

Training manual for Enzyme linked immunosorbent assay for the quantitation of *Streptococcus pneumoniae* serotype specific IgG (Pn PS ELISA).

(89SF Version)

A guide to procedures for qualification of materials and analysis of assay performance.

This manual is now OBSOLETE. Click [here](#) for the current manual (utilizing 007sp as a standard).



Prepared by the World Health Organization Pneumococcal Serology Reference Laboratories at the Institute of Child Health, University College London, London, England and the Department of Pathology at the University of Alabama at Birmingham, Birmingham Alabama, USA.

Index

Introduction	3
SOP 1: Good laboratory practice guidelines	4
SOP 2: Standard enzyme-linked immunosorbent assay for quantitation of human IgG antibodies specific for <i>Streptococcus pneumoniae</i> capsular polysaccharides by enzyme linked immunosorbant assay (Pn PS ELISA)	5
SOP 2A: Adsorption of type-specific capsular polysaccharide antigen to microtiter plates	9
SOP 2B: Procedure for testing human sera with unknown anti-Pn PS antibody concentrations	9
SOP 3: Procedures for selecting ELISA plates	13
SOP 3A: Determine the optimal Pn PS antigen coating concentration for a lot of ELISA plates	
SOP 3B: Determine the variability of results between different lots of ELISA plates	15
SOP 3C: Side by side comparison of the antibody concentrations and lower limits of antibody detection between old and new lots of ELISA plates	17
SOP 4: Procedures for selecting a new lot of Pn PS antigen	18
SOP 4A: Determine the optimal antigen coating concentration for a new lot of Pn PS antigen	18
SOP 4B: Determine the variability of antigen coating between old and new lots of Pn PS antigen	20
SOP 4C: Side by side comparison of the lower limits of antibody detection between old and new lots of Pn PS antigen	21
SOP 5: Selection of a new lot of enzyme-labeled secondary antibody specific for all human IgG subclasses	23
SOP 6: Determine the optimal dilution for enzyme-labeled secondary antibody	26

Introduction

To develop and evaluate the efficacy of pneumococcal vaccines it is important to have an accurate method for measuring the concentration of human antibodies directed against pneumococcal capsular polysaccharides (Pn PS). In 2000, representatives from academia, government, and industry met at the WHO in Geneva, Switzerland, and selected an enzyme-linked immunosorbent assay protocol for quantitation of human IgG antibodies specific for *Streptococcus pneumoniae* capsular polysaccharides (Pn PS ELISA). The protocol was selected to guide assay development within individual laboratories.

The selected protocol is as follows. Human serum samples are mixed before analysis with an absorbent containing C-polysaccharide (C-PS) and 22F capsular PS to neutralize antibody binding to C-PS and other common contaminants present in the Pn PS coating antigens. 22F PS is used because it is a rare serotype, not present in any conjugate vaccine, and is thought to contain contaminating non-Pn PS epitopes. ELISA plates are coated with Pn PS by adsorbing individual Pn PS serotype antigens to micro-titer plates. Dilutions of absorbed human sera are then added to the ELISA plates. The serotype specific antibody bound to the ELISA plate is detected with anti-human IgG antibody conjugated with alkaline phosphatase, followed by addition of the substrate, *p*-nitrophenyl phosphate. The optical density of each well is measured at 405 nm and 690 nm (reference) using an ELISA plate reader. By comparing the optical density of the sample wells to that of the standard (human anti-pneumococcal reference serum, lot 89-SF) the level of antibody in the human serum can be calculated.

To facilitate the development of Pn PS ELISA with uniform assay performance throughout the world, we have prepared this training document describing the selected assay in detail and have explained how the assay can be set up in a laboratory. General good laboratory practices are described as SOP 1. In addition, this training document provides a set of standard operating procedures (SOP's) that describe the procedures for the basic Pn PS ELISA assay (SOP 2), selecting the lot of ELISA plates (SOP 3), determining the optimal antigen coating concentration (SOP 4), selecting the enzyme conjugated polyclonal anti-human antisera with proper specificity, sensitivity and high enzyme activity (SOP 5) and determining the titer of the enzyme conjugated polyclonal anti-human antisera (SOP 6). This document and additional information can be found in a website (<http://www.vaccine.uab.edu>) and a review article (CM Wernette et al, Clinical and Diagnostic Laboratory Immunology 10(4): pages 514-519).

This manual was prepared with the financial support of the Vaccines, Immunization and Biologicals Department of WHO in Geneva Switzerland; by Dr. David Goldblatt and Ms. Lindsay Ashton of University College, London, England; and Drs. Moon H. Nahm and William H. Benjamin at the University of Alabama at Birmingham, Birmingham, Alabama, USA with technical assistance from Wyeth Lederle Vaccines, Rochester, NY, USA. We are indebted to Mr. Keith Friedman, Dr. Dan Sikkema and Dr. Dace Madore of Wyeth Lederle Vaccines, Rochester, NY and Dr. Luis Jodar and Dr. Elwyn Griffiths at WHO, Geneva.

Comments and queries should be addressed to:

Dr. Moon H. Nahm
WHO Pneumococcal Serology
Reference Laboratory
Department Of Pathology
University Of Alabama
BBRB 614,
845, 19th St. South
Birmingham, AL 35294

Telephone: 205-934-0163
Fax: 205-975-2149
Email: nahm@uab.edu

Dr. David Goldblatt
WHO Pneumococcal Serology
Reference Laboratory
Immunobiology Unit
Institute of Child Health
30 Guilford St London
WCIN 1EH
United Kingdom

Telephone: +44(0)207 813 8491
Fax: +44(0)207 813 8494
Email: d.goldblatt@ich.ucl.ac.uk

SOP 1: Good Laboratory Practice Guidelines

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of Revision: July 31, 2002; November 26, 2002; June 9, 2004
Referenced SOP: SOP 1

Safety first: All serum samples are potentially infectious and all chemicals are potentially toxic. Wear gloves, protective clothing, and/or eye protection when handling human sera or chemicals. Prepare only the minimum amounts necessary and discard any remaining solutions or samples properly. Any potentially contaminated materials should be disinfected and discarded properly. Disinfect laboratory benches after work.

Water: Use a water purification system such as a Synergy 185 from Millipore (Bedford, MA). This water is defined as reagent grade water type 1. Avoid prolonged storage since microorganisms can grow and contaminate the water. In some critical cases (e.g., preparation of antigen coating solution), use commercially available, bottled, pyrogen-free water (for example, sterile water for irrigation distributed by Abbott Laboratories, North Chicago, IL).

Equipment: Equipment must be in good working order (e.g., pH meter, single and multi-channel micropipettors).

Supplies: Store supplies properly. ELISA plates should not be stored in areas exposed to heat, direct sunlight or excessive humidity. Also, minimize dust, particulates or fingerprints on plates. Solution storage containers must be free of contaminants as residual amounts of detergent on glassware can inhibit the binding of antibody to antigen or could strip adsorbed polysaccharides from the microtiter plates. All glassware/plastic ware used for preparing buffers/solutions must be depyrogenated by heating in an oven at 180°C for a minimum of 2 hours to remove endotoxin. The decontaminated glassware/plastic ware must be stored aseptically. For critical applications, use brand new magnetic stir bars, glassware or plastic ware.

Labels: All containers for solutions prepared in-house should be labeled with reagent name, date prepared, name of technician, and expiration date. Discard all solutions after the expiration date. When necessary, the preparation date can be used as the lot number.

Contamination: Before use, check buffers/solutions for signs of contamination, which may include flocculence or cloudiness. Discard the solution if there are signs of contamination. Also, all reagents and antigen-coated plates should be equilibrated to room temperature prior to use to reduce variability in daily assay performance.

Temperature and humidity: Conditions in the laboratory can affect day-to-day performance of ELISA methods. Very low humidity (often found in winter) can cause evaporation of components during incubation steps, which can result in an increase in blank values or overestimation of the titer for unknown or control sera. Care should be taken to minimize evaporation of assay components during incubation steps.

SOP 2: Quantitation of human IgG antibodies specific for *Streptococcus pneumoniae* capsular polysaccharides by enzyme linked immunosorbent assay (Pn PS ELISA)

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of revision dates: June 30, 2002; November 26, 2002; June 9, 2004
Referenced SOP: SOP 1

Purpose

To quantitate human IgG antibody to *S. pneumoniae* capsular polysaccharide in human serum using a standardized ELISA assay.

Principle of the assay

The ELISA measures type specific IgG anti-*S. pneumoniae* capsular polysaccharide (PS) antibodies present in human serum. When dilutions of human sera are added to type-specific capsular PS-coated microtiter plates, antibodies specific for that capsular PS bind to the microtiter plates. The antibodies bound to the plates are detected using a goat anti-human IgG alkaline phosphatase-labeled antibody followed by a *p*-nitrophenyl phosphate substrate. The optical density of the colored end product is proportional to the amount of anti-capsular PS antibody present in the serum.

Materials

- Microtiter plates for ELISA: 96 well, flat bottom, polystyrene, medium binding plate (Greiner 655001/Costar 9017, or equivalent)
- Plate lids (Greiner 656161/Costar 3931, or equivalent)
- Serum dilution plates: Deep well, large capacity microtiter plates, 96 well conical bottom (BD Falcon 353966, or equivalent) or cluster tube strips (Costar 4408, or equivalent)
- Disposable polypropylene containers: 50 ml, 110 ml (Falcon 354014, or equivalent)
- Tissue culture media bottles with screw cap and pouring ring: 250 ml, 500 ml, 1000 ml (Pyrex or Duran, brands that can be heated to 200°C, or equivalent)
- Plastic beakers: 1000 ml, 2000 ml
- Weighing boats
- Pipette tips for pipettors
- Sterile 0.2 µm filter units (Millipore, SCGPT05RE, or equivalent)
- Sterile disposable serological pipettes: 10 ml, 20 ml, 50 ml
- Freezer pen (Sanford Sharpie, extra fine point permanent markers or equivalent)
- Graduated cylinders: 100 ml, 250 ml, 1000 ml, 2000 ml
- Micropipettors : Gilson P20, P200, P1000 or equivalent
- Multichannel pipettors (12 channel): 50 µl-200 µl, 50 µl-300 µl
- Microtiter 12 well washing device (Nunc ImmunoWash 12 or Automated ELISA microtiter plate washer or equivalent). (Note 1)
- Mixer (Tube Rotator, Scientific Equipment Products, Cat. No: 60448 or equivalent)
- Magnetic stirrer (Corning, PC- 353, or equivalent)
- Bench top vortex mixer (Scientific products, S-8220, or equivalent)
- pH meter (Tris-compatible) (Orion Research, model- 601A/analyzer, or equivalent)
- Analytical balances (Ohaus, Galaxy-400 and Galaxy-110 or equivalent)
- ELISA microtiter plate reader with 405 nm and 690 nm filters
- Cryovials, 1.5 ml (Sarstedt, 72.694.005; Corning, 430489, or equivalent)
- 37°C incubator
- 2°C to 8°C refrigerator for storage of sera and reagents
- -70°C freezer for storage of sera and reagents
- 180 °C oven

Reagents and Chemicals

- Pneumococcal capsular polysaccharide (Pn PS) – American Type Culture Collection (ATCC)

Detailed instructions on re-suspending lyophilized materials

Remove the vial of lyophilized material from the freezer and allow it to come to room temperature (approximately one hour). Remove the septum and take care not to let any lyophilized material escape. Reconstitute the lyophilized antigen to 1 mg/ml by adding the necessary volume of sterile reagent grade water (type 1) to the vial and replace the septum. Gently shake the vial to moisten any lyophilized material along the top of the vial and place the vial in a mixer (rotator) at 4°C overnight. The mixer turns the vials slowly and helps the polysaccharide dissolve. Aliquot the polysaccharide into 1.5 ml cryovials, label with reagent name, date, lot number, initials and store at -70°C.

Ordering information for capsular Pn PS from ATCC			
Danish Pn PS Serotype	Catalogue #	American Serotype #	Order size
1	164-X	Type 1	200 mg
2	168-X	Type 2	200 mg
3	172-X	Type 3	200 mg
4	176-X	Type 4	200 mg
5	180-X	Type 5	200 mg
6B	228-X	Type 26	200 mg
7F	240-X	Type 51	200 mg
8	188-X	Type 8	200 mg
9V	256-X	Type 68	200 mg
9N	192-X	Type 9	200 mg
10A	232-X	Type 34	200 mg
11A	236-X	Type 43	200 mg
12F	196-X	Type 12	200 mg
14	200-X	Type 14	200 mg
15B	244-X	Type 54	200 mg
17F	204-X	Type 17	200 mg
18C	248-X	Type 56	200 mg
19A	252-X	Type 57	200 mg
19F	208-X	Type 19	200 mg
20	212-X	Type 20	200 mg
22F	216-X	Type 22	200 mg
23F	220-X	Type-23	200 mg
33F	260-X	Type 70	200 mg

Note: A typical 9 valent conjugate vaccine may have capsular PS of serotypes 1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F.

- Pneumococcal cell wall polysaccharide (C-PS, Statens Serum Institut, 3459; Distributed in the US by MiraVista Diagnostics, Indianapolis, IN). Reconstitute lyophilized antigen to 1 mg/ml with sterile reagent grade water (type 1) and store in aliquots at -70°C as described above for capsular polysaccharide.
- Sterile reagent grade water (type 1) (See Note 1)
- Alkaline phosphatase-conjugated goat anti-human IgG (binding all 4 IgG subclasses, Southern Biotech, Birmingham, AL, catalog number 2040-04, or equivalent), store at 4°C.
- US Reference Pneumococcal antiserum (89-SF). To obtain this, contact Dr. Mustafa Akkoyunlu at US FDA (Mustafa.Akkoyunlu@fda.hhs.gov).
- Quality control (QC) sera: (QC sera, which are often referred to as “in house control sera”, are human sera with known ranges of acceptable results. Store in aliquots at -70°C. (Note 2)
- Diethanolamine (Fisher D45-500)
- *p*-nitrophenyl phosphate powder (Sigma 104-0)
- Brij-35 solution (30% w/v, Polyoxyethylene 23 lauryl ether, Sigma, 430AG-6)
- Disodium hydrogen phosphate, hydrate, (Na₂HPO₄·7H₂O; Sigma S-9390 or Fisher S373)
- Sodium azide (NaN₃; Sigma, S-2002)
- Sodium chloride (NaCl; BDH,10241AP or Sigma, S-9625)

- Potassium phosphate, monobasic, (KH₂PO₄; BDH 102034B or Sigma P-0662)
- Potassium chloride (KCl; BDH 101984L or Fisher P-217)
- Tween-20 (Polyoxyethylenesorbitan monolaurate; Sigma P-5927)
- Magnesium chloride, hydrate, (MgCl₂.6H₂O; Sigma M-9272 or Fisher BP-214-500)
- Concentrated hydrochloric acid (HCl, Fisher A-144-500)
- Sodium hydroxide (NaOH; BDH 102524X or Sigma S-0899)
- Trizma HCl (Sigma T-3253)
- Trizma Base (Sigma T-1503)

Solutions

Instructions herein are for commonly used volumes as indicated in square brackets. The solution volume can be adjusted depending on specific needs. Use only reagent grade water (type 1) or equivalent. **DO NOT USE OTHER TYPES OF WATER** (Note 1). Use disposable plastic ware for all containers or dedicate glassware to use with these solutions. Never use glassware that has been washed with detergent. See Note 9 for more information on containers. Use sterile technique whenever possible to prolong the shelf life of the solutions.

Substrate stop solution: 3M NaOH [1 liter]

Preparation of this solution generates extreme heat and can be dangerous. Detailed steps are provided here.

Place 800 ml of water and a magnetic stir bar in a new 2000 ml plastic beaker. Place the beaker on top of a magnetic stirrer. Weigh 120 gm of NaOH pellets and slowly add NaOH pellets. If the water becomes too hot, stop adding the NaOH pellets and wait for it to cool. After adding all the NaOH pellets, mix the solution thoroughly, let it cool and bring the solution to 1 liter with water using a 1000 ml graduated cylinder. Mix well and store in a bottle at room temperature for up to 12 months.

6M HCl [200 ml]

Place 100 ml of water in a beaker in a fume hood. Add 100 ml of concentrated HCl slowly with stirring. Extreme care should be taken when handling concentrated HCl.

Coating Buffer: 10 X PBS /0.2% NaN₃ [1L] (Note 3)

Dry chemical	Weight (grams)
NaCl	80.00
KH ₂ PO ₄	3.14
Na ₂ HPO ₄ ·7H ₂ O	20.61
KCl	1.60
NaN ₃	2.00

Add 800 ml of **Abbott Laboratories** water to a 2-liter beaker placed on a magnetic stirrer. Weigh out the dry chemicals listed above and add them to the water. Dissolve the chemicals and bring the volume to 1000 ml with **Abbott Laboratories** water. Do not adjust pH (see instructions for preparing 1X coating buffer below). Sterilize the solution by filtering it with a 0.22 µm filter and store it in a sterile container at room temperature.

Coating Buffer: 1X PBS / 0.02% NaN₃ [1L]

Add 900 ml of sterile **Abbott Laboratories** water to 100 ml 10X PBS/0.2% NaN₃ prepared above. Check the pH of a small aliquot (do not insert the pH probe into the entire solution to avoid possible contamination). The pH should be 7.2 ± 0.2. If the pH is not within this range, discard the solution, and prepare new 10X buffer.

Antibody Buffer: 10 X PBS /0.2% NaN₃/0.5% Tween-20 [1L] (Note 3)

Dry chemical	Weight (grams)
NaCl	80.00
KH ₂ PO ₄	3.14
Na ₂ HPO ₄ ·7H ₂ O	20.61
KCl	1.60
NaN ₃	2.00

Add 800 ml of reagent grade water type 1 to a 2-liter beaker placed on a magnetic stirrer. Weigh out the dry chemicals listed above and add them to the water. Dissolve the chemicals and bring the volume to 1000 ml with reagent grade water type 1. Add 5 ml Tween-20. Do not adjust pH (see instructions for preparing 1X antibody buffer below).

Antibody Buffer: 1X PBS /0.02% NaN₃/0.05% Tween-20 [1L]

Add 900 ml of reagent grade water type 1 to a 1000 ml graduated cylinder and add 100 ml 10X PBS/0.2% NaN₃/0.5% Tween-20. Check the pH of a small aliquot (do not insert the pH probe into the entire solution to avoid possible contamination). The pH should be 7.2 ± 0.2. If the pH is not within this range, discard the solution, and prepare new 10X buffer.

10X TBS/1% Brij solution [1 liter]

Dry chemical	Weight (grams)
NaCl	80
KCl	1.60
Trizma Base	0.94
Trizma HCl	14.56

Weigh out dry chemicals and dissolve in approximately 800 ml of reagent grade water (type 1) in the appropriate size container. Mix the solution thoroughly using a magnetic stir plate and a stir bar. Add 33 ml of Brij-35 (30% w/v) and mix thoroughly. Bring the solution to 1 liter with reagent grade water (type 1). Store at room temperature for up to 12 months.

Wash buffer: 1X TBS /0.1% Brij solution

Mix one part of 10X TBS/1.0% Brij-35 with 9 parts of Reagent grade water type 1 in an appropriate size container. The pH should be 7.2 ± 0.2.

Substrate Buffer: 1M diethanolamine, 0.5 mM MgCl₂ [1 liter]

Calibrate the pH meter before making this solution. Details for preparing the solution are given below because the pH of the substrate buffer is very important.

1. Place a 2000 ml plastic beaker with a magnetic stir bar on a magnetic stirrer in a fume hood. (Use a beaker with 1 liter calibration marker.)
2. Add about 800 ml of water.
3. Add 97 ml of diethanolamine to the water using a 100 ml graduated cylinder and mix the solution well with the magnetic stirrer.
4. Add 0.1 gm of MgCl₂·6H₂O to the solution.
5. While thoroughly mixing with a magnetic stirrer, adjust the pH to 9.8 ± 0.05 by slowly adding 6M HCl. (~60 ml.)
6. Allow the solution to cool to room temperature.
7. Check the pH again and adjust to pH 9.8 ± 0.05 with 6M HCl if necessary.
8. Bring the solution to 1 liter with water and a 1000 ml graduated cylinder.

9. Since pH is critical, recheck the pH. If the pH is not 9.8 ± 0.05 , discard the diethanolamine buffer.
10. This buffer may be stored sealed in a light protected container at room temperature for up to 6 months.

SOP 2A: Adsorption of type-specific capsular polysaccharide antigen to microtiter plates (“Coating” the plates with the antigen)

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of revision dates: June 30, 2002; November 26, 2002
Referenced SOP: SOP 2

1. Retrieve the appropriate frozen aliquot of capsular PS that was stored at -70°C at 1 mg/ml. Thaw the aliquot at room temperature.
2. Dilute the capsular PS to a predetermined concentration (generally 1-10 $\mu\text{g/ml}$) with sterile antigen-coating buffer (1X PBS/0.02% NaN_3). (See SOP 4 for additional information).
3. Pipette 100 μl of capsular PS antigen into each well of the microtiter plate(s) and cover with a lid.
4. Incubate the plates at 37°C for 5 hours in an appropriate tissue culture grade incubator. Plates should not be stacked more than 2 layers high, and no more than 18 plates per shelf (using a standard tissue-culture grade incubator of 5.7 cu ft). This precaution is taken in order to maintain the air circulation in the incubator, to allow the heat penetration to microtiter plates, and to warm the plates evenly. Stacking plates higher or overloading an incubator can lead to ‘edging’ or poor quality coating of pneumococcal polysaccharide antigens.
5. Store the plates in a humidified chamber kept at 4°C . If sodium azide is used, coated plates may be stored for several months at 4°C , provided that storage conditions are suitable to prevent evaporation and contamination of the coating solution.

SOP 2B: Procedure for testing human sera with unknown anti-Pn PS antibody concentrations

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of revision dates: June 30, 2002; November 26, 2002; June 9, 2004;
August 2010
Referenced SOP: SOP 2

This protocol assumes that each serum sample is analyzed for 9 Pn PS serotypes. If this is not the case, adapt this protocol for the particular situation.

1. Plan the sample layout.

The example of a plate layout (shown below) has 4 samples with unknowns (Unknowns 1-4), one QC, and one standard (89-SF). All samples are analyzed in duplicate (e.g. columns 1 and 2 for Unknown 1, etc). Place the least diluted samples in row A and the most diluted in row H. The reference serum, 89-SF, is used as the standard and is placed in rows A through G of columns 7 and 8. Wells H7 and H8 (marked Blank) are used as “blank wells”. Serial dilutions of a QC are placed in columns 11 and 12. See Note 2 for additional description of QC.

Typical microtiter plate layout for Pn PS ELISA

	Unknown 1		Unknown 2		Unknown 3		Standard 89-SF		Unknown 4		QC	
Serum dilutions	1	2	3	4	5	6	7	8	9	10	11	12
A 1:50												
B 1:125												
C ↓ etc.												
D												
E												
F												
G												
H							Blank	Blank				

2. Retrieve all serum samples, controls and reference sera stored frozen and place them at room temperature to thaw (~30 minutes). Samples should not be freeze/thawed more than 3 times and markings on the lid can be used to track the number of freeze/thaw cycles. After using the samples, mark the remainder on the lid with a freezer pen and immediately return them to the freezer.
3. Retrieve all the required antigen-coated plates from the refrigerator. Place them on the bench at room temperature. Prepare wash and antibody buffers.
4. Prepare the absorption solution by adding enough C-PS and 22F capsular PS to antibody buffer to make 5 µg/ml of C-PS and 5 µg/ml of 22F capsular PS. Typically, 55 ml of the absorption solution is needed for a 9-microtiter plate assay. Also one needs about 12 ml of the 89-SF absorption solution, which has only C-PS and no 22F capsular PS. (Note 11)
5. Mix the thawed samples, QC, and the standard (89-SF) thoroughly. Remove appropriate amounts of the samples (or QC) and mix them with the appropriate amounts of the absorption solution. Mix the appropriate amount of reference serum with the 89-SF absorption solution (Note 11). For instance, to prepare a 50-fold diluted serum sample for 9 serotypes, one may mix 20 µl of the sample with 980 µl of the absorption solution. It is convenient to dilute the samples in the “serum dilution plates”.
6. Perform serial dilutions (usually 2- or 3-fold; see Note 10) as necessary with absorption solution. A 2.5-fold serial dilution can be performed as follows. First, add 600 µl of absorption solution to 7 blank tubes in the “serum dilution plates”. Then, add 400 µl of the initial dilution sample (from step 5) to the first tube and mix the first tube well. Transfer 400 µl to the second tube and mix the second tube well. Repeat these steps for 7 tubes. Incubate the diluted samples at room temperature for 30 minutes.
7. Wash the antigen-coated microtiter plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells. (Note 4) (Note 5) (Note 6)
8. Using a multichannel pipettor, transfer 50 µl of each serum dilution from the dilution plates to the washed antigen coated plates. Add only antibody buffer to two wells (H7 and H8) in each plate to serve as blanks. Cover plates with lids and incubate at room temperature for 2 to 18 hours (see Note 12). For the longer incubations or in low humidity situations, use a humidified sealed box (with an anti-microbial agent), to prevent excessive evaporation.
9. Prepare the appropriate dilution of goat anti-human IgG conjugate in antibody buffer 15 minutes before its use (SOP 6).
10. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
11. Add 100 µl of diluted enzyme conjugate to all microtiter plate wells. Cover plates with lids and incubate for 2 hours at room temperature.
12. Prepare a 1 mg/ml solution of *p*-nitrophenyl phosphate in the diethanolamine substrate buffer 15 minutes before it is required. Mix the substrate solution on the shaker while wrapped in a paper towel to protect it from light.
13. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
14. Add 100 µl of substrate solution to all microtiter plate wells. Put lids on plates and incubate for 2 hours at room temperature.
15. Add 50 µl of 3M NaOH to all wells to stop the enzyme reaction.

16. Wait at least 5 minutes, before reading the optical density of the plates on a microtiter plate reader at 405 nm and 690 nm (Note 7).

Data analysis

1. Optical density data is converted to antibody concentrations with a computer program like Genesis, Softmax PRO, Revelations, or "ELISA". Acceptable results are obtained with computer programs using a standardized curve-fitting four parameter logistic method, a weighted log-logit method, or a logistic/log linear regression analysis. See Note 8 for obtaining the programs mentioned here.
2. The program calls for a "calculation template", which tells the calculation-program the location of samples, standards, QC, the initial dilutions and serial dilutions. Wells H7 and H8 should be labeled as "blank wells" in the template. The program should subtract the average OD of the two "blank wells" from the OD of other wells.
3. The anti-Pneumococcal capsular PS antibody concentration for each sample is found by calculating the mean of the serial dilutions of the sample. The following data inspection rules should be followed to ensure that the assay results are valid.

Data inspection rules

1. Blanks -The optical density of blank wells should be less than 0.1 OD units for all assay plates. In general, the optical density will be less than 0.05.
2. Duplicates - If the absorbance values of replicates at one dilution vary widely and have a coefficient of variation (CV) >15%, the concentrations obtained with the absorbance data at that dilution should be excluded from the calculation of the antibody concentration of the sample.
3. Standards - The slope of the linear portion of the reference standard curve (e.g., OD 0.1 to 2.0) should be very near 1.0 (0.9-1.1) when the log of the OD signal is graphed against the log of the standard concentration.
4. Sensitivity - Calculate the lower detection limit for the assay and confirm that the detection limit is within the established range.
5. Quality control sera - Control sample values must be within the established range (which is 3 SD), or the plate is rejected and samples are reanalyzed.
6. Rules for the samples with unknown concentrations:
 - a. If a sample has OD readings greater than 2.0 at the highest dilution used in the assay, the sample should be reassayed after additional dilution.
 - b. If the antibody concentration of the sample was calculated by averaging the data from multiple dilutions and the CV of the concentration exceeds 30%, then the data should be examined for inappropriate results (e.g., poor duplications, and/or non-linearity) and should be reassayed if no apparent causes are found.
 - c. If upon reassay, the %CV value is greater than 30% due to the fact that the titration slope is not parallel to the assay reference standard sera, the median predicted value should be reported. For additional information on data analysis see CM Wernette et al, Clinical and Diagnostic Laboratory Immunology 10(4): pages 514-519.
 - d. If the lower limit of detection is equal to or less than the established value and a sample has undetectable antibody concentration, report one half of the established assay lower limit as the concentration for the sample. If the lower limit of detection is more than the established value and a sample has undetectable antibody concentration, do not report the result for the sample and reanalyze the sample.

Assay notes

Note 1: Water is extremely important. We use fresh water from Synergy 185 water purification system from Millipore (Bedford, MA). If the water is stored for a prolonged period, microorganisms grow and contaminate the water. To prepare solutions used in coating the plates with Pn PS, we use pyrogen-free water from Abbott (North Chicago, IL, catalog# NDC 0074-7139-36).

Note 2: QC (Quality control) refers to in-house controls used to monitor inter-assay variation. Human serum pools are commonly used as QC after their antibody levels have been very well characterized. Some control sera should have high values and some should have low values. All QC samples should have known ranges of acceptable results. There should be one QC per plate and at least one QC with high values for every 3 assay runs. A set of serum samples with known concentrations (referred to as “calibration sera”) is available from Dr. David Goldblatt (consult <http://www.vaccine.uab.edu/refer/qc3.pdf> for additional information on the sera). The calibration sera are to validate a procedure and each laboratory will need to obtain their own standard sera for routine QC checks. These can be made from Red Cross plasma or from sera collected from subjects immunized with a vaccine. Usually screening 30 plasmas from expired units high and low titer sera can be found. If plasma is used, it needs to be converted to serum.

Note 3: To simplify making PBS buffers, there are PBS tablets, which contain pre-measured amounts of all the required chemicals. However, these PBS tablets often include stabilizers or other components, which can affect ELISA results. Instead of tablets, use of ACS grade chemicals in preparation of buffers improves assay performance.

Note 4: ELISA plates can be washed manually by repeatedly filling the wells with the buffer and removing the buffer. Alternatively, one can wash the plates with a 12-well washing device or a machine that washes 96 wells simultaneously. A popular model of 96-well washing machine is ELx 405 Microplate washer from Bio-Tek (Princeton, NJ).

Note 5: Follow the manufacturer’s recommendations to remove proteins from plate washers. Alternate use of HCl and NaOH, following with several rinses with deionized water. Use of alcohol for the cleaning or decontamination of the plate washers should only be considered once proteins have been removed from the system as alcohol can further cement the proteins to the hardware.

Note 6: Do not allow microtiter plates to dry between wash steps and reagent addition; they should not be left empty for more than 10 minutes.

Note 7: Signal for each well = $OD_{405} - OD_{690}$. The wavelength for the reference may vary from 600 to 690 nm.

Note 8: “ELISA” is a computer program developed by Mr. Brian Plikaytis at The Centers for Disease Control and Prevention in Atlanta, Georgia. To obtain this free program, obtain the necessary contact information from: <http://www.cdc.gov/ncidod/dbmd/bimb/elisa.htm>. Softmax PRO can be purchased from Molecular Devices Corp. (Sunnyvale CA).

Note 9: Glassware that is detoxified by heating at 180°C can be used. Glassware should never be contaminated with detergent, as this will affect binding of some of the Ps antigens more than others (type 14 is particularly sensitive).

Note 10: Some high titer sera will not be sufficiently diluted in 7 wells to use 2-fold dilutions, but by turning the plate the other way, 11 dilutions may allow sufficient dilution. Some low titer sera may not give enough usable points with 3-fold dilutions. Thus, 2.5-fold dilutions have been found to be the best except under special circumstances.

Note 11: The values for 89-SF were assigned with the C-PS absorption alone. Thus, it was decided that 89-SF will be absorbed with C-PS only whereas the samples and QC are absorbed with both C-PS and 22F PS. Also some use 10 µg/ml instead of 5 µg/ml.

Note 12: If measuring antibodies to serotype 3, this incubation should be done for only 2 hours. For the other serotypes tested (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F), incubation times of 2 to 18 hours are acceptable.

OBSOLETE

SOP 3: ELISA plate lot selection

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of Revision dates: July 31, 2002; November 26, 2002; June 9, 2004; June 12, 2006
Referenced SOP: SOP 1, SOP 2

To qualify a new lot of plates, establish that:

- 1) The optimal antigen coating concentration is within the acceptable limit by determining the optimal concentration for Pn PS serotypes (eg, 9 serotypes) using an antigen titration experiment.
- 2) The "plate CV" is within the acceptable limit by testing 3 or more plates from different locations in one box with Pn PS ELISA
- 3) The plates produce comparable results by performing a side-by-side comparison with a minimum of 10 control sera and at least 4 QC sera for all serotypes with old and new plates and show that the new plates yield similar values and lower limits of detection.

Tests designed to establish these aspects are described below as SOP3A, SOP 3B, and SOP 3C.

SOP 3A: Determine the optimal Pn PS antigen coating concentration for a lot of ELISA plates

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of Revision: July 31, 2002; November 26, 2002; June 12, 2006
Referenced SOP: SOP1, SOP 2

Purpose

To determine whether the optimal antigen-coating concentrations of the Pn PS for the new lot of ELISA plates are similar to those for the old lot.

Principles of the test

Antigen is adsorbed on to the surface of a microtiter plate in increasing concentrations. Reference serum is added at one dilution across the plate and the ELISA is completed using the type specific Pn PS ELISA (SOP 2). The optimal coating concentration of an antigen lot is determined by inspecting optical density values vs. antigen concentration. Try to achieve the maximum signal without increasing the OD of the blank wells. The antigen concentration should not be greater than 10 µg/ml.

Materials and reagents

Materials and reagents are outlined in type specific Pn PS ELISA (SOP 2).

Methods

1. Dilute the Pn PS to 20 µg/ml in coating buffer in polypropylene tubes. Add 200 µl to the wells in the first column (8 wells).

ANTIGEN CONCENTRATION (µg/ml)

	20	10	5	2.5	1.25	0.625	0.312	0.156	0.078	0.039	0.020	Buffer
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Rows A, B, C and D: optimum dilution of the reference serum (89-SF).

Rows E, F, G, and H: buffer (“background wells”)

Column 12 contains “blank wells”

- Fill the wells in columns 2-12 with 100 µl of the coating buffer. Perform 2-fold serial dilutions from column 1 to column 11 by repeatedly mixing and transferring 100 µl to the wells in the next column. After the 11th column, discard 100 µl from the 11th column (so that the wells in column 11 have only 100 µl). Leave column 12 undisturbed. Put a lid on each plate.
- Incubate the plate at 37°C for 5 hours and then keep the plates at 4°C for storage.
- Dilute the human anti-pneumococcal PS standard reference serum, 89-SF, in the antibody buffer so it is expected to give an OD of 1.0 under conditions explained in the Pn PS ELISA (SOP 2). The dilution is dependent on the serotype but is typically 1:1500.
- Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
- Add the diluted reference serum to all wells in rows A, B, C and D and the antibody buffer to all wells in rows E, F, G, and H. Incubate as specified in the Pn PS ELISA (SOP 2).
- Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
- Add goat anti-human alkaline phosphatase-labeled secondary antibody at the optimal dilution to each well and incubate, as specified in the Pn PS ELISA (SOP 2).
- Wash the plates 5 times with wash buffer. Allow the wash buffer to soak on the plate 30 seconds to 1 minute after the first filling.
- Add substrate, incubate, and read the optical density of the plate as specified in the type specific Pn PS ELISA (SOP 2).

Data analysis

- All blank wells must have optical density values lower than 0.1. If any blank wells are ≥ 0.1 , the assay must be repeated (Note 1). All background wells should have optical densities less than 0.1.
- Obtain average OD of rows A, B, C and D, and E, F, G, and H for each column.
- For each antigen concentration, obtain the “signal” by subtracting the average OD value of the background wells (antigen and 2° antibody) from the average OD value of the corresponding wells that contain the reference serum (89-SF).
- Plot the signal on the Y-axis and the logarithm of antigen concentration on the X-axis.
- Possible outcomes:
 - a sigmoid curve with a plateau occurring at high antigen concentrations,
 - a sigmoid curve where the plateau falls to base-line (X-axis, sometimes the signal decreases when the antigen coating concentration is too high).
 - an increasing semi-linear plot with no observable plateau or peak in the range of antigen concentrations.

6. For outcomes a) and b), pick the antigen concentration yielding the maximum signal because any higher concentrations of antigen will not yield a higher signal.
7. For outcome c), pick a coating concentration yielding the maximum possible signal with a low background.
8. The optimal coating concentration chosen in this manner provides maximum sensitivity, consistent ELISA performance, and acceptably low signals from blank wells (Note 2).

Assay notes

Note 1: If optical density values are repeatedly higher in the blank wells in the new lot of plates but not in the old lot of plates, there may be a problem with the new lot of microtiter plates.

Note 2: Antigen titration should yield similar optimal coating concentrations. If marked differences in optimal coating concentrations are found, a different lot of plates should be requested, and the testing procedure should be repeated.

SOP 3B: Determine the variability of results between different lots of ELISA plates

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
 History of Revision: July 31, 2002; November 26, 2002; June 9, 2004; June 12, 2006
 Referenced SOP: SOP 1, SOP 2

Purpose

To select a new lot of microtiter plates producing results with minimal well-to-well variation.

Principle of the assay

1. A single dilution of serum is added to 92 out of 96 wells of an antigen-coated plate with 4 wells acting as blank controls with buffer only. Mean absorbance values and their variation from the 92 wells are calculated between wells on a single plate and between each of the plates. Standard deviation and % CV are assessed where the inter-plate CV should be $\leq 20\%$ and the average intra-plate CV should be $\leq 10\%$ for acceptability. Although this procedure can be performed using only one Pn serotype, it is desirable to test all serotypes that will be used in the ELISA. If select Pn serotypes are used, choose ones that give consistent results with controls (e.g., serotype 1).

Materials and reagents

- 3 microtiter plates (for each serotype) from a new lot number from different positions in the box. (See Note 1)
- 3 microtiter plates (for each serotype) from a previously qualified lot number
- Materials and reagents listed in SOP 2

Methods

1. Obtain 3 plates of the qualified lot and 3 plates of a new lot.
2. Add 100 μ l of the Pn PS antigen to each of the 6 microtiter plates (SOP 4).
3. Prepare a dilution of reference serum 89-SF that will give an OD of approximately 1.0 within 2 hours of addition of substrate.
4. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
5. Add the serum dilution to all wells of the plates, excluding 4 blank wells where diluent alone is added. The blank wells are typically B2, B9, F4 and F11 (see template below).

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

- Incubate according to the type-specific Pn PS ELISA procedure (SOP 2).
- Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
- Add goat anti-human IgG alkaline phosphatase labeled secondary antibody at the optimal dilution (SOP 6) to each well and incubate as specified in the Pn PS ELISA procedure (SOP 2).
- Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
- Add substrate to each well and incubate until OD is approximately 1.0 (check OD frequently). After OD has reached approximately 1.0, stop the reaction, and read optical density in the ELISA plate reader as in SOP 2.

Data analysis

- Calculate the mean optical density of the 4 blank wells (i.e. buffer only wells) and subtract this from the optical density of all 92 other wells. Average optical densities for the test wells must be between 0.8 and 1.5 units.
- Calculate the intra-plate mean, standard deviation, and %CV of the absorbance for the 92 wells of all 6 plates to determine well-to-well variation. If the CV of each of the previously qualified plates is not within the specification for acceptance, the assay must be repeated.
- Calculate the inter-plate mean, standard deviation, and %CV of the absorbance for the three plates in each group. If the CV of the previously qualified plates is not within the specification for acceptance, the assay must be repeated.
- Evaluation criteria for intra-plate variation:*
 - OD values of blank wells must be consistently ≤ 0.1 (better if they are ≤ 0.05).
 - Intra-plate well-to-well CV must be $\leq 10\%$.
 - Trends should not be observed in a single plate (e.g., spotting, edge effects). Note any trends in locations of the wells with standard deviations greater than 20% from the intra-plate mean OD.
- Evaluation criteria for inter-plate variation:*
 - OD values of blank wells must be consistent and ≤ 0.1 (better if they are ≤ 0.05).
 - Inter-plate CV for the 3 new plates must be less than 20%.
 - No significant inter-plate trends should be observed from the intra-plate mean OD.

Assay notes

Note 1: Medium binding ELISA-grade microtiter plates should be used for the Pn PS ELISA (NUNC, Greiner, Costar, Dynatech etc.).

SOP 3C: Side by side comparison of antibody concentrations and lower limits of antibody detection between old and new lots of ELISA plates

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of Revision: July 31, 2002, November 26, 2002
Referenced SOP: SOP1, SOP 2

Purpose

To demonstrate that the new lot of ELISA plates produces comparable results and comparable lower limits of detection as the old lot of ELISA plates using real serum samples.

Principles of the assay

Perform the assay for anti-Pn PS antibody with a set of serum samples and compare the results.

Materials and reagents

- ELISA plates: new and old lots.
- Test serum samples: Use a minimum of 12 unknown samples (testing 24-32 samples is better) and at least 4 QC sera or any well-established samples. They should have antibody levels in all ranges (high and low).
- QC serum samples: Use different sera with high and low antibody levels for each of the 9 serotypes.
- All other materials and reagents as described in SOP 2.

Methods

1. Perform the Pn PS ELISA for all 9 serotypes with the 10 (or more) serum samples and also for QC serum samples on both the new and old ELISA plates as described in SOP 2.
2. Plot the concentrations obtained with the new plates (Y-axis) against those obtained with the old plates (X-axis). The axes should be in log scale. Obtain the best-fit line using the least squares method and log-transformed results. The slopes of the best-fit lines should be 1 ± 0.1 and the intercept must be <0.1 and the r^2 should be >0.95 .
3. Calculate the lower limits of detection for new and old ELISA plates. It should be <0.01 for both lots of plates.

Data analysis

1. Perform a separate linear regression analysis on the optical density values from the 7 dilutions of reference serum (89-SF) for both new and old ELISA plates.
2. Calculate the 'absolute detection limit' as determined by the antibody concentration present in the dilution of reference serum yielding two times the assay background.
3. Calculate the 'lower limit of detection' by multiplying the 'absolute detection limit' by the lowest dilution of serum tested in the assay (i.e., 1:50 for human serum).
4. The 'lower limit of detection' should be comparable ($\pm 20\%$) between old and new lots of ELISA plates. To be practical, more than 99% of the samples should be above the 'lower limit of detection'. This is about 0.01 $\mu\text{g/ml}$ in the case of the Pn PS ELISA.

SOP 4: Procedures for selecting a new lot of Pn PS antigen

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of Revision: July 31, 2002, November 26, 2002
Referenced SOP: SOP 1, SOP 2, SOP 3

When new lots of antigen are purchased from the ATCC, they must be tested as discussed earlier for new lots of ELISA plates. The tests are:

1. To determine the antigen titration curve with all 9 serotypes.
2. To determine uniformity of antigen coating by calculating %CV.
3. To determine lower limit of detection (LLQ), and to perform a side-by-side comparisons for acceptability of results using at least 10 serum samples representing the test population.

Methods for testing these aspects are described in SOP 4A, SOP4B, and SOP4C.

SOP 4A: Determine the optimal antigen coating concentration for a new lot of Pn PS antigen

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of Revision: July 31, 2002, November 26, 2002
Referenced SOP: SOP 1, SOP 2, SOP 3, SOP 4

Purpose

To determine the optimal antigen-coating concentration for a new Pn PS antigen lot.

Principle of the assay

Antigen is adsorbed on to the surface of a microtiter plate in increasing concentrations. Reference serum is added at one dilution across the plate and the ELISA is completed using the Pn PS ELISA (SOP 2). The optimal coating concentration of an antigen lot is determined by inspecting optical density values vs. antigen concentrations.

Materials and reagents

- Materials and reagents are outlined in type specific Pn PS ELISA (SOP 2).

Methods

1. Dilute the new Pn PS to 20 µg/ml in coating buffer in polypropylene tubes. Add 200 µl to the wells in the first column. If the old, established lot of antigen is available, wells A1, B1, C1, and D1 are filled with the old qualified antigen, and wells E1, F1, G1 and H1 are coated with the new antigen (see the plate layout figure below). If the old, qualified antigen is not available, all 8 wells are filled with the new antigen.
2. Fill the wells in columns 2-12 with 100 µl of the coating buffer. Perform 2-fold serial dilutions from column 1 to column 10 by repeatedly mixing and transferring 100 µl to the wells in the next column. After the 10th column, discard 100 µl from the 10th column (so that the wells in column 10 have only 100 µl). Leave columns 11 and 12 undisturbed. Put a lid on each plate.
3. Incubate the plate at 37°C for 5 hours and then keep the plates at 4 °C for storage.
4. Dilute the human anti-pneumococcal standard reference serum, 89-SF in the diluent buffer to give an OD of 1.0 under conditions explained in the Pn PS ELISA (SOP 2). The dilution is dependent on the serotype but is typically 1:1500.
5. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.

ANTIGEN CONCENTRATION (µg/ml)

	20	10	5	2.5	1.25	0.625	0.312	0.156	0.078	0.039	Buffer	
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Rows A, B, C, D: Old qualified capsular Ps antigen (if old antigen is not available, these rows are coated with new antigen)
 Rows E, F, G, H: New capsular Ps antigen
 Columns 11 and 12 are “blank wells”.

Rows A and B: optimum dilution of the reference serum (89-SF).
 Rows C and D: Buffer (“background wells”)
 Rows E and F: optimum dilution of the reference serum (89-SF).
 Rows G and H: Buffer (“background wells”)

- Add the diluted reference serum to all wells in rows A, B, E and F and the diluent buffer to all wells in rows C, D, G and H. Incubate as specified in the Pn PS ELISA (SOP 2).
- Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
- Add goat anti-human IgG alkaline phosphatase-labeled secondary antibody at the optimal dilution to each well and incubate, as specified in the Pn PS ELISA (SOP 2).
- Wash the plates 5 times with wash buffer. Allow the wash buffer to soak on the plate 30 seconds to 1 minute after the first filling.
- Add substrate, incubate, and read the absorbance of the plate as specified in the Pn PS ELISA (SOP 2).

Data analysis

- All blank wells must have OD values lower than 0.1. If blank wells are ≥ 0.1 the assay must be repeated (Note 1). (If new antigen-coated background wells have high OD, then the new antigen lot is not acceptable. Old antigen was tested and should give low OD.)
- Obtain average OD of rows A and B, C and D, E and F, and G and H for each column.
- For each antigen concentration, obtain the “signal” by subtracting the average OD value of the background wells (antigen and 2° antibody) from the average OD value of the corresponding wells that contain the reference serum (89-SF).
- Plot the signal on the Y-axis and the logarithm of antigen concentration on the X-axis.
- Possible outcomes:
 - a sigmoid curve with a plateau occurring at high antigen concentrations,
 - a sigmoid curve where the plateau falls to base-line (X-axis, sometimes the signal decreases when the antigen coating concentration is too high),
 - an increasing semi-linear plot with no observable plateau or peak in the range of antigens.
- For outcomes a) and b), pick the antigen concentration yielding the maximum signal because higher concentrations of antigen do not yield a higher signal.
- For outcome c), pick a coating concentration yielding the maximum possible signal with low background.
- The optimal coating concentration chosen in this manner provides maximum sensitivity, consistent ELISA performance, and acceptably low signals of blank wells (Note 2).

Assay notes

Note 1: If OD values are repeatedly higher in the blank wells, there may be a problem with the microtiter plate. However, this should not occur since the plates that were tested in the past are being used in this test.

Note 2: Antigen titration should yield similar (to the previous) optimal coating concentrations. If marked differences in optimal coating concentrations are found, the new lot of the antigen should be evaluated for differences in purity and for differences in composition with other analytical techniques (e.g., NMR, chemical analysis) or with serological analysis (e.g., competition ELISA using homologous and heterologous antigens).

SOP 4B: Determine the variability of antigen coating between old and new lots of Pn PS antigen

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of Revision: July 31, 2002, November 26, 2002
Referenced SOPs: SOP 1, SOP 2

Purpose

To determine whether the new lot of Pn PS antigen coats the ELISA plates uniformly.

Principle of the assay

A single dilution of serum is added to 92 out of 96 wells of an antigen-coated plate with 4 wells acting as blank controls with buffer only. Mean absorbance values and their variation from the 92 wells are calculated between wells on a single plate and between each plate. Standard deviation and % CV are assessed where an average CV should be equal to or less than 10% for acceptability.

Materials and reagents

- Pn PS of old and new lot numbers
- Materials and reagents listed in SOP 2

Methods

1. Coat three ELISA plates with the new antigen. Coat the other ELISA plate with the old antigen. (See SOP 4A for determining the optimal coating concentration.)
2. Prepare a dilution of reference serum 89-SF that will give an OD of approximately 1.0 with the particular Pn PS serotype.
3. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
4. Add the serum dilution to all wells of the plates, excluding 4 blank wells where diluent alone is added. The 4 blank wells are typically B2, B9, F4 and F11 (see template below).
5. Incubate according to the Pn PS ELISA procedure (SOP 2).
6. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
7. Add goat anti-human IgG alkaline phosphatase labeled secondary antibody at the optimal dilution (SOP 6) to each well and incubate as specified in the ELISA procedure (SOP 2).
8. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
9. Add substrate to each well and incubate, stop the reaction, and read absorbance in the ELISA plate reader as in SOP 2.

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

Data analysis

1. Find the mean optical density of the 4 blank wells (i.e., buffer only wells) and subtract this from the optical density values of all 92 other wells. The average optical density for the test wells must be between 0.8 and 1.5 units.
2. Calculate the intra-plate mean, standard deviation, and %CV for the 92 wells to determine well-to-well variation. If the CV of the previously qualified Pn PS lot is not within the specification for acceptance, the assay must be repeated.
3. *Evaluation criteria for intra-plate variation:*
 OD values of blank wells must be “consistent” and ≤ 0.1 (preferably < 0.05).
 Intra-plate well-to-well CV must be $\leq 10\%$. Trends should not be observed in a single plate (e.g., spotting, edge effect). Note any trends in locations of the wells with deviations greater than 20% from the intra-plate mean optical density.

SOP 4C: Side by side comparison of the antibody concentrations and the lower limits of antibody detection (LLQ) between old and new lots of Pn PS antigen

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of Revision: July 31, 2002, November 26, 2002
Referenced SOPs: SOP 1, SOP 2

Purpose

To demonstrate that the new lot of Pn PS produces comparable results and comparable lower limits of detection as the old lot of Pn PS using real serum samples.

Principle of the assay

Perform the assay for anti-Pn PS antibody with a set of serum samples using new and old lots of Pn PS antigens and compare the results.

Materials and reagents

- Pn PS: new and old lots.
- Test serum samples: Use at least 10 QC samples or any well established samples (testing 24-32 samples is better). They should have antibody levels in all ranges (high and low).

- All other materials and reagents are described in SOP 2.

Methods

1. Analyze at least 10 samples with new and old Pn PS for anti-Pn PS IgG antibody as described in SOP 2.
2. Plot the results with new and old Pn PS. Plot the concentrations with the new lot (Y-axis) against those with old lot (X-axis). The axes should be in log scale. Obtain the best-fit line using the least squares method. The slope of the best-fit line should be 1 ± 0.1 . The scatter among the data points should be within 3-fold.
3. Calculate the lower limits of detection for new and old lots of Pn PS.

Data analysis

1. Perform a separate linear regression analysis on the OD values from the 7 dilutions of reference serum (89-SF) for both new and old Pn PS lots.
2. Calculate the 'absolute detection limit' as determined by the units of antibodies present in the dilution of reference serum yielding two times the assay background.
3. Calculate the 'lower limit of detection' by multiplying the 'absolute detection limit' by the lowest dilution of serum tested in the assay (i.e., 1:50 for human serum). The 'lower limit of detection' should be comparable ($\pm 20\%$) between old and new lots of PnPS. To be practical, more than 99% of the samples should be above the 'lower limit of detection'. This is about $0.01 \mu\text{g/ml}$ in the case of the anti-Pn PS assay.

SOP 5: Selection of a new lot of enzyme-labeled secondary antibody specific for all human IgG subclasses.

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
Effective date: June 30, 2002, November 26, 2002
Applicable to: The Pn PS assay, SOP2

Purpose

Polyclonal secondary antibodies (e.g., goat anti-human IgG) are more sensitive than monoclonal antibodies in the Pn PS ELISA. However, properties of polyclonal antisera vary from lot to lot, and each lot of polyclonal antisera must be tested for its binding specificity. The polyclonal antisera for human IgG should bind to all human IgG subclasses (IgG1, IgG2, IgG3, and IgG4) equivalently with minimal cross-reactivity to IgA or IgM (less than 5%). This SOP describes how to evaluate the isotype specificity of the secondary antibody specific for human IgG.

Materials and reagents

- Purified human IgG1 (Sigma I-5154, or equivalent)
- Purified human IgG2 (Sigma I-4264, or equivalent)
- Purified human IgG3 (Sigma I-4389, or equivalent)
- Purified human IgG4 (Sigma I-4639, or equivalent)
- Bovine serum albumin (RIA grade, Sigma A2934, or equivalent)
- Enzyme labeled secondary antibody to be tested (“new secondary antibody”)
- Enzyme labeled secondary antibody currently in use (“old secondary antibody”)
- Sodium carbonate (Na_2CO_3 , BDH #10240, or equivalent)
- Sodium bicarbonate (NaHCO_3 , BDH #102474V, or equivalent)

All other reagents and materials are itemized in the Pn PS ELISA (SOP 2).

Solutions

- **Sterile carbonate/bicarbonate buffer pH 9.6:**

0.76g Na_2CO_3
1.50g NaHCO_3
0.10g NaN_3

Dissolve the dry chemicals in 400 ml reagent grade water (type 1) and bring up to 500 ml with reagent grade water (type 1). Adjust the pH to 9.55-9.65 with 6M HCl/6 M NaOH and sterile filter the solution using a 0.2 μm filter. Remove aliquots from this stock solution using sterile technique. Store at 4°C for a maximum of 1 week.

- **2% BSA in carbonate/bicarbonate buffer**

Dissolve 2.0 g of RIA grade bovine serum albumin (BSA) in 100 ml of sterile carbonate/bicarbonate. Must be made fresh on day of use. All other solutions are described in SOP 2.

Procedure

Conditions used for all steps below should conform to those used in the Pn PS ELISA as defined in SOP 2. This procedure is used for testing one lot of polyclonal antiserum.

1. Dilute each of the human proteins (IgG, IgA, IgM, IgG1, IgG2, IgG3, and IgG4) to 1 $\mu\text{g}/\text{ml}$ in carbonate/bicarbonate buffer. Add 100 μl of diluted human proteins to appropriate wells of the microtiter

plates as shown in Diagram 1 (to show specificity for IgG) and Diagram 2 (to analyze Ig isotypes). Cover the plate with a lid and incubate for 90 minutes at 37°C. (NOTE THE SHORT INCUBATION TIME.)

Diagram 1

	Old qualified 2° antibody						New candidate 2° antibody					
	1	2	3	4	5	6	7	8	9	10	11	12
A 1:250	IgG	IgG	IgA	IgA	IgM	IgM	IgG	IgG	IgA	IgA	IgM	IgM
B 1:500	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C ↓ etc.												
D												
E												
F												
G												
H												

Diagram 2

	1	2	3	4	5	6	7	8	
A 1:250	IgG1	IgG1	IgG2	IgG2	IgG3	IgG3	IgG4	IgG4	Old qualified 2° antibody
B 1:500	↓	↓	↓	↓	↓	↓	↓	↓	
C 1:1000									
D 1:2000									
E 1:250									New candidate 2° antibody
F 1:500									
G 1:1000									
H 1:2000									

- Without removing the above antigens, add 100 µl of 2% BSA in carbonate/bicarbonate buffer to all wells. Cover the plates with lids and incubate for 60 minutes at room temperature.
- Prepare serial 2-fold dilutions for the new and old secondary antibodies in antibody buffer (SOP2), in 50 ml polystyrene tubes, starting at 1:250 and ending at 1:16000 as shown in Diagram 2. Typically prepare about 20 ml for each dilution.
- Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
- Add 100 µl of the dilutions of the secondary antibody to the appropriate wells (rows A through G) of the plate, and antibody buffer as a blank (see Diagram 2). Incubate at room temperature for 1 hour.
- Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
- Add 100 µl of *p*-nitrophenyl phosphate solution to all wells of the plate. Incubate for 15 minutes at room temperature and then add 50 µl 3M NaOH to stop the reaction (SOP 2).
- After 5 minutes, read optical density values at 405 nm and 690 nm (SOP 2).

Data analysis

- Average the duplicate optical density values.
- Subtract the average background (OD of blank wells) from each of the wells for each of the immunoglobulin antigens. Background should be less than 0.1. If not, reject the secondary antibody.

- To test for IgG specificity, select the dilution that produces an optical density of roughly 1 in the wells coated with IgG. Obtain the optical density of IgG, IgA and IgM isotype coated wells at the dilution of the secondary antibody. Calculate the % cross reactivity as shown below. A specific reagent should have cross reactivity less than 3%.

[% cross reactivity=(Average OD of IgA wells (X100) / (average OD of IgG wells)]

- To test for equivalent binding of all IgG subclasses, select the dilution of secondary antibody that produces OD of approximately 1 for IgG2. Obtain the optical density for IgG1, IgG2, IgG3 and IgG4 wells at the dilution. Calculate the binding ratio for all IgG subclasses as shown below by using OD of different IgG subclass in the numerator. A balanced reagent should have a binding ratio close to 1 for all IgG subclass combinations.

[binding ratio of IgG1= (Average OD of IgG1 wells)/(Average OD of IgG2 wells)]

SOP 6: Determine the optimum working dilution for the enzyme labeled secondary antibody

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
Effective date: June 30, 2002, November 26, 2002
Applicable to: Pn Ps ELISA (SOP 2)

Purpose

The purpose is to determine the optimal working dilution of the enzyme-labeled secondary antibody, which is used for the Pn PS ELISA.

Materials and reagents

- Reagents and materials specified in the Pn PS ELISA for which the enzyme-labeled secondary antibody will be used.
- Enzyme-labeled secondary antibody to be tested (“new secondary antibody”)
- Enzyme-labeled secondary antibody currently used (“old secondary antibody”)

All other reagents and materials are specified in SOP 2.

Procedure

Conditions for all steps below should conform to those used in the antigen specific Pn PS ELISA as defined in SOP 2. Also it is assumed that one new secondary antibody is being tested with one old secondary antibody. Depending on the situation, the reagent volumes can be changed.

1. Coat 4 microtiter plates with Pn PS (one plate each of serotypes 4, 6B, 14 and 18C) at the optimal antigen concentration (SOP 4A) for each lot of enzyme-labeled secondary antibody to be tested.
2. Prepare 4 ml of “absorption buffer” by mixing antibody buffer and C-PS stock (1 mg/ml) at 100:1 ratio. Dilute the standard serum (89SF) with 4 ml of the “absorption buffer” such that the optical density value at the end of the assay would be about 2.0. Although the exact dilutions are serotype specific, this dilution is usually about 1:1500 for 89SF (the reference standard). Transfer 200 µl of the diluted serum to each well in row A of the “dilution plate”.
3. Add 100 µl of the antibody buffer to wells in rows B through H in the “dilution plate”. Prepare six 2-fold serial dilutions of the standard serum in the “dilution plate” by transferring 100 µl from the wells in row A to those in row B etc. These dilutions should yield a linear range of optical density values between 0.1 and 2.0. Incubate for 30 minutes.
4. Wash the antigen-coated assay plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
5. Transfer 50 µl from each well of the “dilution plate” to that of the assay plate. Start transferring from row G then move up the next row etc. Add only the antibody buffer to the wells in row H. Incubate the plate for 2 hours (SOP 2).

Diagram 1

Dilution of 89SF ↓	New candidate 2° antibody										Old qualified 2° antibody at in use dilution		
	D1 1:500		D2 1:1000		D3 1:2000		D4 1:4000		D5 1:8000				
	1	2	3	4	5	6	7	8	9	10	11	12	
1:500	A												
1:1000	B												
1:2000	C												
1:4000	D												
1:8000	E												
1:16000	F												
1:32000	G												
Antibody buffer	H												

- In 15-ml polystyrene tubes, prepare five 2-fold serial dilutions (dilutions D1, D2, D3, D4, D5 and D6) of the new secondary antibody. Generally, 2 ml of the new antibody dilutions are prepared at 1:500, 1:1000, 1:2000, 1:4000, 1:8000 and 1:16000 dilutions, but the exact dilutions may vary with the new secondary antibody type, source, and lot. If the old secondary antibody is available, prepare the working dilution of the old secondary antibody and use it instead of D6.
- Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
- Add serial dilutions of enzyme-labeled secondary antibodies in duplicates (100 µl/well) to appropriate columns as shown in Diagram 1. For instance, D1 dilution of the new antibody will be added to wells in columns 1 and 2 (including rows G and H), D2 to columns 3 and 4, etc. The old antibody (or D6) will be added to wells in columns 11 and 12. Incubate the plates according to antigen specific Pn PS ELISA (SOP 2).
- Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
- Add substrate to the plates, incubate the plates, and obtain the OD of the plates using an ELISA reader as described in SOP 2.

Data analysis

Choose the dilution of new secondary antibody that satisfies all 5 requirements described below.

- Signal strength:** Examine OD values of the wells in row A and determine the dilution of the new secondary antibody with OD values close to 2. Usually, 1:1000 or higher dilutions would provide the high OD values. Occasionally, a working dilution as low as 1:250 may be required for a very poor secondary antibody to achieve the required high OD. At these low dilutions, the secondary antibody may have high background binding.
- Background binding:** Examine OD values of the wells in rows G and H (antigen + enzyme labeled secondary antibody) and determine the dilutions of the new secondary antibody with OD values less than 0.1. If a new secondary antibody has a working dilution 1:1000 or higher, it usually has low background binding. Occasionally, the secondary antibody with high working dilution may bind the antigen and may have high background binding. The overall performance of the secondary antibody is determined by the signal to noise ratio. Therefore, select the optimal working dilution, which maximizes the signal and minimizes the noise.

3. Lower limit of detection: Perform a separate linear regression analysis on the absorbance from the 6 dilutions of reference serum for each dilution of enzyme labeled secondary antibody. Calculate the 'lower limit of detection' as determined by the units of antibodies present in the dilution of reference serum yielding two times the assay background. Calculate the units/ml detectable at the lowest dilution of serum tested in the assay (i.e., 1:50 for human serum). The optimal dilution chosen must yield values on more than 99% of cases. In the case of the Pn PS ELISA assay, the sensitivity limit is about 0.01 µg/ml.
4. Comparability: Select the dilution of new secondary antibody that yields equivalent OD (i.e., ± 10% of the current lot) while meeting the lower limit of assay sensitivity. A side-by-side analysis of a panel of sera should confirm that results obtained using the 'new' lot of the secondary antibody is equivalent to those attained with the old, previously qualified antibody.
5. Enzyme development time: If robotic applications of the ELISA are planned, the substrate development time for the alkaline phosphatase enzyme is often made similar to typical ELISA antibody incubation times (e.g. 1-2 hours). Incubation periods less than one hour may severely limit the number of plates the robot can process.