test should be done for both penicillin and ceftriaxone/cefotaxime.

Stenotrophomonas maltophilia

DRUGS	**Disc concentration**	S ≥	I	R ≤
Cotrimoxazole (SXT)	1.25/23.75 µg	16	11-15	10

v 2.0 Updates:

1. Appendix: *Pseudomonas aeruginosa* zones of inhibition interpretations were updated for Imipenem according to the 2012 CLSI guidelines.
2. Appendix: Enterobacteriaceae zone of inhibition interpretations were updated for ciprofloxacin according to the 2012 CLSI guidelines.

1. Definitions:

2. Purpose / Background:
   2.1 Purpose: To give guidelines on how to carry out an antibiotic bioassay to determine usage of antibiotics by study participants prior the collection of the specimen.

   2.2 Principle: Serum and urine containing antimicrobial substances will inhibit growth of susceptible microorganisms. When a blank filter paper disc is immersed in serum or urine and placed onto an agar surface seeded with bacteria and incubated, a zone of inhibition surrounding the disc indicates the presence of antimicrobial activity in the sample.

3. Scope / Applicability:
   3.1 This SOP is applicable to all trained laboratory technicians/technologists/scientists working in the *[name]* microbiology laboratory.

4. Roles / Responsibilities
   4.1. *[Site specific]*

5. Specimen:
   5.1 Serum or urine

6. Prerequisites / Supplies Needed:
   6.1 Equipment
      6.1.1 Incubator at 35-37°C
      6.1.2 Refrigerator at 2-8°C

   6.2 Materials
      6.2.1 Forceps
      6.2.2 Vernier callipers or ruler
      6.2.3 Pipette and tips, 20uL capacity
      6.2.4 Fine tip pastettes
      6.2.5 Sterile 10uL loops
      6.2.6 Sterile cotton swabs
      6.2.7 6 mm sterile filter paper disc (e.g. Whatman 2017-006)
      6.2.8 S. aureus ATCC #25923
      6.2.9 Mueller Hinton Agar (e.g. BD 221275 or equivalent)
      6.2.10 Normal Saline (0.9%)

6.2.11 Commercial antibiotic susceptibility discs (penicillin 10 units, gentamicin 10 µg, cotrimoxazole 1.25/23.75 µg, chloramphenicol 30 µg).

7. Safety/Risk Assessment:
   7.1. Wear PPE and use standard precautions.
   7.2. Process all samples in BSL-1 containment

8. Procedural Steps
   8.1. Take serum or urine out of the freezer (-20°C) and allow to thaw to room temperature. Vortex approx 10-20 seconds

   8.2. Prepare a 0.5 MacFarland suspension of the *S.aureus ATCC 25923* in sterile normal saline (0.9%).

   8.3. Using a sterile cotton swab inoculate onto a labeled Mueller Hinton agar plate; dip the swab into the suspension of *S.aureus*. Gently squeeze swab against the inside of the tube to remove excess fluid. Use the swab to inoculate the entire surface of the Mueller Hinton agar plate three times, rotating the plate 60 degrees between each inoculation to achieve even coverage.

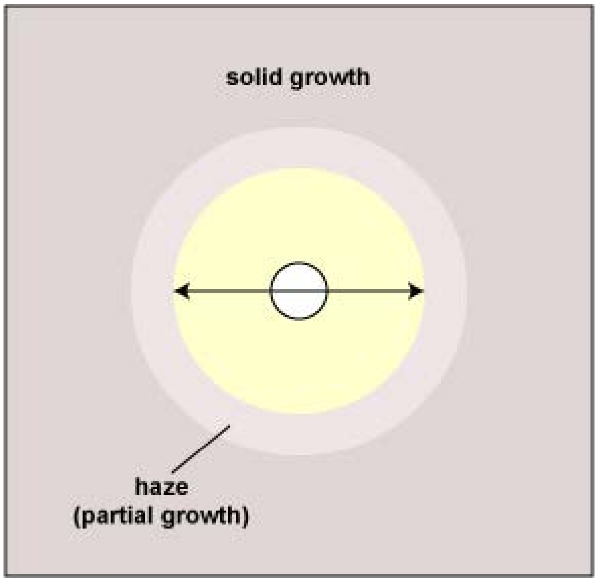
   8.4. Allow the inoculum to dry before placing discs on the plates (5-15 minutes). Keeping within this time limit is important.

   8.5. Place a single sterile 6 mm filter paper disc on the agar plate to act as sensitivity disc. Discs from at least 2 patients can be placed on each plate as long as sufficient space is allowed and they are labeled clearly.

   8.6. Pipette 20µl of the test serum or urine sample and inoculate carefully onto the disc.

   8.7. Incubate the plates overnight (18-24 hours) at 35-37°C in ambient air, agar side up.

   8.8. Measure the zone of inhibition in mm. If two zones of inhibition are observed, measure zone of complete inhibition. If a few colonies are observed within an obvious zone of inhibition, measure the zone of inhibition. Isolated colonies within a zone of inhibition can be ignored. If there is a haze of growth, measure the zone of complete inhibition.



8.9. Record the zone diameter in the PERCH database. **Note:** MINIMUM zone diameter will be 6 mm (the size of the disk). Therefore, if no growth is present, 6mm should be entered into the CRF results field.

8.10. Interpretation of zone diameter: the presence of a zone of inhibition indicates the presence of antimicrobial activity in the test serum or urine sample

9. Quality Assurance / Quality Control:

During Procedure:

9.1. Prepare a 0.5 MacFarland suspension of the *S.aureus* ATCC 25923 in normal saline
9.2. Using a sterile cotton swab pick the organism and inoculate onto a Mueller-Hinton agar plate in the same way as for the procedure above to achieve an even lawn of growth
9.3. Allow the agar to dry for 5-15 minutes maximum.
9.4. Place commercial antibiotic susceptibility discs on the Mueller-Hinton agar plate. This is the positive control. Also place a blank filter paper disc and pipette 20ul of sterile normal saline to the disc - this is the negative control.
9.5. Incubate the plates overnight (18-24 hrs) at 35-37°C.
9.6. Measure the zone of inhibition in mm and record on a chart.
9.7. Confirm that results for the positive (commercial antibiotic) and negative discs lie within acceptable standards:
penicillin 26-37 mm
gentamicin 19-27 mm
cotrimoxazole 24-32 mm
chloramphenicol 19-26 mm

10. Record Management:

10.1 *Access, location, and retention of records pertaining to the SOP – site specific*

11. References:

11.1 Thailand Ministry of Public Health – U.S. Centers for Disease Control and Prevention. Population-based surveillance for microbial agents of pneumonia and sepsis with detection of *Streptococcus pneumoniae*. Standard operating procedures for clinical and laboratory staff. 2008

11.2 Kenya Medical Research Institute Wellcome Trust Research Program, Kilifi. Standard Operating Procedures for the Detection of Antibiotic Activity. 2010

1. Definitions

   1.1 PPE: Personal Protective Equipment

2. Purpose / Background

   2.1 Quantitative lytA RT-PCR is a real-time molecular method for detection not only of the presence of pneumococcal DNA, but also the amount of pneumococcal DNA present. This method is based on the U.S. Centers for Disease Control (CDC) method as described by Carvalho et al., and adapted from the National Institute of Communicable Diseases (NICD) in South Africa. The assay uses primers specific for *lyt*A, a single-copy gene present in all *S. pneumoniae* strains.

3. Scope / Applicability

   3.1. This SOP is applicable to all trained laboratory technicians/technologists/scientists working in the *[name]* microbiology laboratory.

4. Roles / Responsibilities

   4.1 *[Site specific]*

5. Specimen

   5.1 Template*:* extracted DNA from whole blood.
   Negative extraction control: water put through the same DNA extraction process as whole blood
   Positive extraction control: whole EDTA blood spiked with pneumococcus at 10^3 copies/mL, put through the same DNA extraction process as whole blood.
   Negative PCR controls: sterile molecular grade water in place of DNA extracts.
   Positive PCR controls: *S .pneumoniae* DNA plasmid standards in 6 serial dilutions of 10^2–10^7 copies/mL to form a standard curve (run in triplicate on every plate)

6. Prerequisites / Supplies Needed

   6.1. Applied Biosystems 7500 real-time PCR instrument.
   6.2. Plate centrifuge
   6.3. Vortex mixer

6.4. Pipettes (8-channel multichannel 10 or 20µL pipette, single channel pipettes)
6.5. Filtered tips for all pipettes
6.6. Applied Biosystems 96-well plates for 7500 (#N8010560)
6.7. Applied Biosystems optical plate seal (#4311971)
6.8. plate-sealer (#4333183)
6.9. powder-free gloves
6.10. specimen racks
6.11. Mastermix room: 1000 µl pipette and filtered tips (tips long enough to fit into Mastermix tube)
6.12. Mastermix room: 200 µl pipette and filtered tips (tips long enough to fit into Mastermix tube)
6.13. Sterile capped tube for making Mastermix
6.14. Molecular grade water aliquoted into sterile eppendorfs
6.15. TaqMan GeneExpression Mastermix (Applied Biosystems #'s 4369016 ,4369514, 4369510, 4369542, 4370074)
6.16. Aluminium foil
6.17. Refrigerator
6.18. Microcentrifuge

6.19. Primer and probes:

Probes and primers should be ordered from Life Technologies (Applied Biosystems) through your local agency. Orders are channeled from the various local agents to the centralized manufacturing plant in the UK. All primers and probes ordered through Life Technologies are purified in a standard way. For primers, there is only one option for purification, and primers should be ordered at the smallest scale of synthesis. They can be re-ordered when as the site runs out, rather than keep a large stockpile that has a high risk of temperature deviations and degradation over time. Probes are synthesized in 6nm, 20nm and 50nm scales. The 6nm is enough to cover approximately 10 plates, or 660 samples. After rehydrating the lyophilized probe, it is best to aliquot it and freeze below -20°C, store the working aliquot at 2-8°C, and thaw another when needed. This is done to prevent repeated freeze thaw cycles which are damaging to the probe. Use the refrigerated probe working aliquot within 3 months and then thaw another aliquot.

Country	Local Applied Biosystems agents
The Gambia & Mali	**Serge Maurin** Regional Dealer Manager Africa, Emerging markets 25, Av de la Baltique B.P. 96 Courtaboeuf B.P. 96 91943, Villebon sur Yvette Cedex, France T +33 (0)1 69 59 85 26 M +33 (0) 682 84 61 08 F +33 (0)1 69 59 88 90 Serge.Maurin@lifetech.com
Kenya	**Africa Biosystems Ltd.** Tel: (+254) 20-3754884, (+254 –20-2680870) Mobile: (+254) 20-2680870/(+254) 721-633300/(+254) 733-631050 Fax: (+254) 20-3754885 Website: www.africabiosystems.com
Zambia	**Seshnee Pillay** **Sales Specialist - South Africa**

	T +27 31 464 5216 • **M** +27 82 068 1475 • **F** +2786 537 3162 • Seshnee.Pillay@lifetech.com 200 Smit Street • Fairland • Johannesburg • 2195 • South Africa
South Africa	**Chantal Drummond** phone +2711 478 0411 • fax +2711 478 0349• mobile +2783 601 3360• Chantal.Drummond@lifetech.com 200 Smit Street • Fairland • Johannesburg • 2195
Bangladesh	bioMérieux India Pvt. Ltd. A - 32, Mohan Cooperative, Industrial Estate 110 044 New Delhi Phone : 91 11 4 209 88 00 Fax : 91 11 4 209 88 50
Thailand	**bioMérieux Thailand Ltd.** Vibulthani Tower, 4th Floor - 3195/9 Rama IV Road, Klongton, Klong Toey 10110 Bangkok Phone : 0066 2 661 56 44 Fax : 0066 2 661 56 45

6.19.1. Forward primer (F373): 5’-acgcaatctagcagatgaagca-3’

6.19.2. Reverse primer (R424): 5’-tcgtgcgttttaattccagct-3’

6.19.3. Probe (Pb400): 5’-tgccgaaaacgcttgatacagggag-3’ (5' FAM; 3' MGB)

7. Safety/Risk Assessment

7.1 Wear PPE at all times, use only powder-free gloves

7.2 Follow standard procedures to avoid risk of contamination between laboratory areas:

7.2.1 Take care not to contaminate mastermix room with amplified DNA product or template

7.2.2 Wash your hands, change gloves and lab coat when you enter each designated area

7.2.3 Never open sealed plates containing amplicons

7.2.4 Clean all pipettes before and after use, use dedicated pipettes for each stage of the process

8. Procedural Steps

8.1. Sample preparation

8.1.1. Refer to the EasyMag SOP on DNA extraction from whole blood specimens using Specific Protocol B (SOP 2.10).

8.2. Reaction Mix:

Reagents	**Working concentration**	**1 Reaction (µl)**	**Final concentration**	**Volume (µl) for 96 well plate (106 reactions)**
TaqMan gene expression mastermix	2x	12.5	1x	1325
Forward primer	10 µM	0.5	200 nM	53
Reverse primer	10 µM	0.5	200 nM	53
Probe	10 µM	0.5	200 nM	53
Molecular grade H_20 (Sterile)	-	1	-	106
Template DNA	-	10	-	-
TOTAL		25		

8.3. PCR Procedure

8.3.1. Take samples (extracted DNA) and plasmid control out to thaw on ice. Wash your hands before proceeding.

8.3.2. In the clean mastermix room: Make up mastermix according to the recipe in 8.2, in a large enough sterile tube, multiplied by the number of reactions you will perform. In general, samples will be batched on a 96-well plate. The reaction mix is made up for 106 reactions to account for dead volume while pipetting.

8.3.3. Cap and vortex the mastermix tube, then take it to the sample prep area.

8.3.4. Transfer 15µl of the mastermix to each well of the 96-well plate. This can be done with either a multidispensing pipette, or an 8-channel pipette by dividing the mastermix into 8x aliquots of 198µl in an 8-tube strip and filling the plate 8 wells at a time. After filling, lift the plate up and check that the volume in each well is consistent (15µl) and that no wells have been missed.

8.3.5. With an 8 channel pipette, in columns, transfer 10µl of nucleic acid sample to each column on the 96well optical plate. Leave the last 3 columns for the pneumococcal control standards. Use the same tip to mix the nucleic acid sample with the reaction mix by pipetting up and down three times. Change the tip after each sample row to prevent cross-contamination.

8.3.6. Cover the plate with clean aluminum foil, and place the plate in the fridge (alternatively use a cool rack plate-holder pre-cooled in the fridge).

8.3.7. **<u>IN A SEPARATE ROOM</u>** prepare the pneumococcal control standards, see Appendix 12. Tap the tubes to make sure the mixture is homogenous, that there is no air bubble at the bottom, and that no liquid remains in the caps before opening them.

8.3.8. With an 8 channel pipette, in columns, mix gently and then transfer 10µl of each standard to column 10, 11 and 12 (triplicates) on the 96-well optical plate.

8.3.9. Notes: Make sure that the numbered orientation of the tubes and rows are correct. When transferring the nucleic acid, ensure that the multichannel draws the correct amount from each sample, and that there are no drops adhering to the sides of the pipette. Once the plate has been filled with nucleic acid samples, view the plate from the side and underneath to ensure that there is a sample in each well, and that there is the correct sample volume in each well (25µl final).

8.3.10. Seal the plate very well, especially around the edges, to prevent evaporation and loss of sample during thermal cycling. Use a plate sealer. Take care only to handle the plate seal at the edges, to avoid fingerprints on it. Label your plate.

8.3.11. Centrifuge the plate briefly at approximately 500 rpm in a plate holder, to concentrate the contents on the bottom.

8.3.12. Place the sealed plate in the fridge, whilst you set up the 7500 PCR instrument.

8.4. Instrument Setup

8.4.1. Open template and confirm the standards have been assigned the correct wells

8.4.2. Define targets and samples, using unique identifiers

8.4.3. Assign targets and samples

8.4.4. Set tasks; “negatives” (extraction neg and PCR neg controls), standard curve wells are “standards”, rest are “unknowns”

8.4.5. Check ROX as the passive reference dye

8.4.6. Check that under Run Method the reaction volume per well = 25 µl

8.4.7. Under Run, Save with standard filename which includes your initials and the date, and Start when ready.

8.5. Universal Cycling conditions:

Stage 1: 95°C for 10 min

Stage 2: 40 cycles of: 95°C for 15 sec; 60°C for 1 min

8.6. <u>Reading the results:</u>

8.6.1. Set the baseline on auto and set threshold manually to early exponential phase (should be about 0.05).

8.6.2. Check the no template controls (NTC), and the negative extraction controls (NEC) are negative. If any NTC are found positive, repeat the PCR runs, if NEC are positive then repeat the extraction and PCR run.

8.6.3. Check the slope and correlation of the standard curves. The slope indicates PCR efficiency and should be between -3.1 to -3.6. Correlation should be >0.9.

8.6.4. Check each test wells for positive tests

8.6.5. Export the results into excel and save with a standard file name which exactly matches the file name on the worksheet and the run name.

8.6.6. Positives with a Ct of 35-40 will be considered positive if there is “proper” amplification (see the raw data file for the sample). Clinical relevance is yet to be established.

8.6.7. File the worksheet and document the run QC including charting the Ct of the positive extraction control, which should not vary by more than 4 Ct’s between runs.

9. Record Management

9.1 Maintain original electronic results on site. In addition, send copies of all results to the EMMES data coordinating center following the instructions in SOP 2.4.1

10. Quality Assurance / Quality Control

10.1 Extraction positive and negative controls and PCR negative controls and positive standards are included. These should be documented for each run, along with the slope and correlation of the standard curve.

11. References

11.1. Carvalho, M. G., M. L. Tondella, K. McCaustland, L. Weidlich, L. McGee, L. W. Mayer, A. Steigerwalt, M. Whaley, R. R. Facklam, B. Fields, G. Carlone, E. W. Ades, R. Dagan, and J. S. Sampson. 2007. Evaluation and improvement of real-time PCR assays targeting lytA, ply, and psaA genes for detection of pneumococcal DNA. J.Clin.Microbiol. 45:2460-2466.

11.2 Standard Operating Procedure: Detection of *Streptococcus pneumoniae* from blood specimens of patients with severe acute respiratory infections in South Africa. RMPRU, National Institute

for Communicable Diseases, South Africa. 2010.

11.3 Standard Operating Procedure: Quantitative Real-Time lytA PCR for *Streptococcus pneumoniae.* Kenya Medical Research Institute/Wellcome Trust Research Programme. 2010.

12. Appendixes

12.1 Preparation of Standards:

12.1.1 The pneumococcal standard is supplied by Fast-Track diagnostics and consists of the LytA gene cloned into a plasmid. The concentration of the standard is 10^8 copies per ml.

12.1.2 Label six sterile reaction tubes with the dilutions 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2.

12.1.3 Add 90µl of molecular grade H_2O to each tube.

12.1.4 Thaw the plasmid standard and vortex mix briefly, centrifuge briefly to get any liquid out of the lid. Alternatively, if a microcentrifuge is not available, mix by pipetting 10-15 times.

12.1.5 Pipette 10µl of the 10^8 standard into the tube labeled 10^7 and MIX WELL! (Preferably vortex and centrifuge, but if you lack this equipment, mix by pipetting at least 10 times)

12.1.6 With a new tip transfer 10µl from the tube labeled 10^7 to the tube labeled 10^6 and MIX WELL as above!

12.1.7 Continue with the serial dilution until you reach 10^2.

12.1.8 Put the standards into an 8-tube strip if you want to be able to add them to the plate using a multichannel pipette.

12.1.9 The standards must be prepared fresh for each batch. Do not store or freeze the standards for any length of time, as the lower concentrations are unstable. Do not prepare the standards before making the mastermix and adding the patient samples to the plate.

12.1.10 Notes: Due to the high concentration of the standard, to prevent contamination, it is better to perform this operation in a laboratory that is different from the pre-PCR and the sample preparation areas. Thorough mixing of each dilution and tip changing after each dilution is essential for a reliable dilution series.

	1	2	3	4	5	6	7	8	9	10	11	12
A										NTC	NTC	NTC
B												
C										STD-10^7	STD-10^7	STD-10^7
D										STD- 10^6	STD-10^6	STD-10^6
E										STD- 10^5	STD- 10^5	STD- 10^5
F										STD- 10^4	STD- 10^4	STD- 10^4
G										STD-10^3	STD- 10^3	STD- 10^3
H										STD-10^2	STD-10^2	STD-10^2

Reagent	Stock concentration	Lot no/Date prepared	Volume (**µl**) 1x	Volume x	Standards
GenExp Mastermix	2x		12.5		Lot no.
F373	10uM		0.5		
R424	10uM		0.5		
Pb400 probe	10uM		0.5		
H_2O			1		Run name/date
Total Mastermix volume			15		
DNA			(10)		
Final volume			25		

1. Definitions
   - 1.1. NPS = Nasopharyngeal Swab
   - 1.2. STGG: Skim milk tryptone glucose glycerol
   - 1.3. PPE = personal protective equipment
   - 1.4. THY: Todd Hewitt Yeast extract broth

2. Purpose / Background
   - 2.1. The purpose of this SOP is to give guidance on isolation and identification of *Streptococcus pneumoniae* found in nasopharyngeal swab specimens

3. Scope / Applicability
   - 3.1. This SOP is applicable to all trained laboratory technicians/technologists/scientists working in the *[name]* microbiology laboratory.

4. Roles / Responsibilities
   - 4.1 *[site specific]*

5. Specimen
   - 5.1. Nasopharyngeal swab in STGG, transported to the laboratory in a cool box containing a freezer pack, within 8 hours of collection.
   - 5.2. Specimen handling: this protocol applies to specimens that are frozen as well as those are fresh. However, there may be differential recovery of pneumococci if the STGG has been frozen before processing. Therefore, it is important that specimens from all cases and controls are handled in a standardized way within each study site. If, for example, control specimens are frozen before processing, case specimens should also be frozen. Otherwise there may be a systematic bias in the recovery of pneumococci between these two groups.
   - 5.3. Specimen Reception: receive the sample according to laboratory reception procedures, process immediately or store at refrigerator temperature for up to 8 hours since the time of collection, or store in a -80C freezer in upright position immediately, for delayed processing.

6. Prerequisites / Supplies Needed
   - 6.1. Equipment Needed:
     Autoclave
     Centrifuge
     CO2 Incubator or candle-jar in aerobic incubator at 35-37°C
     Bunsen burner

80C freezer
Vortex
Refrigerator
Pipette and tips, 200µL capacity
Biosafety cabinet

6.2 Supplies/Materials Needed:
STGG Medium
Todd Hewitt medium (e.g. Oxoid # CM0189 or equivalent)
0.5% sheep blood (BAP) with 5 µg of gentamicin per ml (e.g. BD# 297457 or equivalent)
Optochin disc (e.g. Oxoid # DD0001 or equivalent)
Sodium desoxycholate
Saline
Rabbit serum
Sterile distilled water
Wire or sterile disposable plastic loops
Screw-capped 1.5 mL vials
Scissors and alcohol wipes for disinfection
Sterile, cotton-tipped swabs

7. Safety/Risk Assessment

7.1 Wear appropriate PPE and observe standard precautions. Always process respiratory specimens in a biohazard cabinet while wearing gloves.

8. Procedural Steps

8.1 Prepare skim milk, tryptone, glucose, glycerol transport medium (STGG) and Todd Hewitt Broth (see Appendices A for STGG Recipe. Todd Hewitt Broth should be procured commercially – e.g BD # 249240)

8.2 Broth enrichment NP swab culture for enhanced pneumococcal growth

8.2.1 If processing from frozen specimens start here:

8.2.1.1 Thaw the NP-STGG specimens at room temperature (25°C) and vortex for approximately 10-20s.

8.2.1.2 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.

8.2.1.3 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.

8.2.2 If processing a freshly received specimen start here:

8.2.2.1 Transfer 200 µl of the NP-STGG to 5 ml enrichment broth that has been combined with 1 ml rabbit serum. (Make the enrichment broth by preparing 100 mL Todd Hewitt broth with 0.5 g dissolved yeast extract (THY)).

8.2.2.2 Vortex and incubate for 4 hours at 35-37°C/CO_2 incubator or candle-jar.

8.2.2.3 Vortex and inoculate one loop (10 µl) of the THY enriched culture on blood agar with 5 µg of gentamicin per ml, streak for isolated colonies and incubate for 18-24 hours at 35-37°C in CO_2-incubator or candle-jar.

8.2.2.4 Transfer 1.0 ml of the THY enriched growth into screw-cap 1.5 ml vials (cryotube) and store at -70°C.

8.3 Pneumococcal isolates detection and identification after overnight incubation:

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

8.3.2 Pick each suspected pneumococcal colony morphotype and subculture to a BAP with an optochin disk, incubate for 18-24 hours at 35-37°C in CO_2-incubator or candle jar. If enough growth proceed to optochin susceptibility and bile solubility tests. Alternatively, an optochin disc can be placed with the initial subculture. When more than one pneumococcal colony morphology is evident, all different morphologies should be tested. **Always subculture from isolated colonies, not from a sweep.**

8.3.3 To perform the optochin susceptibility test:

8.3.3.1 Streak the suspect alpha-hemolytic colony into BAP in confluent lines

8.3.3.2 Place 5 µg optochin disk with 6 mm diameter in the streaked area

8.3.3.3 Incubate in CO2-incubator or candle-jar at 35-37°C for 18-24 h

8.3.3.4 If susceptible to optochin (zone of inhibition diameter ≥14 mm) it is identified as *S. pneumoniae*

Note: a bile solubility test should be done on any suspected *S. pneumoniae* with an optochin zone of 9-13 mm.

8.3.4 To perform the bile solubility:

8.3.4.1 Prepare a milky suspension (McFarland No.1) from an overnight culture in 1ml of 0.5% saline

8.3.4.2 Divide the suspension in two tubes (test and control) of 0.5 ml

8.3.4.3 Add 0.5 ml of 2% sodium desoxycholate (bile salts) to the test tube and 0.5 ml normal saline to the control tube (include preparation of 2% sodium desoxycholate in sterile distilled water or equivalent – Sandra)

8.3.4.4 Vortex and, incubate in CO_2-incubator or candle-jar at 35-37°C for up to 2 h

8.3.4.5 *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.

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

Note: the bile solubility test should be performed only for optochin resistant isolates.

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

8.4 Inoculate for Permanent Storage

1 blood agar plate for -70°C storage

8.4.1 Examine this plate after overnight incubation.

8.4.2 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 or other freezing mixture.

8.4.3 Label and store as soon as possible at -70°C.

9. Quality Assurance / Quality Control

9.1 Ensure that quality control measures are taken when preparing the STGG medium, as described in Appendix A.

10. Record Management

[Site Specific]

11. References

11.1 **Carvalho, M. G., Pimenta, F.C., Jackson, D., Roundtree, A., Ahmad, Y., Millar, E.V., O'Brien, K.L., Whitney, C.G., Cohen, A.L., and Beall, B.W.** Revisiting Pneumococcal carriage by use of broth enrichment and PCR techniques for enhanced detection of carriage and serotypes. J. Clin. Microbiol. **48:**1611-1618

11.2 **O'Brien, K. L., M. A. Bronsdon, R. Dagan, P. Yagupsky, J. Janco, J. Elliott, C. G. Whitney, Y. H. Yang, L. G. Robinson, B. Schwartz, and G. M. Carlone.** 2001. Evaluation of a medium (STGG) for transport and optimal recovery of *Streptococcus pneumoniae* from nasopharyngeal secretions collected during field studies. J. Clin. Microbiol. 39**:**1021-1024

12. Appendices

Appendix A. Preparation of STGG

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	2 g
- Tryptone soya broth	3 g
- Glucose	0.5 g
- Glycerol	10 ml
- Distilled water	100 ml

1. Mix to dissolve all ingredients.
2. Autoclave 10 minutes at 15 pounds/6.8kg
3. Dispense in 1.0 ml amounts in screw-capped 1.5-ml vials in a sterile BSL II hood
4. Loosen the screw-cap tops and autoclave for 10 minutes (at 15 pounds/6.8 kg).
5. Tighten caps after autoclaving.
6. Store STGG frozen at -20°C or refrigerate until use. Use STGG medium within 6 months of preparation. Label box of vials with date of preparation/expiry. Ensure that clinicians will be able to distinguish vials of STGG from vials of VTM (put identifying marker on the individual vials if needed).
7. Quality control test for sterility of the STGG medium:
    - Plate 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.
    - Ensure that ATCC 49619 can grow from STGG after being inoculated into it and frozen for 24-48 hrs.

8. Provide STGG to the clinic in pre-labeled vials

SOP updates:

12 September 2011

- The instructions for the broth enrichment step in section 8.2.2.1 were made clearer.

1.0 Definitions:

1.1 BA - Blood Agar
1.2 CHOC -Chocolate Blood Agar
1.3 MAC -MacConkey agar
1.4 BHB - Bacitracin Heated Blood
1.5 CO_2 - Carbon dioxide
1.6 O2 – Oxygen
1.7 NG- No growth
1.8 LPF - low power field (x10 objective)
1.9 OPF – oropharyngeal flora (some laboratories use the term "normal respiratory flora (NRF)" or similar terms instead).
1.10 PPE -Personal protective equipment
1.11 HPF - High power field (x100 objective, oil immersion)
1.12 Predominant = present in a greater quantity than other organisms, e.g. 1+ compared to scanty, 3+ compared to 2+ of growth on agar plates or numbers of organisms seen per high power field on Gram stain.

2. Purpose / Background:

2.1 The diagnosis of the causative agent of pneumonia is not straightforward. Lower respiratory tract specimens such as sputum and induced sputum are frequently contaminated with oropharyngeal flora and several organisms are capable of either carriage or pathogenicity. It may be difficult to determine which is the pathogen among the many organisms present in the specimen. Gram-stained smear assessment is performed to ensure that excessively contaminated sputum specimens are identified, to predict the result of culture and to assist in the interpretation of culture results. Use of both selective and non-selective culture is required to select potential pathogens from normal flora.

Respiratory specimens should be transported to the laboratory immediately since even a moderate amount of time at room temperature can result in overgrowth of contaminants. Storage in a refrigerator can lead to loss of temperature-sensitive infectious agents such as *Streptococcus pneumoniae.*

2.2. The purpose of this SOP is to give guidance on isolation and identification of organisms found in respiratory specimens, in particular the following organisms as potential causative agents of pneumonia:

- *Streptococcus pneumoniae*
- *Haemophilus influenzae*
- Beta-haemolytic Streptococci A, B, C or G
- *Cryptococcus neoformans*
- *Moraxella catarrhalis*

- *Staphylococcus aureus*
- Coliforms eg. *Klebsiella pneumoniae*
- *Pseudomonas aeruginosa*

2.3 Oropharyngeal flora (OPF) consists of a mixed culture of organisms, including viridans streptococci, commensal *Neisseria*, coagulase-negative staphylococci, yeasts, diphtheroids, *Capnocytophaga*, enterococci, coryneform bacteria*,* and anaerobes. In addition, some potential respiratory pathogens (*M. catarrhalis*, Enterobacteriaceae, *Pseudomonas* species, as well as *S. pneumoniae* and *H. influenzae*) may be carried as part of OPF.

3. Scope / Applicability:

3.1 This SOP is applicable to all trained laboratory technicians/technologists/scientists working in the *[name]* microbiology laboratory.

4. Roles / Responsibilities

4.1. *[Site specific]*

5. Specimen:

5.1 Induced sputum or expectorated sputum

6. Prerequisites / Supplies Needed:

6.1Equipment

6.1.1 5% CO2 incubator
6.1.2 Aerobic incubator , or 3.5L gas jar and gas generating kit for laboratories that do not have such incubators.
6.1.3 - 80°C freezer
6.1.4 -20°C freezer
6.1.5 1.5-sterile disposable culture plates
6.1.6 Microscope
6.1.7 Biohazard cabinet
6.1.8 Slide warmer
6.1.9 Box slide holder labeled with the day of the week

6.2 Materials

6.2.1 Wire loop
6.2.2. Screw top freezer vials
6.2.2 Bunsen burner
6.2.3 Oil immersion
6.2.4 Microscope slides
6.2.5 Sterile disposable loop
6.2.6 Sterile disposable Pasteur pipette
6.2.7 Blood agar
6.2.8 Chocolate agar (or Bacitracin Heated Blood (BHB)/Chocolate bacitracin agar)
6.2.9 MacConkey agar

6.2.10 10 IU bacitracin disks

7. Safety/Risk Assessment:

7.2. **All SPUTUM, INDUCED SPUTUM, ASPIRATES OR FLUID RESPIRATORY SPECIMENS MUST BE PROCESSED IN A BIOHAZARD CABINET**

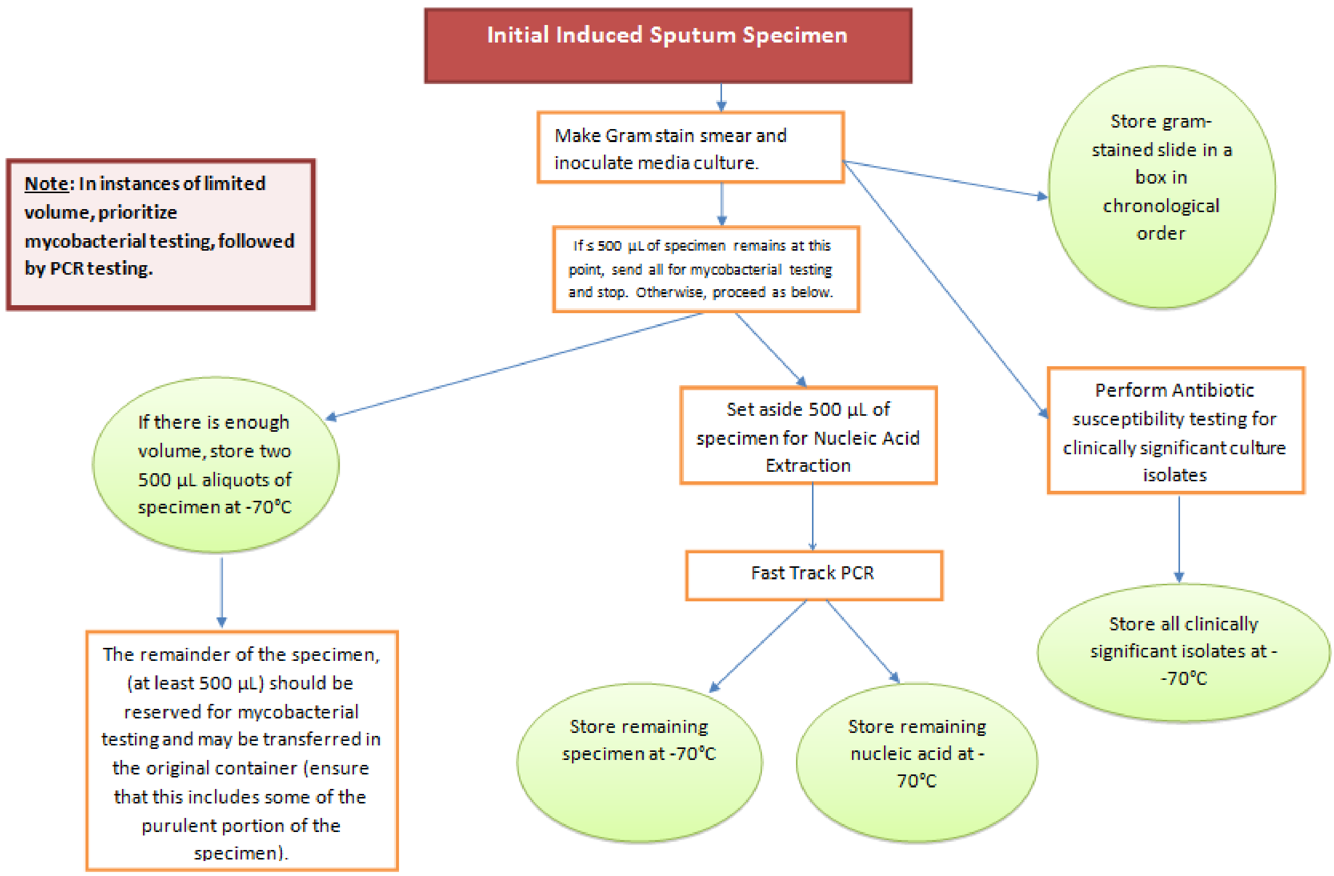
7.3. **Appropriate PPE must be worn by laboratory technologists**

8. Procedural Steps

8.1 Pre-processing specimen handling

Process specimen immediately on arrival in the laboratory. Store at 4°C for a maximum of 24 hours after collection if the specimen cannot be processed immediately.

Processing of Induced Sputum Specimen:



8.2. **Preparation of smear and Gram staining**

8.2.1 Using sterile cotton swab, make a smear on labeled clean, dry glass slide, **using the most**

purulent portion of the specimen. The smear should not be so thick that it is difficult to read after it has been stained, but not so thin that pathogens are missed.

8.2.2. The material placed on the slide to be stained is allowed to air dry within the biosafety cabinet and then heat-fixed.

8.2.3. Do a Gram stain (**Ref: Relevant Site Specific SOP**)

8.2.4. Use the Bartlett sputum grading system to assess the quality of sputum by assessing the numbers of squamous epithelial cells and neutrophils per representative low-power field. Read the Gram stain results, quantifying the relative numbers of each type of bacteria seen per representative high-power field. Compare it with culture results the following day. **(Refer appendix 1 and 2)**

8.2.5. **Do not reject any specimen because of the Bartlett score.** All specimens should be cultured and have results recorded.

8.2.6. Store the Gram-stained slide in a slide box in chronological order (do not discard).

8.3. **Culture procedure**

8.3.1 Respiratory fluid specimens should be processed as soon as possible after collection.

8.3.2. All respiratory fluid specimens should be transported at room temperature in a leak proof container and be processed within 2 hours of collection. If there is an unavoidable delay, store at 4-8°C for up to 24 hours, but this will lead to loss of sensitivity of the culture.

8.3.3. Ensure all inoculating instruments are available in the biohazard cabinet; disposable plastic loops, all required media, slides, aliquoting vials etc.

8.3.4. Select the appropriate media (BA, MAC and CHOC (or BHB)). The media to be inoculated should be labeled indicating the specimen identification and the date of inoculation.

8.3.5. Inoculate media with a drop of sputum, induced sputum or aspirate using the most purulent portion of the sample and streak out using four quadrant streaking method. Make sure some of the purulent portion is reserved for tuberculosis culture. It is important to use a standardized streaking method because this will aid in the interpretation of culture results. Always use an entire plate for each specimen.

8.3.6. Streak as per the template in **appendix 3** without turning the loop.

8.3.7. If plain chocolate agar is used, place a 10 IU bacitracin disk on the second quadrant of the chocolate agar plate. This inhibits most OPF and improves detection of *H. influenzae* (there is no need to do this if BHB agar is used).

8.3.8. After the gram stain has been performed and culture plates have been inoculated, make the following aliquots in labeled sterile containers:

Table 1

Specimen Aliquot		Priority (in instances of decreased volume)
500 µL of specimen	Nucleic Acid Extraction for Fast Track PCR testing **(ensure some of the purulent portion is included if it is a purulent specimen)**	In instances of limited volume, prioritize mycobacterial testing, then PCR testing.
500 µL of specimen (two aliquots of 500 µL if sufficient specimen volume)	Storage/Future Testing	
Remainder of specimen (at least 500 µL). May transfer specimen in original container rather than aliquoting.	Mycobacterial culture and staining **(ensure some of the purulent portion goes for mycobacterial culture if it is a purulent specimen)**	

8.3.9. Incubate the seeded plates in the appropriate incubator; BA and CHOC in the CO_2 incubator and MAC in the aerobic incubator.

8.3.10. Plates are examined at 24 hours and 48 hours.

NOTE: comparing Gram stain result to culture result is an excellent internal method for monitoring quality assurance.

8.4 **Examination of Culture**

DAY 1.

8.4.1 Examine each plate for significant growth, referring to sections 2.2 and 2.3

8.4.2 Describe, number and record the colonial morphology, and quantify the individual morphotypes based on their growth distribution along the lines of streak e.g **scanty growth** on 1st streak, **1+ growth** on the 2nd streak**, 2+ growth** as far as the 3rd streak and **3+ growth** right out onto the 4th streak (see Appendix 3). Purity plate any suspected significant organisms.

8.4.3 Proceed with Gram stain and identification and antibiotic susceptibility testing for significant pathogens according to the schema in Table 2.

Table 2: Guidelines for Reporting Pathogens in Sputum

	Organism(s)	Action
a	*Streptococcus pneumoniae* *Haemophilus influenzae* *Streptococcus pyogenes* *Cryptococcus neoformans*	• Always record and report, regardless of quantity or predominance. • Record and report the quantity (scanty, 1+, 2+, or 3+) • Work up (identify, antibiotic susceptibility testing (except *Cryptococcus*), and archive (freeze)).
b	*Moraxella catarrhalis* *Staphylococcus aureus* Other β-haemolytic streptococci Single morphotype of gram-negative rods* Any other organism that is predominant	• For mixed growth, record and report only if ≥1+ growth (i.e. 2nd quadrant or greater). For pure growth record and report at any quantity. • Record and report the quantity (1+, 2+, or 3+) • Work up (identify, antibiotic susceptibility testing, and archive (freeze)) only those organisms that are predominant over OPF on Gram stain or culture.
c	More than one morphotype of gram-negative rods (i.e. mixed coliforms or pseudomonads)	• Record and report as "mixed gram-negative rods" (organism code MGNR) • Record and report the quantity (scanty, 1+, 2+, or 3+) • Do not work up further
d	Viridans streptococci Commensal *Neisseria* Coagulase-negative staphylococci Yeasts (unless *Cryptococcus*) Diphtheroids *Capnocytophaga* Enterococci	• Record and report as oropharyngeal flora • Record and report the quantity (scanty, 1+, 2+, or 3+) as a group. There is no need to list each organism or to give the individual quantities • Do not work up further

*Including coliforms, *Pseudomonas aeruginosa*, *Acinetobacter*, *Stenotrophomonas maltophilia*, and *Burkholderia*.

If in doubt whether to identify or report a particular isolate, record its relative quantity from the original plates, make a purity plate or freeze it, and discuss with a clinical microbiologist/senior scientist. Any predominant organism in sputum specimens, except those listed in group b in Table 2, should be regarded as a potential pathogen, not just the bacteria listed in section 2.2.

Describe mixed growth of respiratory bacteria without a predominating potential pathogen in the gram stain as "Oropharyngeal flora" (OPF) scanty/1+/2+/3+, using the maximum zone of growth to describe the quantity.

8.4.4 If there is no growth after 24hrs, re-incubate for another 24 hours.
8.4.5 Compare the culture plates with the original sputum or aspirate smear Gram stain.

DAY 2.

8.4.6 Examine re-incubated plates, if no growth after 48 hours record as “No Growth.”

8.4.7 Read and describe colonial morphology, size and topography of re-incubated plates and purity plate if there are mixed growth.

8.4.8 Do Gram stain and identification from previously purified plates.

8.4.9 Perform antibiotic susceptibility testing on all significant organisms in accordance with CLSI guidelines. (Ref #)

8.4.10 Once organism identification and antibiotic susceptibility have been read, the results will be entered into the database. Record the Bartlett grades and total score, the morphology and quantity of organisms seen in the gram-stain, the cultured quantity of all potentially pathogenic bacteria and OPF, the antibiotic susceptibility test results for pathogenic isolates, and freezer positions of all frozen isolates. Do not go out of your way to scrutinize the *S. pneumoniae* isolates looking for different morphotypes, but if there are very clearly two morphotypes, freeze only one isolate where there is clearly a dominant one. If they are both present in the same amount and obviously different morphotypes, freeze both separately.

9 Record Management:

9.1 *Access, location, and retention of records pertaining to the SOP – site specific*

10 Quality Assurance / Quality Control:

10.1 Initial training and competency assessment of all appropriate staff in this SOP.

10.2 Subsequent periodic refresher training and observation by senior laboratory staff.

10.3 At least one in ten (10%) of Gram stain smears will be reviewed by a second senior technologist/scientist.

10.4 Ongoing monitoring of reports

11 References:

11.1 Standards: WHO/CDC/CSR/RMD/2003-6. Manual for the identification and Antimicrobial Susceptibility Testing of Bacterial pathogens of Public Health importance in the Developing world.

11.2 Kenya Medical Research Institute/ Wellcome Trust Research Programme, Center for Geographic Medicine -Coast. Microbiology Laboratory SOP Manual M; 2008.

11.3 Washington Winn Jr , Stephen Allen et al. Koneman's color atlas and textbook for diagnostic microbiology (sixth edition) pg 78. Publishers: Lippincott William & Wilkins;2006

11.4 Patrick R. Murray, Ellen Jo Baron et al. Manual of Clinical microbiology; 9th Edition, 2007

11.5 Lahti E, Peltola V, Waris M, et al, Induced sputum in the diagnosis of childhood community-acquired pneumonia. Thorax, 2009;64:252–257

11.6 Culture of Respiratory Specimens, Pathology Queensland Central Laboratory, Queensland Government, Australia, 2008.

11.7 Sharp SE, Robinson A, Saubolle M, Santa Cruz M, Carroll K, Baselski V. Cumitech 7B, lower respiratory tract infections. Washington, DC: ASM Press, 2004

11.8 Isenberg HD (Ed). Clinical microbiology procedures handbook. 2nd Ed. Washington, DC: ASM Press, 2004

APPENDIX 1: BARTLETT GRADING SYSTEM for evaluating sputum samples

Number of neutrophils per representative LPF (x10 objective)	**Grade**
< 10	0
10-25	+ 1
> 25	+ 2
Presence of mucus	+ 1
Number of epithelial cells per representative LPF (x10 objective)	**Grade**
<10	0
10-25	- 1
>25	- 2
Total Bartlett Score	

Note: A minimum of 20 fields should be observed to ensure that you are observing a representative field.

Negative (-ve) numbers are assigned to a smear when squamous epithelial cells are observed indicating contamination with oropharyngeal secretion (saliva).

Positive (+ve) numbers are assigned for the presence of segmented Neutrophils (indicating the presence of active inflammation).

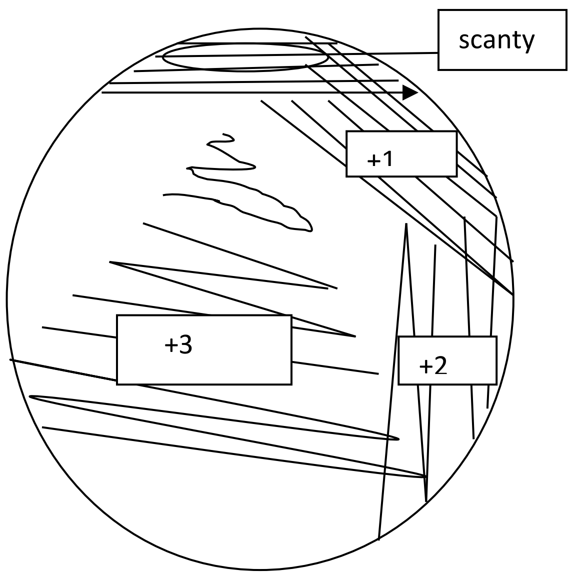
The magnitude of the –ve and +ve determination depends on the relative number of epithelial cells and segmented neutrophils.

A final score of zero (0) or less indicates either the lack of inflammatory response or the presence of significant salivary contamination.

APPENDIX 2: Relative quantity of bacteria seen in gram-stained sputum

Bacteria per representative HPF (x100 objective, oil)	Quantity
1	scanty
1-9	1+
10-99	2+
$\geq$100	3+

APPENDIX 3: Streaking template



Appendix 4: Tests required for identification of selected organisms

Streptococcus pneumoniae

- Gram-positive diplococci
- α-hemolytic colonies on blood agar with draughtman-like or mucoid appearance
- Optochin susceptible
- Bile solubility (*S. pneumoniae* are bile soluble). Should always be performed on isolates that are optochin intermediate (i.e. 9-13 mm zone).
- Serotyping

Haemophilus influenzae

- Gram-negative coccobacilli
- Growth on chocolate agar (grey colonies), but poor or no growth on blood agar (unless as satellitism around *S. aureus* colonies)
- X and V factors (requires both)
- Serotyping

Moraxella catarrhalis

- Gram-negative diplococci
- Growth on blood and chocolate agar as grey or whitish opaque colonies
- Easily pushed across agar surface (push test)
- Produces butyrate esterase and is DNAse positive. Either of these tests can be used to confirm identification along with the above characteristics.
- Does not utilize glucose, sucrose, lactose or maltose.

Staphylococcus aureus

- Gram positive cocci and clusters
- Growth on blood and chocolate as white to yellow colonies, often with zone of β-hemolysis
- Catalase test (positive)
- Coagulase test (positive)
- DNAse test (positive) can be used for additional confirmation

B-Hemolytic streptococci

- Gram-positive cocci in chains
- B-hemolytic on blood agar
- Catalase test (negative)
- Lancefield grouping
- PYR test (positive) for group A streptococci

Gram-negative rods

- Identification is based on standard algorithms (such as in the Manual of Clinical Microbiology) using Gram stain and colonial morphology, lactose fermentation on MacConkey agar, oxidase test, TSI or KIA, and biochemical tests such as indole, urea, and citrate (as individual tests or part of commercial identification panels).

Cryptococcus neoformans

- Yeast-like appearance on gram stain
- Cream-coloured colonies on blood agar
- Presence of capsule demonstrated by India ink or other method
- Urease test (positive)

PERCH Pneumonia Etiology Research for Child Health	►Standard Operating Procedure◄
Section: Laboratory	**Version: FINAL** **Initials: ______**
Title: 2.7 Processing of pleural fluids	**Revision Date:** 20 April 2011

1. Definitions:
    1. BA: Blood Agar
    2. BHIB: Brain Heart Infusion Broth
    3. CHOC: Chocolate Agar
    4. MAC: MacConkey Agar
    5. TSB: Tryptone Soy Broth

2. Purpose / Background:

    2.1 Principle: Fluid aspirated from the pleural space, when correctly analyzed, can provide information on the type of pleural effusion. Organisms causing empyema can be isolated and identified.

    The presence of micro-organisms in normally sterile body fluids may be representative of life-threatening infections. Furthermore, the concentration of micro-organisms may be low due to dilution by the large volume of fluid that may be present in the given site. This means it is important to collect and submit as large a volume of specimen as possible, to transport to the laboratory and process immediately, using techniques designed to detect small numbers of organisms in large volumes of fluid specimens.

3. Scope / Applicability:

    3.1 This SOP is applicable to all trained laboratory technicians/technologists/scientists working in the *[name]* microbiology laboratory.

4. Roles / Responsibilities

    4.1. *[Site specific]*

5. Specimen:

    5.1 Pleural fluid collected in a sterile screw-capped container. The specimen may be received already inoculated into a blood culture bottle but if so, there must also be sufficient specimen in the sterile container as well. The specimen is best processed in the lab within 15 minutes but transport at room temperature if $\leq$2 hours, or store at 4°C for up to 24 hours if processing within2 hours is unavoidable.

6. Prerequisites / Supplies Needed:

    6.1 Equipment

    6.1.1 O_2 incubator at 35-37°C

    6.1.2 CO_2 incubator at 35-37°C or candle jar

    6.1.3 Anaerobic jar or gas packs

6.1.4 Centrifuge that can go up to 1500 *g*
6.1.5 Biosafety cabinet
6.1.6 Sterile Pasteur or sterile disposable pipettes

6.2 Media
6.2.1 5% sheep blood agar – aerobic (Tryptic soy agar (TSA) + 5 % sheep blood (e.g. Remel R01198 or equivalent) or Columbia + 5% sheep blood (e.g. Remel R01215 or equivalent))
6.2.2 Blood agar - anaerobic (any anaerobic agar, e.g. brucella blood agar (e.g. Remel R01254 or equivalent), CDC anaerobic blood agar (e.g. R01036), TSA +5% sheep blood)
6.2.3 MacConkey Agar (MAC) (e.g. Remel R01293 or equivalent)
6.2.4 Chocolate Agar (CHOC) (e.g. Remel R01550 or equivalent)
6.2.5 Broth (e.g. blood culture bottles, tryptone soy broth (TSB), brain heart infusion (BHI), or brucella broth)

6.3 Other Materials
6.3.1 Wire inoculating loop (or disposable plastic sterile loops)
6.3.2 Bunsen burner
6.3.3 Glass slides
6.3.4 Gram staining reagents
6.3.5 BinaxNOW *S. pneumoniae* test kit

7. Safety/Risk Assessment:

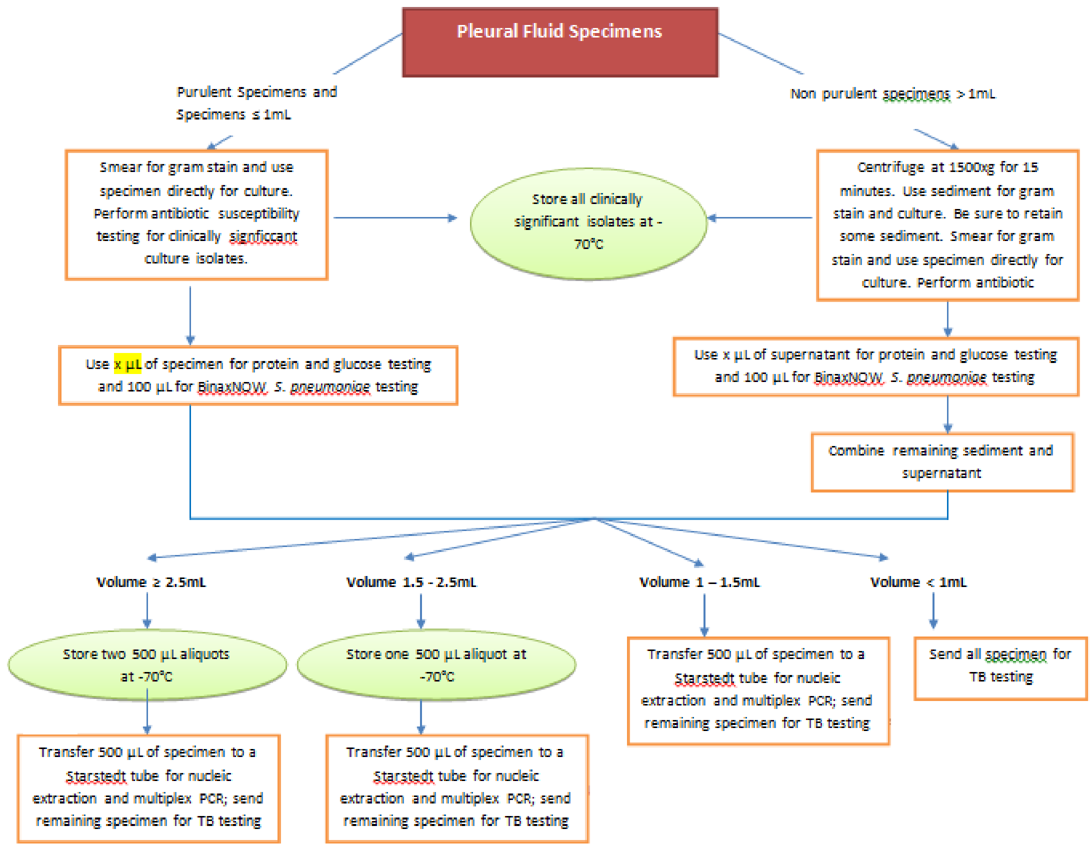
7.1 Always process pleural fluid specimens in biohazard cabinet wearing gloves.

8. Procedural Steps

8.1 Pre-processing specimen handling

Process specimen immediately on arrival in the laboratory. Store at 4°C for a maximum of 24 hours after collection if the specimen cannot be processed immediately.

Processing of Pleural Fluid Specimen:



8.2 Description

Note volume sent and appearance (e.g. purulent, bloody, clear)

8.3 Protein/glucose

8.3.1 Aliquot 100 µL of specimen and send for protein and glucose testing. *[volume may vary between labs dependent on local assays].* This aliquot may be obtained after whole specimen has been centrifuged (see below).

8.4 Bacterial Culture

8.4.1 For a purulent aspirate, make a smear for a gram stain and use the specimen directly for culture.

8.4.2 For a non-purulent aspirate that is >1mL in volume, centrifuge at 1500xg for 15 min before processing and use the sediment for the Gram stain and culture. Ensure some of the sediment remains to go for mycobacterial culture.

8.4.3 Use a sterile Pasteur pipette or a sterile disposable pipette to inoculate onto BA (aerobic and anaerobic), CHOC and MAC. Streak plates with separate sterile disposable loops or sterilize wire loop in between plates. Inoculate into an appropriate broth known to support the growth of both fastidious aerobic organisms and anaerobes in order to detect small numbers of organisms. Appropriate broths include blood culture bottles, tryptone soy broth (TSB), brain heart infusion (BHI), and brucella broth. In order to inoculate a blood culture bottle, aspirate approximately 0.5mL of fluid with a needleless syringe. Disinfect the rubber seal of a pediatric blood culture bottle. Attach a needle to the syringe and carefully inject the fluid through the rubber seal while holding the blood culture bottle at its base.

8.4.4 Incubate inoculated media in appropriate incubator at 35-37°C: BA and CHOC in the CO_2 incubator or candle jar, MAC and broth in the aerobic incubator, anaerobic BA in an anaerobic jar, and blood culture bottles (if used) in the blood culture instrument.

8.5 Gram stain

8.5.1 Leave smears to air dry in cabinet. Remove when dry, heat fix, Gram stain and then examine.

8.5.2 Report presence or absence of organisms and leucocytes seen in Gram stain.

8.5.3 Use the following system to report the number of leucocytes and bacteria seen per low-(10x) and high (100x)-power field respectively.

Number of leucocytes per representative LPF (×10 objective)	Grade
0	nil
1-9	1+
10-24	2+
≥25	3+

Bacteria per representative HPF (×100 oil objective)	Quantity
<1	Scanty
1-9	1+
10-99	2+
≥100	3+

8.6 BinaxNOW *S. pneumoniae* testing

8.6.1 This rapid immunochromatographic assay is designed to detect *S. pneumoniae* antigens in the urine of patients with pneumonia and cerebrospinal fluid from patients with meningitis, but the test can be modified to detect pneumococcal antigens in pleural fluid.

8.6.2 Procedure

8.6.2.1 Unwrap one testing device for each specimen to be tested without touching the reaction area of the testing device.

8.6.2.2 Remove one Binax swab per sample from the kit, and use the foil package from the testing device as a tray for the swab. Do not use other swabs for this test.

8.6.2.3 Transfer drops of pleural fluid with a transfer pipette or similar onto a Binax swab until the swab head is completely soaked, but not lying in a puddle of excess media. If the swab head drips when picked up, remove the excess liquid by pressing against inside edge of the foil package.

8.6.2.4 Insert the swab into the bottom hole (swab well) on the inner right panel of the testing device. Firmly push upwards so that the swab tip is fully visible in the top hole. Do not remove the swab.

8.6.2.5 Hold the Reagent A vial vertically (straight up and down) 1-2 cm above the device. Slowly let 3 drops of Reagent A fall into the bottom hole.

8.6.2.6 Immediately remove the adhesive liner from the right edge of the test device, and close and seal the device. Repeat all steps for each apparent false positive bottle.

8.6.2.7 Read the result in the window 15 minutes after closing the device. Results read after 15 minutes may not be accurate; strongly positive samples may produce a visible sample line in less than 15 minutes.

8.6.2.8 One or two lines should appear in the window on the testing device. A single pink-to-purple colored Control Line in the top half of the window means that the test was performed correctly, but no pneumococcus antigens were detected. The appearance of two pink-to purple colored lines, the Control Line and a Sample Line, indicated a positive result even if the sample line is very faint. If no lines appear, or only the bottom Sample Line appears, the test results are invalid. If this happens, the test should be repeated using three samples: the pre-packaged positive and negative control swabs, and the pleural fluid again.

8.7 Aliquoting

After the gram stain, culture, BinaxNOW, and protein/glucose testing has been completed,, make the following aliquots in labeled sterile containers:

Specimen Volume	**Aliquoting Instruction**
≥2.5 mL:	transfer 500 µl of specimen into a Sarstedt tube for nucleic acid extraction and multiplex PCR; store two additional 500 µL aliquots at -70°C; send remaining specimen for TB testing.
1.5-2.5 mL	transfer 500 µl of specimen into a Sarstedt tube for nucleic acid extraction and multiplex PCR; store one additional 500 µL aliquot at -70°C; send remaining specimen for TB testing.
1-1.5 mL	transfer 500 µl of specimen into a Sarstedt tube for nucleic acid extraction and multiplex PCR; send remaining specimen for TB testing.
<1mL	send all for TB testing.

8.8 Examination of Culture

8.8.1 Examine all plated and broth media (except the anaerobic plate or blood culture bottle if it is in an automated instrument) for macroscopic evidence of growth at 24 hr.

8.8.2 If no visible growth is observed on the culture media, re-incubate

8.8.2.1 Read aerobic plates at 48, 72 and 96 hr.

8.8.2.2 Read anaerobic plates at 48 hr

8.8.2.3 Subculture broth cultures onto BA and CHOC only if there is evidence of turbidity up to 96 hr.

8.8.2.4 Incubate blood culture bottles for 5 days

8.8.3 Correlate culture results with those of the direct Gram stain

8.8.4 Identify any organisms present, note their relative quantities and whether they were found on solid media, in broth or both, and perform susceptibility testing of significant pathogens as per CLSI protocols. Mixed skin flora eg coagulase-negative staphylococci, Bacillus species and corynebacteria do not need to have susceptibility testing or to be frozen but should be reported as skin flora. Any organism present in pure heavy growth on primary SOLID MEDIA should be stored, even if it is a potential member of skin flora. Skin flora from broth culture should not be preserved.

8.8.5 Store any significant isolates in -70°C freezer.

NB Pleural infections are often mixed, therefore examine carefully for multiple bacterial populations. Foul odour and obvious turbidity are important indicators of infected pleural fluid.

9. Quality Assurance / Quality Control:

9.1. Initial training and competency assessment of all appropriate staff in this SOP.

10. Record Management:

10.1 *Access, location, and retention of records pertaining to the SOP – site specific*

11. References:

11.1. Sharp SE, Robinson A, Saubolle M, Santa Cruz M, Carroll K, Baselski V. Cumitech 7B, lower respiratory tract infections. Washington, DC: ASM Press, 2004

11.2. Isenberg HD (Ed). Clinical microbiology procedures handbook. 2nd Ed. Washington, DC: ASM Press, 2004

1. Definitions:

   1.1 BA: Blood Agar
   1.2 BHIB: Brain Heart Infusion Broth
   1.3 CHOC: Chocolate Agar
   1.4: MAC: MacConkey Agar
   TSB: Tryptone Soy Broth

2. Purpose / Background:

   2.1 Principle: Transthoracic lung aspiration is a sensitive and proven diagnostic method that aids the management of pneumonia by identifying potential pneumonia pathogens.

   The presence of micro-organisms in normally sterile body fluids may be representative of life-threatening infections. Furthermore, the concentration of micro-organisms in such specimens may be low. These observations underscore the absolute need to collect and submit as large a volume of specimen as possible, to transport immediately to the laboratory and to process such specimens expeditiously in the laboratory using techniques designed to detect small numbers of organisms in large volumes of fluid specimens.

3. Scope / Applicability:

   3.1 This SOP is applicable to all trained laboratory technicians/technologists/scientists working in the *[name]* microbiology laboratory.

4. Roles / Responsibilities

   4.1. *[Site specific]*

5. Specimen:

   5.1 Lung aspirate (in 2.5mL normal saline)

6. Prerequisites / Supplies Needed:

   6.1 Equipment

   6.1.1 O_2 incubator at 37°C
   6.1.2 CO_2 incubator at 37°C
   6.1.3 Anaerobic jar (or gas packs)
   6.1.4 Centrifuge that can go up to 1500 *g*
   6.1.5 Biosafety cabinet
   6.1.6 Sterile Pasteur pipette

6.2 Media

6.2.1 Blood agar – aerobic (Tryptic soy agar (TSA) + 5 % sheep blood (e.g. Remel R01198 or equivalent) or Columbia + 5% sheep blood (e.g. Remel R01215 or equivalent))

6.2.2 Blood agar – anaerobic (any anaerobic agar, e.g. brucella blood agar (e.g. Remel R01254 or equivalent), CDC anaerobic blood agar (e.g. R01036), TSA +5% sheep blood)

6.2.3 MacConkey Agar (MAC) (e.g. Remel R01293 or equivalent)

6.2.4 Chocolate Agar (CHOC) (e.g. Remel R01550 or equivalent)

6.2.5 Broth (e.g. blood culture bottles, tryptone soy broth (TSB), brain heart infusion (BHI), or brucella broth)

6.3 Other Materials

6.3.1 Wire Inoculating loop

6.3.2 Bunsen burner

6.3.3 Glass slides

6.3.4 Antibiotic susceptibility disks

6.3.5 Gram staining reagents

7. Safety/Risk Assessment:

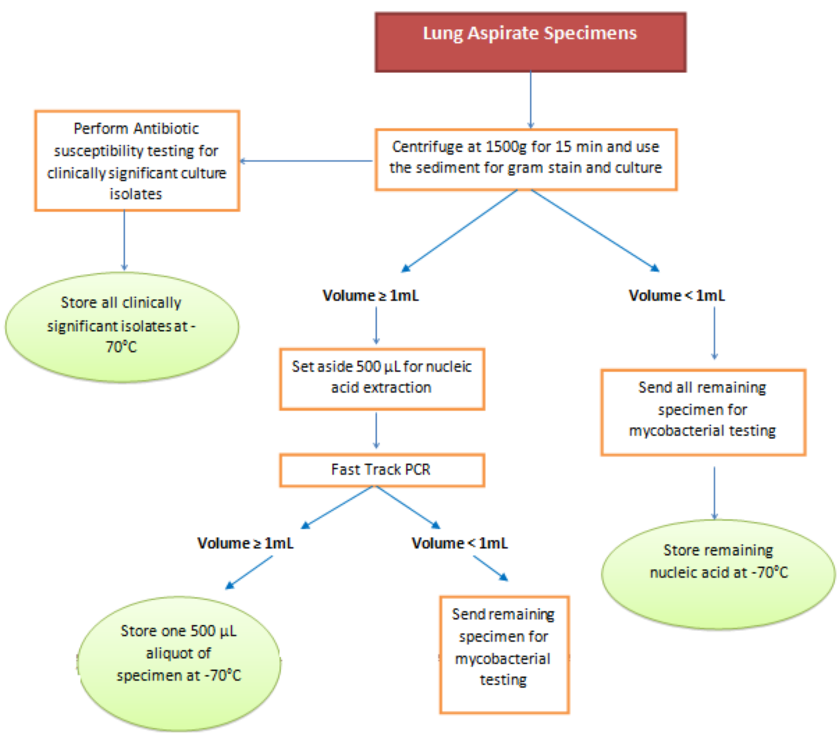
7.1 Always process respiratory specimens in biohazard cabinet wearing gloves.

8. Procedural Steps

8.1. Pre-processing specimen handling

Process specimen immediately on arrival in the laboratory. Store at 4°C for a maximum of 24 hours after collection if the specimen cannot be processed immediately.

Processing of Lung Aspirate Specimens:



8.2 Culture

8.2.1 Centrifuge at 1500g for 15 min before processing and use the sediment for the Gram stain and culture.

8.2.2 Use a sterile Pasteur pipette or a sterile disposable pipette to inoculate onto BA (aerobic and anaerobic), CHOC and MAC. Spread plates with separate sterile disposable loops. Inoculate into an appropriate broth known to support the growth of both fastidious aerobic organisms and anaerobes in order to detect small numbers of organisms. Appropriate broths include blood culture bottles, tryptone soy broth (TSB), brain heart infusion (BHI), and brucella broth.

8.2.3 Incubate inoculated media in appropriate incubator at 35-37°C overnight: BA and CHOC in the CO_2 incubator, MAC and broth in the aerobic incubator, anaerobic BA in an anaerobic jar, and blood culture bottles (if used) in the blood culture instrument.

8.3 Gram stain

8.3.1 Take one loopful of sediment and make a smear on glass slide

8.3.2 Leave smears to air dry in cabinet. Remove when dry, heat fix, Gram stain and then examine.

8.3.3 Report presence or absence of organisms and leucocytes seen in Gram stain.

8.3.4 Use the following system to report the number of leucocytes and bacteria seen per low- (10x) and high-power (100x) field respectively.

Number of leucocytes per representative LPF (×10 objective)	**Grade**
0	nil
<1	scanty
1-9	1+
10-24	2+
≥25	3+

Bacteria per representative HPF (×100 oil objective)	**Quantity**
<1	Scanty
1-9	1+
10-99	2+
≥100	3+

8.4 After the gram stain has been performed and culture plates have been inoculated, make the following aliquots in labeled sterile containers:

<table>
<tr><th colspan="2">Specimen Aliquot</th><th>Priority (in instances of decreased volume)</th></tr>
<tr><td>500 µL of specimen</td><td>Fast Track PCR testing</td><td rowspan="3">In instances of limited volume, prioritize TB testing, then PCR testing.</td></tr>
<tr><td>500 µL of specimen</td><td>Storage/Future Testing</td></tr>
<tr><td>Remainder of specimen (at least 500 µL). May transfer specimen in original container rather than aliquoting.</td><td>Mycobacterial culture (ensure some of the purulent portion goes for Mycobacterial culture if it is a purulent specimen)</td></tr>
</table>

8.5 Examination of Culture

8.5.1 Examine all plated and broth media (except the anaerobic plate) for macroscopic evidence of growth at 24 hr.

8.5.2 If no visible growth is observed on the culture media, re-incubate

8.5.2.1 Read aerobic plates at 48, 72 and 96 hr.

8.5.2.2 Read anaerobic plates at 48 hr.

8.5.2.3 Subculture broth cultures onto BA and CHOC only if there is evidence of turbidity up to 96 hr.

8.5.2.4 Incubate blood culture bottles for 5 days

8.6 Correlate culture results with those of the direct Gram stain.

8.7 Identify any organisms present and perform susceptibility testing as per CLSI protocols.

8.8 If the culture is pure, scrape all of the growth using a sterile cotton tipped swab into a cryotube containing 1.0mL of STGG medium

8.9 Label and store any isolates in -70°C freezer as soon as possible.

9. Quality Assurance / Quality Control

10. Record Management:

10.1 *Access, location, and retention of records pertaining to the SOP – site specific*

11. References:

11.1 Sharp SE, Robinson A, Saubolle M, Santa Cruz M, Carroll K, Baselski V. Cumitech 7B, lower respiratory tract infections. Washington, DC: ASM Press, 2004

11.2 Isenberg HD (Ed). Clinical microbiology procedures handbook. 2nd Ed. Washington, DC: ASM Press, 2004

1. Definitions:

   1.1 MGP- Magnetic Glass Particle
   1.2 TNA- Total nucleic acid
   1.3 GuSCN-Guanidine thiocyanate
   1.4 PPE-personal protective equipment
   1.5 MSDS-Material safety data sheet

2. Purpose / Background:

   2.1 The NucliSens easyMAG platform is a second-generation system for automated isolation of nucleic acids from clinical samples based upon silica extraction technology. The extraction method is universal and can be applied to a broad range of different specimens such as blood, sputum, serum, throat and nasal swabs. The isolation of nucleic acid is a key step in the molecular process as poor sample preparation or impure nucleic acid will impact on the quality of results. It is important that the sample is pre-processed to remove PCR inhibitors and release nucleic acid for isolation. The sample is then diluted in lysis buffer to further remove any PCR inhibition effects and then isolation of total nucleic acid (TNA) on the easyMAG extractor (BioMerieux).

3. Scope / Applicability:
   3.1 This SOP is applicable to all trained laboratory technicians/technologists/scientists working in the *[name]* microbiology laboratory.

4. Safety/ Risk Assessment
   4.1. All patient samples, reagents, as well as all waste should be treated as potentially bio-hazardous materials. Use of appropriate (PPE) is mandatory.
   4.2. NucliSens easyMAG Lysis Buffer, NucliSens easyMAG Extraction Buffer 1 or waste from the instrument should **NOT** come into contact with acidic materials. NucliSens easyMAG Lysis Buffer can potentially release **Poisonous** cyanide gas on contact with acid, (Refer to the MSDS for NucliSens easyMAG Lysis Buffer and NucliSens easyMAG Extraction Buffer 1 for further information).
   4.3. NucliSens easyMAG lysis Buffer and NucliSens easyMAG Extraction Buffers contain **Guanidine thiocyanate** (GuSCN) which is known to cause eye, skin and respiratory tract irritation. Avoid contact with these by having the appropriate PPE.

5. Roles / Responsibilities
   5.1. *[Site specific]*

6. Equipment /Materials / Reagents

6.1. EasyMAG sample strip
6.2. Aspirator disposables
6.3. NucliSENS lysis buffer 2.0 mL tube
6.4. BioHit pipette
6.5. Vortex
6.6. Molecular grade water
6.7. NucliSENS easyMAG Extraction Buffer 1
6.8. NucliSENS easyMAG Extraction Buffer 2
6.9. NucliSENS easyMAG Extraction Buffer 3
6.10. NucliSENS easyMAG Magnetic Silica
6.11. Barrier tips (1000, 200 µl)
6.12. Pipettes (1000, 200 µl)
6.13. Pasteur pipettes
6.14. 8 trip PCR reaction tubes and 8 strip caps for 0.2 mL tubes
6.15. 70% ethanol
6.16. Powder free gloves

7. Specimen:

7.1 Whole Blood (EDTA) sample:

7.1.1 Disinfect the hood and clean the working surface and pipettes with 70% ethanol.

7.1.2 Depending on the number of samples available, label each set of EIGHT NucliSENS lysis buffer 2.0 mL tubes with numbers (A1-A8, B1-B8, C1-C8) see Appendix 9.1.

7.1.3 Confirm that the sample I.D is the same as the one on the sample layout sheet, and matches with the EIGHT NucliSENS lysis buffer 2.0 mL tubes positions. **N.B** make note of any sample that is less than 200µL on the sample layout sheet

7.1.4 Place the sample strips on the sample strip carrier.

7.1.5 Gently invert tubes to ensure sample is homogeneous (**do not vortex**)

7.1.6 Add 200 µL whole blood sample to the respective eppendorf tubes containing 800 ul of Extraction Buffer 3.

7.1.7 Add 1.0 mL of whole blood sample/Extraction buffer 3 mix to the NucliSENS lysis buffer 2.0 mL tube and vortex immediately. **N.B** Ensure that the pipette shaft does not touch the rim of the 2.0 mL tube.
To prevent splashing of samples onto the 2.0 mL tube caps, hold the middle part of the tube.

7.1.8. Add 140 µL of vortexed undiluted silica to the sample-lysis buffer mix and vortex immediately.

7.1.9 Transfer the lysed samples from the NucliSENS lysis buffer 2.0 mL tubes (total volume 3.0 mL) to the sample strips using a Pasteur pipette **N.B** Ensure that all the lysed sample is transferred to the corresponding sample strip position without causing foam or droplets

7.1.10 Have positive (10^3 pneumococcus) controls at the end (see Appendix 9.2)

7.1.11 Incubate the samples at room temperature for 10 minutes.

Note: These samples and sample strips will be used in **8.2**

7.1.12. **Proceed to the Specific B protocol for "off board" workflow**

8. Procedural Steps

8.1 **Nucilsens EasyMag Off board workflow**

The following procedure is based on Nuclisens Easymag user manual v 2 (2007-07) and applies to the Easymag 3.2 v3 system.

8.1.1 Switch the easyMAG machine on.

8.1.2 Wait until the orange light on the right front side of the machine turns green then switch on the computer, and log in.

8.1.3 Select Daily use icon on the menu bar.

Define extraction request

8.1.4 Select extraction run parameters for whole blood samples

Matrix: <u>Whole blood,</u>

Protocol: <u>Specific B 2.0.1</u>

Volume: <u>1 mL</u>

Elute : <u>100 µl</u>

Type: <u>Lysed</u>

Priority: <u>Normal</u>

Lot number: (for the 2mL lysis buffer)

8.1.5 Key in the sample ID and save.

8.1.6 Click on the 'new run' button and type in the subsequent Sample ID and save.

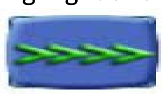
8.1.7 Press the Enter button on the keyboard to move to the next sample.

8.1.8 Assign samples to a run press (organise runs) icon

8.1.9 Click on 'new run' button  and enter run name on the pop up window using the format; daymonthyear_PERCH_run for example 29032011_PERCH_run1

8.1.10 In the workflow diactivate the on board lysis incubation and press OK

8.1.11 Highlight and move unassigned samples into the run by pressing the 'autofill' icon



This will move all unassigned and move unassigned samples to the current run in order of sample entered and each sample can now be seen on the layout screen.

8.1.12 Load the run progress view by pressing  (load run) icon.

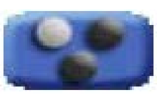
8.1.13 Load the run work area – process view by pressing  button.

8.1.14 Load the sample strips containing lysed samples from and aspirator disposables onto the instrument.

Select barcode type input  and scan the reagent ID position barcode (A, B, or C)and the sample strip ID barcode using the barcode reader (number that starts with Z).

Note: the indicator lights should turn green for each scanned item.

8.1.15 Using the barcode reader, scan the LOT NUMBER for the magnetic silica, aspirator disposables, sample strips, reagents, , and reagents ID position barcode (A,B, C and D).

8.1.16 Select  to enter barcode for current batch of silica and scan the Z number on the silica box.

8.1.17 Assign the silica to each sample by pressing  icon on menu bar and entering the batch number for the silica for each sample well; by selecting the silica batch number and highlight each sample position and press  button to assign.

8.1.18 Start nucleic acid isolation run by pressing  icon

Note: A pop-up screen will appear needing user input to confirm that the silica has been added click **YES** to continue the run.

8.1.19 Once run has finished the Nucleic acid is in the sample strip. Transfer the nucleic acid to another vail for storage.

Suggestion: Axygen 48 well plate Cat. No PCR-48-C or Axygen 8 trip PCR reaction tubes and 8 strip caps for 0.2mL tubes

Storage NA: 48 hours at 4ºC

1 month at -20ºC

Long-term at -80ºC

Note: Do not leave NA in the sample strip tubes for > 30 minutes as silica particles may fall back into the TNA elute.

Store any extracted nucleic acid remaining after downstream application (i.e. PCR) at -70°C

9 APPENDICES:

9.1 Sample layout sheet

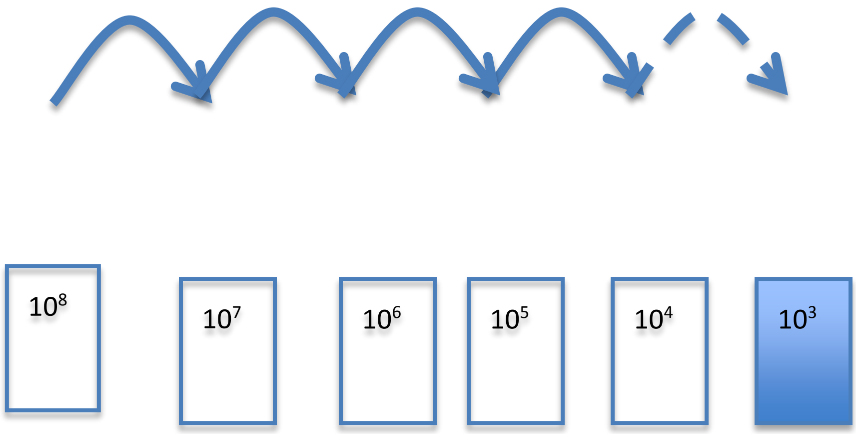
	Work area A	Sample I.D	Work area B	Sample I.D	Work area C	Sample I.D
1	A1		B1		C1	
2	A2		B2		C2	
4	A3		B3		C3	
4	A4		B4		C4	
5	A5		B5		C5	
6	A6		B6		C6	
7	A7		B7		C7	
8	A8		B8		C8	

9.2 Preparation of positive controls for LytA PCR

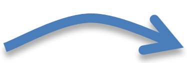
Procedure.

9.2.1 Prepare 0.5 MacFarland of SPN (this contains ~10^8 cells/mL) from a young overnight growth of *S. pneumoniae* on a blood agar plate.

9.2.2 Do a 1:10 serial dilution using sterile normal saline to obtain a concentration of 10^4 by serially pipetting 50 µl of each concentration into 450 µl of normal saline as shown below. The final serial dilution should be 420 µl of 10^4 suspension into 3780 µl of EDTA whole blood for a final concentration of 10^3 cells/mL. N.B. vortex mix each concentration before proceeding to the next, except for the final dilution into whole blood, which should be mixed gently by inversion 20 times to avoid haemolysis.



KEY



50 µl of SPN suspended in saline

420 µl of SPN suspended in saline

450 µl of normal saline

3780 µl of whole blood

9.2.3 Make aliquots of the final 10^3 cells/mL suspension for use as EasyMag positive controls. These can be kept refrigerated for up to a week, or stored at -20 to -80ºC.

10.0 REFERENCES:

10.1 NucliSENS easyMAG User Manual version 2.0

10.2 **K. Loens, K. Bergs, D. Ursi, H. Goossens, and M. Ieven** (2006) Evaluation of NucliSens easyMAG for Automated Nucleic Acid Extraction from Various Clinical Specimens. *Journal of clinical microbiology*. P.421–425

1. Definitions:

    1.1 MGP- Magnetic Glass Particle
    1.2 TNA- Total nucleic acid
    1.3 GuSCN-Guanidine thiocyanate
    1.4 PPE-personal protective equipment
    1.5 MSDS-Material safety data sheet

2. Purpose / Background:

    2.1 The NucliSens easyMAG platform is a second-generation system for automated isolation of nucleic acids from clinical samples based upon silica extraction technology. The extraction method is universal and can be applied to a broad range of different specimens such as blood, sputum, serum, throat and nasal swabs. The isolation of nucleic acid is a key step in the molecular process as poor sample preparation or impure nucleic acid will impact the quality of results. It is important that the sample is pre-processed to remove PCR inhibitors and release nucleic acid for isolation.).

3. Scope / Applicability:

    3.1 This SOP is applicable to all trained laboratory technicians/technologists/scientists working in the *[name]* microbiology laboratory.

4. Safety/ Risk Assessment

    4.1. All patient samples, reagents, as well as all waste should be treated as potentially bio-hazardous materials. Use of appropriate (PPE) is mandatory.
    4.2. NucliSens easyMAG Lysis Buffer, NucliSens easyMAG Extraction Buffer 1 or waste from the instrument should **NOT** come into contact with acidic materials. NucliSens easyMAG Lysis Buffer can potentially release **Poisonous** cyanide gas on contact with acid, (Refer to the MSDS for NucliSens easyMAG Lysis Buffer and NucliSens easyMAG Extraction Buffer 1 for further information).
    4.3. NucliSens easyMAG lysis Buffer and NucliSens easyMAG Extraction Buffers contain **Guanidine thiocyanate** (GuSCN) which is known to cause eye, skin and respiratory tract irritation. Avoid contact with these by having the appropriate PPE.

5. Roles / Responsibilities

    5.1. *[Site specific]*

6. Equipment /Materials / Reagents

    6.1. EasyMAG sample strip

6.2. Aspirator disposables
6.3. BioHit pipette
6.4. Vortex
6.5. Molecular grade water
6.6. NucliSENS easyMAG Extraction Buffer 1
6.7. NucliSENS easyMAG Extraction Buffer 2
6.8. NucliSENS easyMAG Extraction Buffer 3
6.9. NucliSENS easyMAG Magnetic Silica
6.10. Barrier tips (1000, 200 µl)
6.11. Pipettes (1000, 200 µl)
6.12. Pasteur pipettes
6.13. 8 strip PCR reaction tubes and 8 strip caps for 0.2 mL tubes
6.14. 70% ethanol
6.15. Powder free gloves

7. Specimen:

Preparation of controls: FTD negative control (NC) and internal control (IC): thaw one NC (white cap) and one IC (blue cap) for a 12 patient setup. Make sure to keep the IC on ice before use. The NC (400µl) is extracted together with the IC. DO NOT extract the FTD positive controls (PCs -red caps). The IC is added directly to the lysis step of each extraction (see 8.1.10).

7.1 **Sputum:**

7.1.1 The 500 µL sputum sample is digested using dithiothreitol (e.g. Sputosol (Oxoid #SR0233)) in a 1:1 ratio and incubated at ambient temperature until sputum dissolved.

Optional: using sterile glass beads and shaking to assist in its break down if required. If sample is still not able to be easily pipetted then add more sputosol to obtain a ratio 1:3 to the sample and shake.

Note: Dithiothreitol breaks down the mucus aiding release of bacteria. The specimen should be completely homogenised prior to separation.

7.1.2 Transfer 400 µL of sputum to the EasyMag sample strip. Store the remaining digested specimen at -70°C. Include one NC in the extraction process

7.1.3 Proceed to the generic “on-board” lysis workflow

7.2 Nasopharngeal/oropharyngeal swab samples:

7.2.1 Resuspend cellular material from swabs by vigorous vortexing

7.2.2 Transfer 400µL of sample suspension to the sample strip well. Include one NC in the extraction process.

7.2.3 **Proceed to the generic "on-board" lysis workflow**

7.3 Pleural fluid and lung aspirate samples:

7.3.1. Transfer 400µL of sample suspension to the sample strip well. Include one NC in the extraction process

7.3.2 **Proceed to the generic "on-board" lysis workflow**

8. Procedural Steps

8.1 Nuclisens Easymag On board lysis workflow

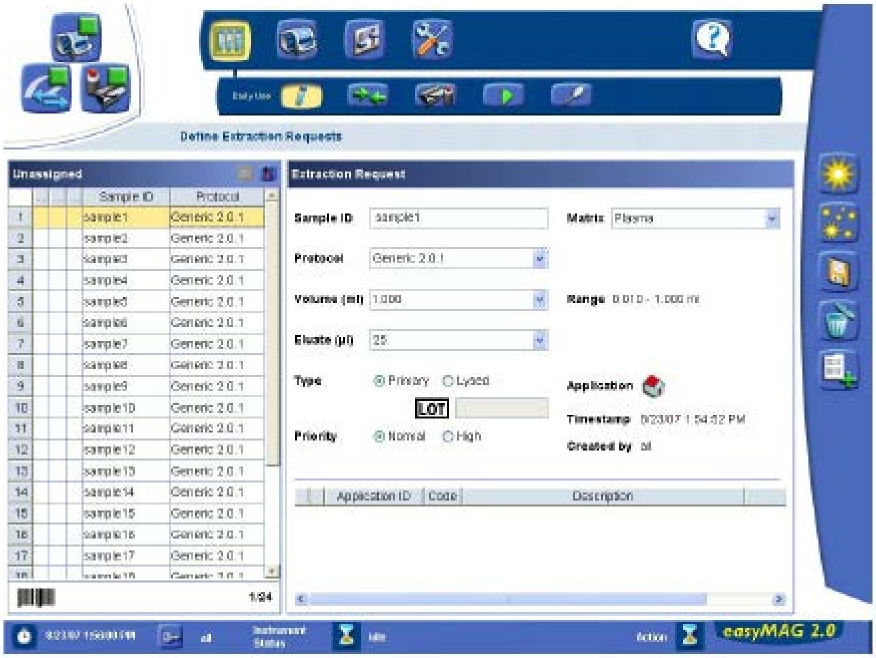
The following procedure is based on Nuclisens Easymag user manual v 2 (2007-07) and applies to the Easymag 3.2 v3 system.

8.1.1 Start the instrument and log in to the software.
Switch the Easymag machine on. Wait for 5 minutes before switching on the computer otherwise you will receive an error message. The Easymag application will start automatically. Wait until the orange light on the right front side of the machine turns green before the log in to ensure that the connection between the module and the computer is established

8.1.2 Select Daily use icon on the menu bar

Daily Use Menu

Define extraction request



8.1.3 Select extraction run parameters

Extraction parameters for Sputum, nasopharyngeal/oropharyngeal swabs, pleural fluid, lung aspirates:

Matrix: Choices are Sputum, Other

Protocol: Generic 2.0.1

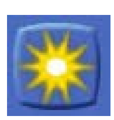
Volume (mL): 0.4

Elute (µL): 60 to110

Type: Primary

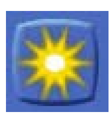
Priority: Normal

8.1.4 Scan in the Bar codes of each sample in the "Sample ID" field

Press the "Enter" icon  after each sample has been entered

Note: Run parameters (Matrix, Volume, Elute, primary or lysed) can be changed only for each block of 8 samples (locations A, B, and C) but the protocol can not be changed

8.1.5 Assign samples to a run press  (organise runs) icon

Click  (new run) button to enter new run and a pop up window will appear

Enter the run name and press OK

Suggestion: Naming a run in the following format daymonthyear_Study_run#

For example 29032011_PERCH_run1

8.1.6 Move unassigned samples into the run by pressing the  (autofill) icon

This will move all unassigned samples to the current run in order of sample entered and each sample can now be seen on the layout screen.

Optional: Samples can be highlighted and moved individually using the  icon

Samples can be unassigned by removing them from a run by highlighting the sample and pressing the  icon.

Refer to the current version of the Nuclisens Easymag user manual for other options available on this screen such as editing runs and deleting.

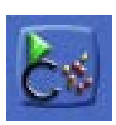
8.1.7. Load the run progress view by pressing  (load run) icon

Load the run work area – process view by pressing  button

8.1.8 Load the sample strips and aspirator disposables onto the instrument. Select Barcode type input  and scan the reagent ID position barcode (A, B, or C)and the sample strip ID barcode using the barcode reader (number that starts with Z).

Note: the indicator lights should turn green for each scanned item.

8.1.9 Load the the samples and the NC to the defined wells, Verify that the samples have been pipetted correctly and the machine cover has been closed.

8.1.10 Start run by pressing the  icon

Important: use this icon to start the on board lysis protocol

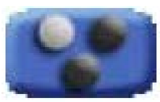
Lysis step takes about 10 minutes. The lysis step is completed when “idle” appears in the bottom of the screan and NOT when the time has run out.

8.1.11 Open the Easymag and add the Internal control: Add 4 µL FTD internal control(s) (IC, blue cap) directly to the EasyMag sample strip well of each extraction (including the NC). To add the internal control to each of your samples and to the negative control is a very important step to review the nucleic acid isolation and to check for possible PCR inhibition!

8.1.12 Prepare pre-mix (silica bead suspension) as follows

Note: one vial is sufficient for 8 samples, ensure that the silica is mixed before adding water

- Using program 1 (P1) on the BioHit pipette add **550 µL sterile molecular grade** water to the **550 µL magnetic silica** solution tube. Mix well.
- Using program 2 (P2) on the BioHit pipette, transfer **125 µL magnetic silica pre-mix** to a 8 well microtitre strip (Griener)
- Using program 3 (P3) on the BioHit pipette transfer the silica suspension to the sample strips containing the lysed samples and properly homogenize the mixture.

8.1.13 Select  to enter barcode for current batch of silica and scan the Z number on the silica box.

8.1.14 Next assign the silica to each sample by pressing  icon on menu bar and entering the batch number for the silica for each sample well; by selecting the silica batch number and highlight each sample position and press  button to assign. The diluted silica beads can only be stored for one week.

8.1.15. Continue Extraction run by pressing  icon

A pop-up screen will appear needing user input to confirm that the silica has been added click YES to continue the run

The instrument performs incubation, washing, elution and particle separation from elution buffer.

Press  (execute run) icon on menu bar and select  to see execute run screen

Note:

Run incidents press  icon for descriptive detail of any problems.

Refer to the Nuclisens Easymag user manual or contact a BioMerieux representative for troubleshooting options.

8.1.16 On the menu bar select  icon to view results work area

Optional: a printout of the run can be done for record keeping. Refer to the Nuclisens Easymag user manual for other available options and functions.

8.1.17 Once run has finished the Nucleic acid is in the sample strip. Transfer the nucleic acid to another vial for storage.

Note: Do not leave TNA in the sample strip tubes for > 20 minutes as silica particles may fall back into the TNA elute.

Be careful not to dislodge the magnetic bead silica when taking out the samples. Transfer the samples as soon as possible to avoid the beads from sliding back into the DNA solution.

Suggestion: Axygen 48 well plate Cat. No PCR-48-C or Axygen 8 trip PCR reaction tubes and 8 s trip caps for 0.2mL tubes

Storage TNA: 48 hours at 4ºC

1 month at -20ºC

Long-term at -80ºC

Note: Do not leave TNA in the sample strip tubes for > 30 minutes as silica particles may fall back into the TNA elute.

8.1.18 Store any extracted nucleic acid remaining after downstream application (i.e. PCR) at -70°C

8.1.19. Maintenance: Fill in the maintenance plan after each use of the machine

Daily: Inspect dispense probe and clean the machine exterior, disinfect the workspace before and after each use, empty waste if needed

Perform weekly, monthly and 6-monthly maintenance as outlined in the Easymag User manual.

8.1.20. Shut down: Exit the program using the key icon. The computer will turn off automatically. After 5 min turn off the machine using the button at the side of the machine.

Storage NA: 48 hours at 4ºC

1 month at -20ºC

Long-term at -80ºC

Note: Do not leave NA in the sample strip tubes for > 30 minutes as silica particles may fall back into the TNA elute.

Store any extracted nucleic acid remaining after downstream application (i.e. PCR) at -70°C

10.0 REFERENCES:

10.1 NucliSENS easyMAG User Manual version 2.0

10.2 **K. Loens, K. Bergs, D. Ursi, H. Goossens, and M. Ieven** (2006) Evaluation of NucliSens easyMAG for Automated Nucleic Acid Extraction from Various Clinical Specimens. *Journal of clinical microbiology*. P.421–425

Changes since last version of the SOP:

V1.1 : In this version, the instructions for extracting the internal control are modified from the previous version to prevent deterioration of the IC during pre-processing steps. Changes are reflected in section 7 and sections 8.1.9 and 8.1.10 of this SOP.

MICROBIOLOGY ORGANISM DICTIONARY

The following codes should be used for reporting all cultures results for PERCH.

Please contact the Amanda Driscoll (adriscol@jhsph.edu) if there is uncertainty about which code to use for a specific organism.

GENERIC

GNBI	AEROBIC GRAM NEGATIVE RODS
GNCI	AEROBIC GRAM NEGATIVE COCCI
GPBI	AEROBIC GRAM POSITIVE RODS
GPCI	AEROBIC GRAM POSITIVE COCCI
ANCO	ANAEROBIC GRAM NEGATIVE COCCI
APCO	ANAEROBIC GRAM POSITIVE COCCI
ASPB	ANAEROBIC GRAM POSITIVE SPORING RODS
ANPB	ANAEROBIC GRAM POSITIVE RODS
ANBA	ANAEROBIC GRAM NEGATIVE RODS
COLI	COLIFORM BACILLI
DIPH	DIPHTHEROID, COMMENSAL
MGNR	MIXED GRAM NEGATIVE RODS
NFGB	NON FERMENTATIVE GRAM NEGATIVE RODS

BACTERIA

ACHR	ACHROMOBACTER SPECIES
AXYL	ACHROMOBACTER XYLOSOXIDANS
ABAU	ACINETOBACTER BAUMANNII
ACAL	ACINETOBACTER CALCOACETICUS
ACHA	ACINETOBACTER HAEMOLYTICUS
AJOH	ACINETOBACTER JOHNSONII
AJUN	ACINETOBACTER JUNII
ALWO	ACINETOBACTER LWOFFII
ACRA	ACINETOBACTER RADIORESISTENS
ACIN	ACINETOBACTER SPECIES
ACSP	ACTINOBACILLUS SPECIES
AISR	ACTINOMYCES ISRAELII
ANAE	ACTINOMYCES NAESLUNDII
AODO	ACTINOMYCES ODONTOLYTICUS
ASPE	ACTINOMYCES SPECIES
AERV	AEROCOCCUS VIRIDANS
AECA	AEROMONAS CAVIAE
AEHY	AEROMONAS HYDROPHILA
AESP	AEROMONAS SPECIES
AEVE	AEROMONAS VERONII VAR SOBRIA
AGAC	AGREGATTIBACTER ACTINOMYCETEMCOMITANS
AGGA	AGREGATTIBACTER (HAEMOPHILUS) APHROPHILUS
AGGP	AGREGATTIBACTER (HAEMOPHILUS) PARAPHROPHILUS
ARAD	AGROBACTERIUM RADIOBACTER
AGSP	AGROBACTERIUM SPECIES
ALFA	ALCALIGENES FAECALIS
ALSP	ALCALIGENES SPECIES
AOTI	ALLOIOCOCCUS OTITIS
ARSP	ARACHNIA SPECIES
ABER	ARCANOBACTERIUM BERNARDIAE
AHAE	ARCANOBACTERIUM HAEMOLYTICUM
APYO	ARCANOBACTERIUM PYOGENES
ARCA	ARCANOBACTERIUM SPECIES

BANT	BACILLUS ANTHRACIS
BCER	BACILLUS CEREUS
BLIC	BACILLUS LICHENIFORMIS
BSPE	BACILLUS SPECIES
BSUS	BACILLUS SUBTILIS
BAFR	BACTEROIDES FRAGILIS GROUP
BASP	BACTEROIDES SPECIES
BARS	BARTONELLA SPECIES
BISP	BIFIDOBACTERIUM SPECIES
BOPA	BORDETELLA PARAPERTUSSIS
BOPE	BORDETELLA PERTUSSIS
BPET	BORDETELLA PETRII
BOSP	BORDETELLA SPECIES
BREV	BREVIBACTERIUM SPECIES
BVSP	BREVUNDIMONAS SPECIES
BVES	BREVUNDIMONAS VESICULARIS
BRAB	BRUCELLA ABORTUS
BRSP	BRUCELLA SPECIES
BCEN	BURKHOLDERIA CENOCEPACIA
BCPE	BURKHOLDERIA CEPACIA
BMUL	BURKHOLDERIA MULTIVORANS
BCPS	BURKHOLDERIA PSEUDOMALLEI
BUSP	BURKHOLDERIA SPECIES
BAGR	BUTTIAUXELLA AGRESTIS
CCOL	CAMPYLOBACTER COLI
CFFE	CAMPYLOBACTER FETUS
CJEJ	CAMPYLOBACTER JEJUNI
CLAN	CAMPYLOBACTER LANIENAE
CLAR	CAMPYLOBACTER LARI
CSPE	CAMPYLOBACTER SPECIES
CUPS	CAMPYLOBACTER UPSALIENSIS
CAPS	CAPNOCYTOPHAGA SPECIES
CHOM	CARDIOBACTERIUM HOMINIS
CHTR	CHLAMYDIA TRACHOMATIS
CHVI	CHROMOBACTERIUM VIOLACIUM
CMEN	CHRYSEOBACTERIUM MENINGOSEPTICUM
CHRY	CHRYSEOBACTERIUM SPECIES
CBRA	CITROBACTER BRAAKII
CITR	CITROBACTER FREUNDII
CKOS	CITROBACTER KOSERI (DIVERSUS)
CISP	CITROBACTER SPECIES
CLBO	CLOSTRIDIUM BOTULINUM
CLCL	CLOSTRIDIUM CLOSTRIDIOFORME
CLDI	CLOSTRIDIUM DIFFICILE
CLNO	CLOSTRIDIUM NOVYI
CLPE	CLOSTRIDIUM PERFRINGENS
CLSE	CLOSTRIDIUM SEPTICUM
CLSP	CLOSTRIDIUM SPECIES
CSPO	CLOSTRIDIUM SPOROGENES
CTER	CLOSTRIDIUM TERTIUM
CLTE	CLOSTRIDIUM TETANI
COTE	COMAMONAS TESTOSTERONI
CACC	CORYNEBACTERIUM ACCOLENS
CAMY	CORYNEBACTERIUM AMYCOLATUM
CDIP	CORYNEBACTERIUM DIPHTHERIAE
COJK	CORYNEBACTERIUM JEIKEIUM
CKRO	CORYNEBACTERIUM KROPPENSTEDTII
CMAC	CORYNEBACTERIUM MACGINLEYI
CMIN	CORYNEBACTERIUM MINUTISSIMUM
CPSD	CORYNEBACTERIUM PSEUDODIPHTHERITICUM
CPTB	CORYNEBACTERIUM PSEUDOTUBERCULOSIS
CORY	CORYNEBACTERIUM SPECIES
COST	CORYNEBACTERIUM STRIATUM
CULC	CORYNEBACTERIUM ULCERANS

COUR	CORYNEBACTERIUM UREOLYTICUM
CXER	CORYNEBACTERIUM XEROSIS
DCON	DERMATOPHILUS CONGOLENSIS
DISP	DIALISTER SPECIES
EDWS	EDWARDSIELLA SPECIES
EDTA	EDWARDSIELLA TARDA
EGGS	EGGERTHELLA SPECIES
EICO	EIKENELLA CORRODENS
EAER	ENTEROBACTER AEROGENES
EAGG	ENTEROBACTER AGGLOMERANS
EASB	ENTEROBACTER ASBURIAE
ECLO	ENTEROBACTER CLOACAE
ESAK	ENTEROBACTER SAKAZAKII
ESPE	ENTEROBACTER SPECIES
ECAS	ENTEROCOCCUS CASSELIFLAVUS
EFAE	ENTEROCOCCUS FAECALIS
ENFA	ENTEROCOCCUS FAECIUM
EGAL	ENTEROCOCCUS GALLINARUM
ENTC	ENTEROCOCCUS SPECIES
ERYS	ERYSIPELOTHRIX RHUSIOPATHIAE
ECOL	ESCHERICHIA COLI
EVUL	ESCHERICHIA VULNERIS
EUSP	EUBACTERIUM SP
FLAM	FLAVOBACTERIUM MENINGOSEPTICUM
FLAV	FLAVOBACTERIUM SPECIES
FLUR	FLUORIBACTER SPECIES
FTUL	FRANCISELLA TULARENSIS
FUNE	FUSOBACTERIUM NECROPHORUM
FUNU	FUSOBACTERIUM NUCLEATUM
FUSP	FUSOBACTERIUM SPECIES
GVAG	GARDNERELLA VAGINALIS
GEMO	GEMELLA MORBILLORUM
GBRO	GORDONA BRONCHIALIS
HAEG	HAEMOPHILUS AEGYPTIUS
HHAE	HAEMOPHILUS HAEMOLYTICUS
HINF	HAEMOPHILUS INFLUENZAE
HINB	HAEMOPHILUS INFLUENZAE type B
HPHA	HAEMOPHILUS PARAHAEMOLYTICUS
HPAR	HAEMOPHILUS PARAINFLUENZAE
HAEM	HAEMOPHILUS SPECIES
HAFN	HAFNIA ALVEI
HCAN	HELICOBACTER CANADENSIS
HPYL	HELICOBACTER PYLORI
HSPE	HELICOBACTER SPECIES
HSOM	HISTOPHILUS SOMNI
ILIM	INQUILINUS LIMOSUS
KKIN	KINGELLA KINGAE
KISP	KINGELLA SPECIES
KOXY	KLEBSIELLA OXYTOCA
KOZA	KLEBSIELLA OZAENAE
KPNE	KLEBSIELLA PNEUMONIAE
KSPE	KLEBSIELLA SPECIES
KINT	KLUYVERA INTERMEDIA
KLUY	KLUYVERA SPECIES
LACT	LACTOBACILLUS SPECIES
LSPE	LEUCONOSTOC SPECIES

LMIC	LEGIONELLA MICDADEI
LEFE	LEGIONELLA FEELEII
LEPH	LEGIONELLA PNEUMOPHILA
LLON	LEGIONELLA LONGBEACHAE
LEGS	LEGIONELLA SPECIES
LRIC	LEMINORELLA RICHARDII
LEPS	LEPTOSPIRA SPECIES
LIST	LISTERIA MONOCYTOGENES
LISP	LISTERIA SPECIES
MISP	MICROBACTERIUM SPECIES
MICR	MICROCOCCUS SPECIES
MMIC	MICROMONAS MICROS
MOBI	MOBILUNCUS SPECIES
MCAT	MOREXELLA (BRANHAMELLA) CATARRHALIS
MOLA	MORAXELLA LACUNATA
MONL	MORAXELLA NONLIQUEFACIENS
MOPH	MORAXELLA PHENYLPYRUVICA
MORA	MORAXELLA SPECIES
MOUR	MORAXELLA URETHRALIS
MOMO	MORGANELLA MORGANII
MOSP	MORGANELLA SPECIES
MYBA	MYCOBACTERIUM SPECIES
MAVI	M. AVIUM/INTRACELLULARE COMPLEX
MBOV	MYCOBACTERIUM BOVIS
MCHE	MYCOBACTERIUM CHELONAE
MABS	MYCOBACTERIUM ABSCESSUS GROUP
MFOR	MYCOBACTERIUM FORTUITUM
MGAS	MYCOBACTERIUM GASTRI
MGOR	MYCOBACTERIUM GORDONAE
MKAN	MYCOBACTERIUM KANSASII
MLEP	MYCOBACTERIUM LEPRAE
MMAL	MYCOBACTERIUM MALMOENSE
MMAR	MYCOBACTERIUM MARIUM
MSCR	MYCOBACTERIUM SCROFULACEUM
MSIM	MYCOBACTERIUM SIMIAE
MSZU	MYCOBACTERIUM SZULGAI
MTER	MYCOBACTERIUM TERRAE COMPLEX
MTUB	MYCOBACTERIUM TUBERCULOSIS COMPLEX
MULC	MYCOBACTERIUM ULCERANS
MXEN	MYCOBACTERIUM XENOPI
NTM	NONTUBERCULOUS MYCOBACTERIA
MHOM	MYCOPLASMA HOMINIS
MYPL	MYCOPLASMA SPECIES
MYRO	MYROIDES SPECIES
NCOM	Commensal Neisseria
NGON	NEISSERIA GONORRHOEAE
NLAC	NEISSERIA LACTAMICA
NMEN	NEISSERIA MENINGITIDIS
NSPP	NEISSERIA SPECIES
NAST	NOCARDIA ASTEROIDES
NBRA	NOCARDIA BRASILIENSIS
NFAR	NOCARDIA FARCINICA
NNOV	NOCARDIA NOVA GROUP
NOTI	NOCARDIA OTITIDISCAVIARUM
NOSP	NOCARDIA SPECIES
PANT	PANTOEA SPECIES
PMIC	PARVIMONAS MICRA
PASM	PASTEURELLA MULTOCIDA
PASP	PASTEURELLA SPECIES
PENS	PENICILLIUM SPECIES
PESP	PEPTOCOCCUS SPECIES
PTSP	PEPTOSTREPTOCOCCUS SPECIES

PLSH	PLESIOMONAS SHIGELLOIDES
PLSP	PLESIOMONAS SPECIES
PBUC	PREVOTELLA BUCCAE
PDEN	PREVOTELLA DENTICOLA
BAME	PREVOTELLA (BACTEROIDES) MELANINOGENICUS
PREV	PREVOTELLA SPECIES
PACN	PROPIONIBACTERIUM ACNES
PAVI	PROPIONIBACTERIUM AVIDUM
PSPE	PROPIONIBACTERIUM SPECIES
PRMI	PROTEUS MIRABILIS
PRPE	PROTEUS PENNERI
PROT	PROTEUS SPECIES
PRVU	PROTEUS VULGARIS
PROR	PROVIDENCIA RETTGERI
PRSP	PROVIDENCIA SPECIES
PROS	PROVIDENCIA STUARTII
PAER	PSEUDOMONAS AERUGINOSA
PFLU	PSEUDOMONAS FLUORESCENS
PPUT	PSEUDOMONAS PUTIDA
PSEU	PSEUDOMONAS SPECIES
PSTU	PSEUDOMONAS STUTZERI
RAQU	RAHNELLA AQUATILIS
REQU	RHODOCOCCUS EQUI
RALS	RALSTONIA SPECIES
ROSE	ROSEOMONAS SPECIES
RHSP	RHODOCOCCUS SPECIES
RMUC	ROTHIA (STOMATOCOCCUS) MUCILAGINOSA
RDEN	ROTHIA DENTOCARIOSA
RORN	RAOULTELLA (KLEBSIELLA) ORNITHINOLYTICA
SARI	SALMONELLA ARIZONAE
SALB	SALMONELLA BRANDENBURG
SCHO	SALMONELLA CHOLERAESUIS
SENT	SALMONELLA ENTERITIDIS
SHAD	SALMONELLA HADAR
SHAI	SALMONELLA HAIFA
SHAV	SALMONELLA HAVANA
SHIN	SALMONELLA HINDMARSH
SIBA	SALMONELLA IBADAN
SINF	SALMONELLA INFANTIS
SMBA	SALMONELLA MBANDAKA
SMGU	SALMONELLA MGULANI
SMIS	SALMONELLA MISSISSIPPI
SNEW	SALMONELLA NEWPORT
SOND	SALMONELLA ONDERSTEPOORT
SAOR	SALMONELLA ORANIENBURG
SOSL	SALMONELLA OSLO
SPAN	SALMONELLA PANAMA
SPAA	SALMONELLA PARATYPHI A
SPAB	SALMONELLA PARATYPHI B
SPEN	SALMONELLA PENSACOLA
SPOO	SALMONELLA POONA
SREA	SALMONELLA READING
SSTP	SALMONELLA SAINTPAUL
SASA	SALMONELLA SANDIEGO
SASC	SALMONELLA SCHWARZENGRUND
SSEN	SALMONELLA SENFTENBERG
SSIN	SALMONELLA SINGAPORE
SASP	SALMONELLA SPECIES
SSTA	SALMONELLA STANLEY
STEN	SALMONELLA TENNESSEE
STYP	SALMONELLA TYPHI
STYM	SALMONELLA TYPHIMURIUM

SVIR	SALMONELLA VIRCHOW
SWAY	SALMONELLA WAYCROSS
SWEL	SALMONELLA WELTEVREDEN
SELI	SERRATIA LIQUEFACIENS
SEMA	SERRATIA MARCESCENS
SEPL	SERRATIA PLYMUTHICA
SESP	SERRATIA SPECIES
PFAC	SHEWANELLA (PSEUDOMONAS) PUTREFACIENS
SHBO	SHIGELLA BOYDII
SHYD	SHIGELLA DYSENTERIAE
SHFL	SHIGELLA FLEXNERI
SHSO	SHIGELLA SONNEI
SHIG	SHIGELLA SPECIES
SEXI	SLACKIA EXIGUA
SOSP	SOLOBACTERIUM SPECIES
SPMU	SPHINGOBACTERIUM MULTIVORUM
SPSP	SPHINGOBACTERIUM SPIRITIVORUM
SPHI	SPHINGOBACTERIUM SPECIES
SPPA	SPHINGOMONAS PAUCIMOBILIS
SAUR	STAPHYLOCOCCUS AUREUS
SCAP	STAPHYLOCOCCUS CAPITIS
STCN	STAPHYLOCOCCUS coagulase negative
SCOH	STAPHYLOCOCCUS COHNII
SEPI	STAPHYLOCOCCUS EPIDERMIDIS
STHE	STAPHYLOCOCCUS HAEMOLYTICUS
SHOM	STAPHYLOCOCCUS HOMINIS
STIN	STAPHYLOCOCCUS INTERMEDIUS
SKLO	STAPHYLOCOCCUS KLOOSII
SLUG	STAPHYLOCOCCUS LUGDUNENSIS
SMUS	STAPHYLOCOCCUS MUSCAE
STSA	STAPHYLOCOCCUS SAPROPHYTICUS
STSC	STAPHYLOCOCCUS SCHLEIFERI
SSCI	STAPHYLOCOCCUS SCIURI
SSIM	STAPHYLOCOCCUS SIMULANS
STAP	STAPHYLOCOCCUS SPECIES
SWAR	STAPHYLOCOCCUS WARNERI
SXYL	STAPHYLOCOCCUS XYLOSUS
STMA	STENOTROPHOMONAS MALTOPHILIA
STOM	STOMATOCOCCUS SPECIES
AHST	STREPTOCOCCUS, Alpha-haemolytic (viridans)
SMIL	STREPTOCOCCUS ANGINOSUS (MILLERI) GROUP
BHST	STREPTOCOCCUS Beta-haemolytic
STBO	STREPTOCOCCUS BOVIS
SCON	STREPTOCOCCUS CONSTELLATUS
SGOR	STREPTOCOCCUS GORDONII
STRA	STREPTOCOCCUS GROUP A (PYOGENES)
STRB	STREPTOCOCCUS GROUP B (AGALACTIAE)
STRC	STREPTOCOCCUS GROUP C
STRD	STREPTOCOCCUS GROUP D
STRF	STREPTOCOCCUS GROUP F
STRG	STREPTOCOCCUS GROUP G
SINT	STREPTOCOCCUS INTERMEDIUS
SMIT	STREPTOCOCCUS MITIS
SMUT	STREPTOCOCCUS MUTANS
NHST	STREPTOCOCCUS, Non-haemolytic
SORA	STREPTOCOCCUS ORALIS
SPAR	STREPTOCOCCUS PARASANGUIS
PNEU	STREPTOCOCCUS PNEUMONIAE
SSAL	STREPTOCOCCUS SALIVARIUS
SSAN	STREPTOCOCCUS SANGUINIS (SANGUIS)
STSP	STREPTOCOCCUS SPECIES
SSUI	STREPTOCOCCUS SUIS
SMYC	STREPTOMYCES SPECIES

TVAG TRICHOMONAS VAGINALIS
TOTI TURICELLA OTITIDIS

URPL UREAPLASMA SPECIES

VESP VEILLONELLA SPECIES
VALG VIBRIO ALGINOLYTICUS
VCHO VIBRIO CHOLERAE
VPAR VIBRIO PARAHAEMOLYTICUS
VISP VIBRIO SPECIES

YENT YERSINIA ENTEROCOLITICA
YFRE YERSINIA FREDERIKSENII
YINT YERSINIA INTERMEDIA
YPES YERSINIA PSEUDOTUBERCULOSIS
YROH YERSINIA ROHDEI
YESP YERSINIA SPECIES

MYCOLOGY

ABSS ABSIDIA SP
ACOR ABSIDIA CORYMBIFERA
ACRS ACREMONIUM SPECIES
ALTS ALTERNARIA SPECIES
ASFL ASPERGILLUS FLAVUS
AFUM ASPERGILLUS FUMIGATUS
ANID ASPERGILLUS NIDULANS
ANIG ASPERGILLUS NIGER
ASPS ASPERGILLUS SPECIES
ATER ASPERGILLUS TERREUS
APUL AUREOBASIDIUM PULLULANS

BEAS BEAUVARIA SPECIES
BIPS BIPOLARIS SPECIES
BDER BLASTOMYCES DERMATITIDIS

CALB CANDIDA ALBICANS
CGLA CANDIDA (TORULOPSIS) GLABRATA
CGUI CANDIDA GUILLIERMONDII
CKER CANDIDA KEFYR
CKRU CANDIDA KRUSEI
CLUS CANDIDA LUSITANIAE
CPAR CANDIDA PARAPSILOSIS
CPSE CANDIDA PSEUDOTROPICALIS
CASP CANDIDA SPECIES
CSTE CANDIDA STELLATOIDEA
CTRO CANDIDA TROPICALIS

CHAS CHAETOMIUM SPECIES
CHRS CHRYSOSPORIUM SPECIES
CHRT CHRYSOSPORIUM TROPICUM
CIMM COCCIDIOIDES IMMITIS
CCAR CLADOSPORIUM CARRIONII
CLAS CLADOSPORIUM SPECIES
CRYA CRYPTOCOCCUS ALBIDUS
CLAU CRYPTOCOCCUS LAURENTII
CNEO CRYPTOCOCCUS NEOFORMANS
CRYS CRYPTOCOCCUS SPECIES
CELE CUNNINGHAMELLA ELEGANS
CUNS CUNNINGHAMELLA SPECIES
CURS CURVULARIA SP

DFUN Dematiaceous fungus
DRES DRESCHSLERA SPECIES

EPIS	EPICOCCUM SPECIES
EFLO	EPIDERMOPHYTON FLOCCOSUM
EJEA	EXOPHIALA JEANSELMEI
EXOS	EXOPHIALA SPECIES
EWER	EXOPHIALA WERNECKII
EXSS	EXSEROHILUM SPECIES
FPED	FONCECAEA PEDROSOI
FONS	FONSECAEA SPECIES
FUSS	FUSARIUM SPECIES
GARG	GEOSMITHIA (PENICILLIUM) ARGILLACEA
GCAN	GEOTRICHUM CANDIDUM
GEOS	GEOTRICHUM SPECIES
GLIS	GLIOCLADIUM SPECIES
GRAS	GRAPHIUM SPECIES
HTOR	HENDERSONULA TORULOIDEA
HCAP	HISTOPLASMA CAPSULATUM
MGRI	MADURELLA GRISEA
MMYC	MADURELLA MYCETOMATIS
MFUR	MALASSEZIA FURFUR
MAUD	MICROSPORUM AUDOUNII
MCAN	MICROSPORUM CANIS
MCOO	MICROSPORUM COOKEI
MEQU	MICROSPORUM EQUINUM
MFER	MICROSPORUM FERRUGINEUM
MGYP	MICROSPORUM GYPSEUM
MNAN	MICROSPORUM NANUM
MICS	MICROSPORUM SPECIES
MORS	MORTIERELLA SPECIES
MWOL	MORTIERELLA WOLFII
MUCO	MUCOR SPECIES
NATS	NATTRASSIA MANGIFERAE
OCHS	OCHROCONIS (DACTYLARIA) SPECIES
PAES	PAECILOMYCES SPECIES
PBRA	PARACOCCIDIOIDES BRASILIENSIS
PHIS	PHIALOPHORA SPECIES
PVER	PHIALOPHORA VERRUCOSA
PHOS	PHOMA SPECIES
PTHS	PROTOTHECA SPECIES
PWIC	PROTOTHECA WICKERHAMII
PBOY	PSEUDOALLESHERIA BOYDII (SCED. APIOSPER)
RHIM	RHIZOPUS MICROSPORUS
RORY	RHIZOPUS ORYZAE
RHIS	RHIZOPUS SPECIES
RPUS	RHIZOMUCOR PUSILLUS
RGLU	RHODOTORULA GLUTINIS
RRUB	RHODOTORULA RUBRA
RHOS	RHODOTORULA SPECIES
SACE	SACCHAROMYCES CEREVISIAE
SACS	SACCHAROMYCES SPECIES
SCOS	SCOPULARIOPSIS SPECIES
SCEA	SCEDOSPORIUM APIOSPERMUM
SCED	SCEDOSPORIUM SPECIES
SCYS	SCYTALIDIUM SPECIES
SCEP	SCEDOSPRORIUM PROLIFICANS
SEPS	SEPEDONIUM SPECIES

SSCH	SPOROTHRIS SCHENCKII
SYNS	SYNCEPHALASTRUM SPECIES
TORS	TORULOPSIS SPECIES
TAJE	TRICHOPHYTON AJELLOI
TCON	TRICHOPHYTON CONCENTRICUM
TEQU	TRICHOPHYTON EQUINUM
TEVA	TRICHOPHYTON EQUI VAR AUTOTROPHICUM
TERE	TRICHOPHYTON ERINACEI
TMEN	TRICHOPHYTON MENTAGROPHYTES
TMVI	TRICHOPHYTON MENT VAR INTERDIGITALE
TINT	TRICHOPHYTON INTERDIGITALE
TMVM	TRICHOPHYT. MENT VAR MENTAGROPHYTES
TMVN	TRICHOPHYTON MENT. VAR NODULARE
TMVQ	TRICHOPHYTON MENT. VAR QUINCKEANUM
TMVE	TRICHOPHYTON MENT, VAR ERINACEI
TRUB	TRICHOPHYTON RUBRUM
TSCH	TRICHOPHYTON SCHOENLEINII
TSOU	TRICHOPHYTON SOUDANENSE
TRIS	TRICHOPHYTON SPECIES
TTER	TRICHOPHYTON TERRESTRE
TTON	TRICHOPHYTON TONSURANS
TVER	TRICHOPHYTON VERRUCOSUM
TVIO	TRICHOPHYTON VIOLACEUM
TCUT	TRICHOSPORON (BEIGELII) CUTANEUM
TCAP	TRICHOSPORON CAPITATUM
TLOU	TRICHOSPORON LOUBIERI
TRIC	TRICHOSPORON SPECIES
TRID	TRICHODERMA SPECIES
TRIT	TRICHOTHECIUM SPECIES
VERS	VERTICILLIUM SPECIES
WDER	WANGIELLA DERMATITIDIS