Automated and Multiplexed Pneumococcus Serotyping Method

(Updated: 1/24/2012)

Part A) Preparation of Bacterial Culture Lysates

The automated serotyping assay requires pneumococcal lysates. The following protocol describes preparation of the lysates. The lysates can be stored frozen and/or shipped for future serotyping assays.

Materials/Equipment

Description	Supplier	Catalog Number
Incubator, 37ºC	Any	Any
Water Bath, 37ºC	Any	Any
Vortex Mixer	Any	Any
Candle Jar	Any	Any
Blood Agar Plates	Remel (or Equivalent)	R01202 (or Equivalent)
Nasal Swabs (if isolating pneumococcus from the nasopharynx)	Remel (or Equivalent)	723025 (or Equivalent)
Blood Agar Plates with Gentamicin (if isolating pneumococcus from the nasopharynx)	Remel (or Equivalent)	R01227 (or Equivalent)
1.1 ml Microtubes in Rack (Sterile)	VWR (or Equivalent)	89092-274 (or Equivalent)
Caps for 1.1 ml Microtubes (Sterile, 8 Caps/Strip)	VWR (or Equivalent)	89092-276 (or Equivalent)
Bromocresol Purple	Fisher	AC22961
Sodium Deoxycholate	Sigma	D6750
Sodium Dodecyl Sulfate	Sigma (or Equivalent)	L4390 (or Equivalent)
Sodium Azide	Sigma (or Equivalent)	S2002 (or Equivalent)
Sodium Citrate Dihydrate	Fisher (or Equivalent)	S279 (or Equivalent)
Todd-Hewitt Broth (BD Biosciences 249240, see Note 2)	Fisher	DF0492-17-6
Yeast Extract (BD Biosciences 212750)	Fisher	DF0127-17-9
0.22 Micron Bottle Top Filter	Fisher	SCGPT05RE

Solutions

For all solutions, high purity water is used (for example, water from a Synergy 185 purification system from MilliPore).

<u>0.8% Bromocresol Purple Stock</u>
To 50 ml water, add 0.8 grams of bromocresol purple. After complete dissolution, add water to 100 ml. Sterile filter and store at room temperature.

1% Sodium Azide
To 50 ml water, add 1 gram of sodium azide. After complete dissolution, add water to 100 ml. Store at room temperature.

1% Sodium Dodecyl Sulfate (SDS)
To 50 ml water, add 1 gram of SDS. After complete dissolution, add water to 100 ml. Store at room temperature.

14X Lysis Buffer
To 30 ml water, add 0.1 grams of sodium deoxycholate, 1 ml SDS (1% stock), 5 ml sodium azide (1% stock), and 5.2 grams sodium citrate dihydrate. After complete dissolution, add water to 50 ml. Adjust pH to 7.8 and store at room temperature for up to 1 year.

Todd-Hewitt Broth with 0.001% Bromocresol Purple (THYB with 0.001% BCP)
To 50 ml of water, add 3 grams of Todd-Hewitt Broth and 0.5 grams of yeast extract. After complete dissolution, add water to 100 ml. Sterilize by filtration. Add 125 microliters of bromocresol purple (sterile 0.8% stock solution) and store at room temperature for up to 1 month.

Procedures

To obtain nasopharyngeal samples

Follow the WHO guidelines for nasopharyngeal swab collection (O'Brien et al. Ped Inf Dis J. 22:e1, 2003) using the BBL CultureSwab Plus Collection and Transport System, catalog number 220126 or 220125 (they differ in charcoal content).

To obtain pneumococci

1. Streak the putative pneumococcal isolates (or nasal swabs) onto individual blood agar plates. If nasal swabs are used, it is better to use blood agar plates containing gentamicin to inhibit growth of non-pneumococcal species.
2. Incubate the blood agar plates overnight in a candle jar at 37ºC.
3. Dispense 500 microliters of THY broth with 0.001% BCP into the appropriate number of sterile microtubes in microtube rack.
4. Inoculate each microtube with a single colony from the blood agar plate. Avoid transferring any agar (Note 1).
5. Close the lid of the rack (or cover tubes with parafilm).
6. Incubate for 5 hr (or overnight) in a candle jar at 37ºC until microtubes look turbid or the medium turns from purple to yellow (see Note 2).

To prepare bacterial lysates

1. Remove the microtubes from the candle jar and add 40 microliters of 14X Lysis Buffer to each microtube.
2. Securely apply caps to microtubes.
3. Carefully vortex the microtubes for 5 seconds (or invert the box several times to mix the lysis buffer and bacteria culture).
4. Incubate microtubes at 37ºC until pneumococci lyse and the suspension becomes clarified. Usually 1 hour is sufficient.
5. At this point, the lysates can be used in the serotyping procedure below or they can

be stored. The lysates are good for more than one year at -20°C, one month at 4°C, two weeks at RT, and several days at 37°C. If the lysates are to be stored frozen, tape the box so that the lid does not open during handling or shipping. Also, add some packing material on top of the tubes to prevent caps coming off the tubes (the caps easily come off the tubes at temperatures below -20°C).

Part B) Multibead Pneumococcal Serotyping assay using mAbs

(Updated: 12/24/2012)

The presence of capsular polysaccharide (PS) in any samples or in the bacterial lysates is determined using a competitive inhibition, flow cytometry-based assay. In this assay, the bacterial lysates and a mixture of color-coded beads coupled to reference capsular PSs are incubated with a mixture of monoclonal antibodies that bind the immobilized capsular PSs. If a capsular PS is in the sample, the PS will inhibit the binding of the monoclonal antibody to the corresponding PS-coated bead. The amount of the monoclonal antibody bound to the beads is determined using a fluorescently labeled anti-mouse immunoglobulin secondary antibody. Currently, the bead set (Set A) consists of beads coated with PS from 26 serotypes: 1, 2, 3, 4, 5, 6A, 6B, 6C, 7F, 8, 9N, 9V, 10A, 11A, 11E, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. See Table 2 for bead regions associated with assay specificity.

Materials/Equipment/Software

Description	Supplier	Catalog/Model Number
Bio-Plex System	Bio-Rad Laboratories	Bio-Plex 200 Analyzer with Bio-Plex Manager V5.0 Software
Plate Shaker (shaking radius ~1-2 mm – ***this is critical***)	Bellco Glass (or Equivalent)	7644-20115 (or Equivalent)
Millipore Vacuum Manifold (or Equivalent)	Thermo Fisher	MSVMHTS00
Vacuum source	See Note 3	See Note 3
Vortex Mixer	Any	Any
Sonicating Water Bath	Bransonic (or Equivalent)	Model Branson 2200, 200 (or Equivalent)
Millipore 96-Well Filter Plates	Thermo Fisher	MSBVN1210 (10 plates) or MSBVN1250 (50 plates)
1.1 ml Microtubes in Rack (Sterile, for preparing dilutions of lysates)	VWR (or Equivalent)	89092-274 (or Equivalent)
5-ml Test Tubes (12 x 75 mm, Falcon 352054)	Fisher	14-959-2A
0.22 Micron Bottle Top Filter	Fisher	SCGPT05RE
Sodium Azide	Sigma (or Equivalent)	S2002 (or Equivalent)
Sodium Chloride	Fisher (or Equivalent)	S271 (or Equivalent)
Potassium Phosphate Monobasic	Sigma (or Equivalent)	P0662 (or Equivalent)
Potassium Chloride	Fisher (or Equivalent)	P217 (or Equivalent)
Sodium Phosphate Heptahydrate	Fisher (or Equivalent)	S373 (or Equivalent)
Polysorbate 20 (Tween 20)	Sigma (or Equivalent)	P-5927 (or Equivalent)

Bovine Serum Albumin (BSA)	Sigma (or Equivalent)	A7030 (or Equivalent)
PE-Conjugated Anti-Mouse Ig	BD Biosciences	550589
3X MabPool D, *Green-Topped Tubes*	UAB	(should have 1 ml.)
Stock Bead Mixture A, *Red-Topped Tubes*	UAB	(should have 1 ml.)
MuSA.03.xlsx (**Mu**ltiplexed **S**erotyping **A**nalyzer)	UAB	An Excel template for processing Bio-Plex data output

Solutions

For all solutions, high purity water is used (for example, water from a Synergy 185 purification system from MilliPore).

<u>10X PBS</u>

Add 800 ml of water to a 2-liter beaker placed on a magnetic stirrer. Weigh out the dry chemicals listed below and add them to the water. Dissolve the chemicals and bring the volume to 1000 ml with additional water. Sterilize the solution by filtering it with a 0.22 micron filter and store it in a sterile container at room temperature.

Dry chemical	Weight (grams)
NaCl	80.00
KH_2PO_4	3.14
$Na_2HPO_4 \cdot 7H_2O$	20.61
KCl	1.60
NaN_3	2.00

<u>1X Wash Buffer</u>

Add 900 ml of water to 100 ml of 10X PBS. Add 1 ml of Tween-20. Store at RT for up to 3 months.

<u>1X Blocking Buffer</u>

Dissolve 1 gram of BSA in 100 ml of 1X Wash Buffer. Filtrate and store at 4°C for up to 1 month.

Procedure

The volumes below are sufficient for ***one*** 96-well plate.

1. Prepare 10-fold and 30-fold dilutions of pneumococcal lysates. It is easiest to prepare the two dilutions in 1.1 ml microtubes in microtube racks such that a multichannel pipettor can be used to transfer the diluted sample to the assay plate below.
2. To prepare a 10-fold dilution, add 40 microliters of lysate to 360 microliters of Blocking Buffer.
3. To prepare a 30-fold dilution, add 80 microliters of the 10-fold dilution to 160 microliters of Blocking Buffer.
4. Thaw one vial of Stock Bead Mixture A (red-capped tube) and transfer the entire contents to a fresh 5 ml test tube. Add 2 ml of Blocking Buffer and vortex thoroughly. Sonicate the mixture for 1 minute in a sonicating water bath (at RT). This breaks up any bead aggregates that may have formed during storage. This solution is the "Working Luminex Bead Mixture" and may be kept at 4°C, protected from light, for more than 2 months (if

the bead mixture is stored for more than 2 hours, make sure to vortex and sonicate again before using).

5. Thaw one vial of 3X MabPool C (green-capped tube) and transfer the entire contents to a fresh 5 ml test tube. Add 2 ml of Blocking Buffer and vortex thoroughly. This is the "Working MabPool D" solution.
6. With the vacuum OFF, place a 96-well filter plate onto the vacuum manifold. Add 100 microliters of Wash Buffer to each well to pre-wet the filters. Turn the vacuum ON and allow all fluid to be aspirated from the wells.
7. Turn off the vacuum, and add 25 microliters of Working Luminex Bead Mixture prepared above.
8. Wash the beads two times using vacuum filtration. (See Note 4 for details.) (See Note 7 for alternative way of washing the beads.)
9. After the final wash (and liquid has been aspirated from all wells), turn the vacuum OFF, and remove the plate from the vacuum manifold.
10. Gently blot the bottom of the plate onto paper towels to remove residual Wash Buffer from the bottom of the plate by capillary action.
11. Add 25 microliters of Blocking Buffer to each well.
12. To wells A1, B1, and C1, add 25 microliters of additional Blocking Buffer. Beginning with well D1, add 25 microliters of appropriately diluted samples/control lysates to the remaining wells. See Table 1.

Table 1: Summary of well contents

Well ID	Control Name	In step 12, add	In step 13, add	In step 18, add
A1	Control 1	Blocking Buffer	Blocking Buffer	Blocking Buffer
B1	Control 2	Blocking Buffer	Blocking Buffer	Anti-mouse Ig PE
C1	Control 3	Blocking Buffer	Mab Pool B	Anti-mouse Ig PE
D1 *etc.*	Samples	Diluted sample	Mab Pool B	Anti-mouse Ig PE

13. To wells A1 and B1, add 25 microliters of Blocking Buffer. To all other wells, add 25 microliters of Working MabPool D.
14. Apply plate lid, wrap plate in aluminum foil (to prevent exposure to light), and incubate for 30 min at RT with shaking (700 rpm using Bellco shaker 7644-20115).
15. During incubation, prepare a 200-fold dilution of the phycoerythrin (PE)-conjugated anti-mouse Ig secondary antibody by adding 30 microliters of the secondary antibody to 6 ml of Blocking Buffer.
16. When incubation is complete, wash the beads four times using vacuum filtration (Note 4).
17. After the final wash (and liquid has been aspirated from all wells), turn the vacuum OFF, remove the plate from the vacuum manifold, and gently blot the bottom of the plate onto paper towels to remove residual Wash Buffer from the bottom of the plate.
18. To well A1, add 50 microliters of Blocking Buffer. To all other wells, add 50 microliters of the diluted secondary antibody prepared above. See Table 1.
19. Apply plate lid, wrap plate in aluminum foil (to prevent exposure to light), and incubate for 30 min at RT with shaking (700 rpm using Bellco shaker 7644-20115).
20. When incubation is complete, wash the beads four times using vacuum filtration (Note 4).
21. After the final wash (and liquid has been aspirated from all wells), turn the vacuum OFF, remove the plate from the vacuum manifold, and gently blot the bottom of the plate onto paper towels to remove residual Wash Buffer from the bottom of the plate.
22. Add 75 microliters of Wash Buffer to all wells.

23. Cover the plate with aluminum foil (to keep out light), and incubate for 2 min at RT with shaking (700 rpm using Bellco shaker 7644-20115) to fully suspend the beads.
24. Leave the plates for 2-18 hours at RT, wrapped in foil (to protect from light).
25. Shake the plate for 5 minutes on shaker. Acquire the data using the Bio-Plex Analyzer, see Note 5 for instrument set up.
26. After acquisition is complete, open the Bio-Plex software. Locate and open the data file (.rbx file).
27. On the left side of the window (under "Results"), there are 4 options. Select the "Raw Data" option and export the data to MS Excel®.
28. Open MuSA.03.xlsx (**Mu**ltiplexed **S**erotyping **A**nalyzer). Note the order of the bead regions (highlighted in green).
29. Enter sample IDs into the 'Sample ID' column (column A) of MuSA.03.
30. Copy the fluorescent values from the raw data file and paste into the appropriate cells in the '**Raw Data**' worksheet of MuSA.03.xlsx. Make sure the beads regions are in the correct order.
31. The results displayed in the '**Report**' worksheet are the normalized fluorescent values of the test samples. Wells that are positive are highlighted red. Wells that are negative are not highlighted. Wells that are indeterminate are highlighted blue.

    Data is interpreted as follows (provisional):

Normalized Fluorescence	**Interpretation**
≤33%	Positive
>33% and ≤67%	Indeterminate
>67%	Negative

Note that for serotype 15B, we interpret a normalized fluorescence value ≤67% as positive.

Table 2: Bead regions and Serotype Specificity of different bead sets

#	Bead regions	specificity	#	Bead regions	specificity
1	101	1	15	115	11A/D/F
2	102	2	16	116	11E/(11A/D/F)
3	103	3	17	117	12F
4	104	4	18	118	14
5	105	5	19	119	15B/(15C)
6	106	6A	20	120	17F/17A
7	107	6B	21	121	18C
8	108	6C/6D	22	122	19A
9	109	-	23	123	19F
10	110	7F/7A	24	124	20
11	111	8	25	125	22F/22A
12	112	9N	26	126	23F
13	113	9V/9A	27	127	33F/33A
14	114	10A/39			

Assay Notes

Note 1 We have found that the presence of agar may affect the binding of some monoclonal antibodies to polysaccharide. Therefore, when transferring colonies, it is important to not pick up any agar from the blood agar plates.

Note 2 Although almost all strains grow well in THYB, some clinical isolates may not grow in THYB. For more fastidious strains, addition of 12~315 units/ml of Catalase (Worthington #1896) in THY may help pneumococci grow.

Note 3 We use the “in house” vacuum supplied for our building. The system has a vacuum pressure of ~55-60 cm Hg.

Note 4 The Wash Cycle used to wash filter plates using vacuum filtration:

1. Turn the vacuum OFF, and place the plate onto the vacuum manifold.
2. Turn the vacuum OFF, and add 150 microliters of Wash Buffer.
3. Turn the vacuum ON, and allow all liquid to be aspirated.
4. Repeat steps 2 and 3 for the indicated number of cycles.

When adding reagents to the plate, the vacuum must be OFF. If the vacuum is on, the wells that receive reagent first may be dry by the time the reagent is added to the last wells.

Note 5 Below is a brief description of the operation of the Bio-Plex software. If there are any questions, please contact Rob Burton (robburton@uab.edu) for additional assistance.

1. Open the Bio-Plex software.
2. Under the File menu, select New.
3. On the left side of the window (under “Protocol Settings”), there are 7 steps. We do not use steps 1 (“Describe Protocol”), 4 (“Enter Standards Info”), 5 (“Enter Controls Info”), or 6 (“Enter Sample Info”), so they can be skipped.
4. Select step 2 (“Select Analytes”). In the Panel pull down menu, select the previously defined panel. First-time users will have to add a new panel (see Note 6).
5. In the Select Analytes window, select “Add All” to copy all regions from the “Available” section to the “Selected” section.
6. Select step 3 (“Format Plate”). Indicate which wells of the plate contain sample. Also, enter all samples as “Unknowns”.
7. Select step 7 (“Run Protocol”). Follow the on-screen instructions to begin acquisition.

Note 6 To enter a new Panel in the Bio-Plex software:

1. On the left side of the screen (under “Protocol Settings”), there are 7 steps. Select step 2 (“Select Analytes”).
2. Select “Add Panel”. In the Add Panel window, enter the name of the new panel (e.g., “Pn Serotyping).

3. Select “Add Analyte”. In the Add Analyte window, enter the regions and names below (enter one region and one analyte name, select “Add Continue”, enter the next region and name, select “Add Continue”, etc. After the last region and analyte have been added, select “Add”). In the Add Panel window, select “OK”. Make sure the regions and names are in the right order.

Note 7

Instead of washing the beads with filter plate, one can wash with 96-well V bottom plate (Fisher #12-565-216, NNI No.:249570) and centrifugation (Sorvall RT-6000). To wash, add 130 ul of Wash Buffer to each well and centrifuge the plate at 1,600 rpm for 5 min at RT. Then, carefully remove the buffer by aspiration and re-suspend the bead in a buffer.

References

1. Lin J, et al. Validation of a Multiplex Pneumococcal Serotyping Assay with Clinical Samples. J Clin Microbiol. 2006 February; 44(2): 383–388
2. Yu J, et al. Rapid multiplex assay for serotyping pneumococci with monoclonal and polyclonal antibodies. J Clin Microbiol. 2005 Jan;43(1):156-62
3. Park MK, et al. A latex bead-based flow cytometric immunoassay capable of simultaneous typing of multiple pneumococcal serotypes (Multibead assay). Clin Diagn Lab Immunol. 2000 May;7(3):486-9
4. Yu et al. Development of an automated and multiplexed serotyping assay for *Streptococcus pneumoniae* Clin. Vacc. Immunol. 18:1900, 2011.

Part C) Multibead Pneumococcal Serotyping assay using *wzy* PCR.

(1/24/2012)

Assay Summary

The assay is designed to detect pneumococcal serotypes by identifying *wzy* gene. *wzy* from a pneumococcal lysate is PCR amplified with a mixture of PCR primers and the resulting PCR product is identified by hybridizing it to luminex beads which are conjugated with a serotype specific probe. One sample is tested for about 30 different serotypes with Primer Pool Reaction B and Stock Bead mixture Reaction B. It is tested for the remaining serotypes with Primer Pool Reaction C and Stock Bead Mixture Reaction C. See table 2 for bead region and assay specificity. In combination with mAb-based multiplex assay, one can test a pneumococcal isolates for all known pneumococcal serotypes.

Warning! PCR is very easy to be contaminated. One should be very careful while performing the assay. The protocol is written for PCR reaction volume of 25 uL.

Materials/Equipment/Solutions

Description	Supplier	Catalog Number
Bio-Plex System	Bio-Rad Laboratories	Bio-Plex 200 Analyzer with Bio-Plex Manager V5.0 Software
Incubator, 48ºC	Fisher (isotemp, 500 series)	11-683-516D
Water Bath, 48ºC	Precision Scientific Co	66557
Vortex Mixer	Sientific Industries Inc	G-560

Microcentrifuge	Kendro	75005522
Plate Shaker (shaking radius ~1-2 mm – ***this is critical***)	Bellco Glass (or Equivalent)	7644-20115 (or Equivalent)
Sonicating Water Bath, room temperature	Bransonic	Model Branson 2200, 200
Thermocycler	Eppendorf	5331
PCR plate	Fisher	08-408-220
PCR tubes	Fisher	08-408-214
PCR Plate seals	Fisher	08-408-240
1.1 ml Microtubes in Rack (Sterile)	VWR	89092-274
Caps for 1.1 ml Microtubes (Sterile, 8 strip)	VWR	89092-276
ExTaq DNA polymerase (5U/uL, 250U/vial)	Takara	RR001
EZ load 100bp marker	Invitrogen	170-8352
Agarose	Fisher	BP1356-100
Primers	Invitrogen	
Bacterial lysates		
2X Primer Pool (Green Capped Tube), 990 uL/vial	UAB	(Reaction B and Reaction C) Store at -20°C
5-ml Test Tubes (12 x 75 mm, Falcon 352054)	Fisher	14-959-2A
1.1 ml Microtubes in Rack (Sterile)	VWR	89092-274
Caps for 1.1 ml Microtubes (Sterile, 8 strip)	VWR	89092-276
Millipore 96-Well Filter Plates	Thermo Fisher	MSBVN1210 (10 plates) or MSBVN1250 (50 plates)
0.22 Micron Bottle Top Filter	Fisher	SCGPT05RE
Millipore Vacuum Manifold	Thermo Fisher	MSVMHTS00
5M TMAC solution	Sigma	T3411
Sodium Azide	Sigma	S2002
Sodium Dodecyl Sulfate	Sigma	L4390
Sodium Chloride	Fisher	S271
Potassium Phosphate Monobasic	Sigma	P0662
Potassium Chloride	Fisher	P217
Sodium Phosphate Heptahydrate	Fisher	S373
Polysorbate 20 (Tween 20)	Sigma	P-5927
Trizma base	Sigma	T1503
0.5M EDTA, pH8.0	Gibco	4925
Bovine Serum Albumin (BSA)	Sigma	A7030
Stock Bead Mixture (Red Capped Tube) (0.4	UAB	**Stock Bead Mixture** Reaction B and Reaction C, Kept frozen.

ml/aliquot)		
PE-Streptavidin (0.5 mg/ml)	BD pharmingen	554061

Solutions

For all solutions, high purity water is used (for example, water from a Synergy 185 purification system from MilliPore).

1. **1.5X TMAC (**tetramethylammonium chloride) **buffer:**
   To make 250 ml of 1.5X TMAC buffer, mix reagent as below:

5M TMAC	225 ml,
20% SDS	1.88 ml,
1M Tris-HCl pH 8.0	18.75 ml,
0.5M EDTA pH 8.0	3.0 ml,
H_2O	1.37 ml

2. **1X TMAC buffer:** dilute 1.5X buffer with H_2O to make 1X TMAC buffer

3. **10X PBS**
   Add 800 ml of water to a 2-liter beaker placed on a magnetic stirrer. Weigh out the dry chemicals listed below and add them to the water. Dissolve the chemicals and bring the volume to 1000 ml with additional water. Sterilize the solution by filtering it with a 0.22 micron filter and store it in a sterile container at room temperature.

Dry chemical	Weight (grams)
NaCl	80.00
KH_2PO_4	3.14
$Na_2HPO_4 \cdot 7H_2O$	20.61
KCl	1.60
NaN_3	2.00

4. **1X PBS with 0.1% Tween 20**: Add 900 ml of water to 100 ml of 10X PBS. Add 1 ml of Tween-20. Store at RT for up to 3 months.
5. **PE-Streptavidin 100X Stock** (Yellow Capped Tubes) – we usually make this 100X stock by diluting the commercially supplied PE-streptavidin 1:50 in PBS pH 7.2.

Procedure I: Multiplexed PCR of Pneumococcal lysates *- Designed for a 96 well assay.*

1. Prepare Pneumococcal lysates as described for Multibead assay with mAbs. Dilute pneumococcal lysates 1:50 by adding 10 uL of lysate to 490 uL of H_2O in Microtubes in Rack. Cap the Microtubes in Rack and store the tubes at 4 ^oC for several days or -20 ^oC for long term storage.
2. Prepare PCR Master Mix as following.
   Obtain a 5 ml test tube.
   Thaw one aliquot of the 2X Primer Pool (Each aliquot has 990 uL)
   Transfer the entire content to the 5 ml test tube.
   Add 990 uL of water.
   Add 20 uL of ExTaq DNA polymerase to the tube.
   Cap the tube and vortex the mixture briefly.

3. Add 20 uL of the PCR Master Mix into each well of the PCR plate.
4. Add 5 uL of the 1:50 diluted pneumococcal lysate to each well of the PCR plate and mix the reagents by gently pipetting up and down. In one tube, add 5 uL of H_2O instead of lysate as a control.

5. Put PCR plate into the thermal cycler. Perform PCR. The PCR condition is: 94°C for 15 min followed by 35 amplification cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. After extending for 10 min at 72°C, keep at 4°C.
6. *(optional) Check the PCR product by electrophoresis using 2% agarose gel (80 volts, 30 minutes). Visualize bands with Ethidium bromide and UV light.*

Procedure II: Analysis of PCR products with Bioplex (Luminex)

- Designed for one 96 well plate.

1. Put 5 ml of 1.5X TMAC buffer and 50 ml of 1XTMAC buffer in 48 °C water bath to warm up. Prepare a rack with minitubes. Fill the minitubes with 380 ul of water.
2. Add 100 µL of water to the PCR plates already containing the PCR products (25 uL). Mix the samples carefully by pipetting up and down.
3. Transfer 20 µL of the diluted PCR product (Step 2) to 380 µL of H_2O in Minitubes in Rack prepared in step 1. Mix the samples carefully by pipetting up and down. The combination of Steps 2 and 3 gives 1:100-diluted PCR product.
4. Get a new PCR plate and add samples as shown in Table 1. To well A1, add 20 uL of H_2O. To well B1, add 20 uL of 1:100 diluted PCR product of the H_2O control. Beginning with well C1, add 20 µL of 1:100-diluted PCR products to each well. Seal the plate with Plate Seals.

Table 1: Summary of contents in each well

Well ID	Control Name	In step 4, add	In step 11, add
A1	Control 1	H_2O	1X TMAC buffer
B1	Control 2	Diluted PCR H_2O control	PE-Streptavidin
C1 *etc.*	Samples	Diluted sample	PE-Streptavidin

5. Place the PCR plate in a thermal cycler at 95 °C for 10 min (to denature the DNA).
6. While the PCR product is being heated, prepare the Working Beads as following:
    - Get one aliquot of Stock Bead Mixture (0.4 mL/aliquot) and thaw at RT.
    - Add 400 uL of 1.0X TMAC buffer (at 48°C) to the Stock Bead Mixture tube.
    - Centrifuge the tube at room temperature at 13,000 rpm for 1 minute.
    - Remove supernatant using vacuum to obtain bead pellet.
    - Add 1 ml of 1.5X TMAC at 48°C to the pellet (to resuspend the beads).
    - Transfer the entire content to a 5 ml test tube.
    - Add 3 ml of 1.5X TMAC at 48°C to make final volume 4 ml.
    - Place the 5 ml tube with beads in a sonicating water bath (RT) for about 20 seconds.
7. Remove the Plate Seal from the PCR plate on the thermal cycler (set at 95 °C), and add 40 µL of Working Beads to each well.
8. Reseal the PCR plate and place the plate at 48 °C incubator for 30 min (for hybridization).
9. Remove the Plate Seal and add 135 µL of 1X TMAC (48 °C) to each well.
10. Transfer the whole content to a filter plate by removing 195 ul from each well of the PCR plate and placing the fluid in the filter plate.
11. Wash the beads two times using vacuum filtration. (See Note 2 for washing details.)
12. Dilute PE-Streptavidin by adding 50 ul of 100X PE-Streptavidin Stock to 4.95 ml of 1X TMAC at 48 °C. (Use 15 ml Tissue Culture Tube.)
13. Add 50 µL of the diluted PE-Streptavidin to each well except A1 (see Table 1).
14. Place the Filter Plate in the Bellco plate shaker (set at 700 rpm) for 30 sec. at RT to suspend beads.
15. Place the Filter plate in 48 °C incubator for 20 minutes (without shaking).
16. Remove the Filter plate from the incubator and remove the fluid by vacuum.
17. Add 135 µL of 1X TMAC (48 °C) to each well to wash the bead. Remove the fluid by vacuum.

18. Add 135 uL of PBS-0.1% Tween 20 to each well at RT. Gently resuspend the beads in the plate. Remove the fluid by vacuum.
19. Resuspend each sample in 80 µL of PBS-0.1% Tween 20 at RT.
20. Analyze the samples with Bioplex. (Note 1)

Assay Notes

Note 1: *cpsA* PCR is generally negative for serotypes 38, 25A and 25F when cpsA1 primer set is used. Consequently cpsA2 primer set was developed to detect these serotypes and is included in Reaction C.

Note 2: Follow the steps below to wash filter plates with vacuum filtration:

1. Turn the vacuum OFF, and place the plate onto the vacuum manifold.
2. Turn the vacuum OFF, and add 150 microliters of Wash Buffer.
3. Turn the vacuum ON, and allow all liquid to be aspirated.

Repeat steps 2 and 3 for the indicated number of cycles.
When adding reagents to the plate, the vacuum must be OFF. If the vacuum is on, the wells that receive reagent first may be dry by the time the reagent is added to the last wells.

Table 2: Bead Regions and Serotype Specificity

Bead Region	Analyte specificity (Reaction B)	Bead Region	Analyte specificity (Reaction C)
101	LytA	122	9N/9L
102	cpsA1	123	28A/28F
103	7B/7C/40	124	10C/10F
104	21	125	11B/11C
105	33A/33F/37	126	13
106	15B/15C	127	24F/24A/24B
107	16A	128	12A/B/F, 44, 46
108	16F	129	19B/19C
109	18A/18B/18C/18F	130	27
110	23A	131	32F/32A
111	23B	132	15A/15F
112	25F/25A/38	133	33B/33D
113	10A/10B	134	31
114	43	135	41A/41F
115	36	136	29
116	48	137	45
117	34	138	47A
118	35A/35C/42	139	33C
119	35F/47F	141	NCC2
120	35B	142	NCC2 & 3
140	6C/6D	143	NCC1
		144	cpsA2

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