

Automation of a Homogeneous Proximity Assay for Immunogenicity Testing of Biological Drug Products

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Introduction

Several challenges have surfaced during clinical evaluation of biological drug products due to a commonly associated immune response in patients. Anti-drug antibodies (ADA) are known to be frequently generated during administration of humanized monoclonal antibody therapeutics. These ADAs are nearly indistinguishable from antibody drug therapeutics thus requiring robust selective methods to determine the extent to which they impact safety and efficacy during treatment¹. A commonly used technology platform for assessment of immunogenicity relies on the bridging immunogenicity assay format typical of the Enzyme-Linked Immunosorbent Assay (ELISA). Other methods have been used to provide simpler work flows and higher sensitivity, such as Electrochemiluminescence (ECL) assays using streptavidin ECL plates to create the classic bridging assay². Here we present a homogeneous assay based on using a bridging assay format where all reagents and sample are in solution. This facilitates automation of reagent addition and simplifies further the work flow without sacrificing sensitivity.

Here we compare the automation of an AlphaLISA[®] ADA assay with a solution ELISA ADA assay using liquid handling and dispensing instrumentation and a high-throughput screening (HTS) multi-mode reader which can be used to detect the presence of ADA activity in a model system³. The AlphaLISA ADA assay utilizes bivalent binding of anti-drug antibodies to biotinylated drug which is then captured on streptavidin (SA)-coated Donor beads and drug antibody immobilized on Acceptor beads (Figure 1). The resulting complex is formed in the presence of ADAs resulting in the two beads coming into close proximity. Laser excitation of Donor beads at 680 nm results in singlet oxygen release and subsequent energy transfer to Acceptor beads and light emission at 615 nm. The formation of the complex in solution eliminates washing steps and secondary detection antibodies typically required with standard sandwich ELISA methods.

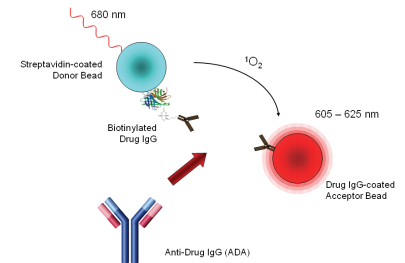
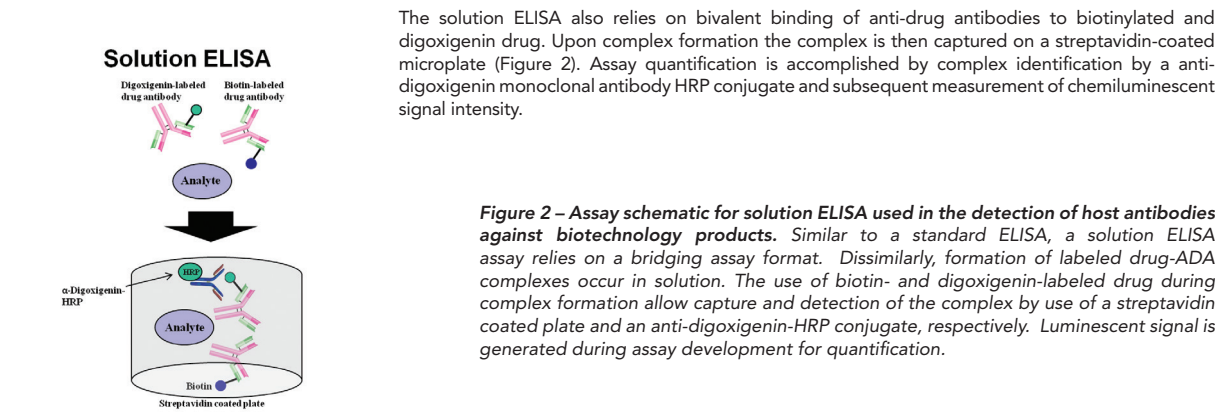


Figure 1 – Assay schematic for AlphaLISA used in the detection of host antibodies against biotechnology products. Upon excitation, the AlphaLISA donor bead releases singlet oxygen molecules that trigger a cascade of energy transfer to an Acceptor bead, providing the Acceptor beads are in close proximity to the Donor beads.



The solution ELISA also relies on bivalent binding of anti-drug antibodies to biotinylated and digoxigenin drug. Upon complex formation the complex is then captured on a streptavidin-coated microplate (Figure 2). Assay quantification is accomplished by complex identification by an anti-digoxigenin monoclonal antibody HRP conjugate and subsequent measurement of chemiluminescent signal intensity.

Figure 2 – Assay schematic for solution ELISA used in the detection of host antibodies against biotechnology products. Similar to a standard ELISA, a solution ELISA relies on a bridging assay format. Dissimilarly, formation of labeled drug-ADA complexes occur in solution. The use of biotin- and digoxigenin-labeled drug during complex formation allow capture and detection of the complex by use of a streptavidin coated plate and an anti-digoxigenin-HRP conjugate, respectively. Luminescent signal is generated during assay development for quantification.

BioTek Instrumentation

Precision[™] Microplate Pipetting System. Precision is an affordable, innovative solution for automated 96- or 384-well microplate liquid handling. The instrument was used to transfer serum samples and controls from a master plate to assay plates, bulk addition of acid dissociation reagent, and replicate sample transfer from 96- to 384-well assay plate formats (AlphaLISA assay).

MultiFlo[™] Microplate Dispenser. MultiFlo Microplate Dispenser offers up to four reagents dispensed in parallel with one compact instrument. The instrument was used to dispense assay specific reagents to the 96- and 384-well assay plates.

ELX50[™] Microplate Strip Washer. The ELX50 Washer is a flexible platform that provides a variety of washing capabilities. The washer was used for all wash steps associated with the solution ELISA assay workflow.

Synergy[™] NEO HTS Multi-Mode Microplate Reader. The new Synergy NEO is an HTS multi-mode microplate reader designed specifically for today's screening and core laboratories. Synergy NEO has all the features you would expect from a screening instrument, including multiple parallel detectors for ultra-fast measurements, laser-based excitation, super-fast plate stacker and high sensitivity on low volume assays. The Synergy NEO also incorporates BioTek's unique patented Hybrid Technology[™] for ultimate wavelength flexibility.

Data Reduction

- AlphaLISA signal is reported as Alpha counts and solution ELISA signal is reported as relative luminescence units (RLUs).
- Data was analyzed using GenS[™] Data Analysis Software (BioTek Instruments, Inc., Winooski, VT, USA), Microsoft[®] Excel[®] (Microsoft, Redmond, WA, USA), and GraphPad Prism[®] (GraphPad Software, LaJolla, CA, USA).

Materials and Methods

Reagents
Pooled neat human serum (PNHS) and individual lots of human serum were purchased from Bioreclation, LLC (Catalog No. HMSRM, Westbury, NY, USA). Anti-DIG-HRP (Catalog No. 200-032-156) and the positive control antibody, polyclonal goat anti-mouse IgG (Catalog No. 115-005-062) was purchased from Jackson ImmunoResearch[®] Labs, Inc. (West Grove, PA, USA). Goat anti-mouse HRP (Catalog No. 12349PM), Pierce ECL substrate (Catalog No. PI-32109), and Zeba Spin desalting columns (Catalog No. 89883) were purchased from Thermo Scientific (Waltham, MA, USA). Carboxymethylamine (Catalog No. C13408), sodium cyanoborohydride (Catalog No. 152159), bovine γ -globulin (Catalog No. G5009) and Proclin-300 (Catalog No. 48912-U) were purchased from Sigma-Aldrich (St. Louis, MO, USA). NHS-Chromalink-biotinylating reagent (Catalog No. B1001-105) and Chromalink Digoxigenin One-Shot Antibody Labeling Kit (Catalog No. B-9014-009K) was purchased from Solulink (San Diego, CA, USA). Mouse monoclonal IgG2b was purchased from AbD Serotec (Raleigh, NC, USA). AlphaLISA Donor (Catalog No. 6760002S) and Acceptor beads (Catalog No. 6772003) were purchased from PerkinElmer (Waltham, MA, USA).

Preparation of Drug-conjugates

A portion of drug was either biotinylated, digoxigenin-labeled or conjugated to AlphaLISA Acceptor beads as per manufacturer's protocol. Briefly, for biotinylation of drug antibody NHS-Chromalink-biotinylating reagent was used in PBS at a 30:1 molar ratio of biotin reagent to antibody to label 25 μ g of mouse monoclonal IgG2b. Purification was performed using standard procedures and analyzed for labeling efficiency by absorbance spectroscopy using an Epoch[™] Microplate Reader and Take3[™] Micro-Volume Plate accessory (BioTek Instruments, Inc., Winooski, VT, USA) as well as assessed for purity by SDS-PAGE with silver staining.

For digoxigenin labeling of drug antibody Chromalink Digoxigenin One-Shot Antibody Labeling Kit was used and analyzed as above by absorbance spectroscopy.

For conjugation of antibody to AlphaLISA Acceptor beads a coupling ratio of 50:1 (w/w) beads to antibody, 1 mg beads to 0.02 mg antibody (drug), was used at an antibody concentration of 0.5 mg/mL.

Assay Plates

AlphaLISA: OptiPlate[™] -384 white opaque 384-well microplates were from PerkinElmer (Catalog No. 6007299, Waltham, MA, USA).

Solution ELISA: Pierce Streptavidin Plates were from Thermo Scientific (Catalog No. 15502, Rockford, IL, USA).

Instrument Components

Synergy NEO HTS Multi-Mode Microplate Reader was used for all determinations.

Synergy Neo Read Parameters (AlphaLISA)		Synergy Neo Read Parameters (Solution ELISA)	
Mode	Alpha	Mode	LUM
Gain	200	Optics position	Top
Delay after plate movement	0 msec		225
Excitation time	80 msec	Integration time	1 sec
Delay after excitation	120 msec	Delay after plate movement	100 msec
Integration time	160 μ sec	Read Height	1.00 mm
Read Height	8.00 mm		

Table 1 – Alpha signal was read on the Synergy NEO. Alpha reading parameters used in GenS Data Analysis Software.

AlphaLISA Assay Setup

The AlphaLISA assay was performed as previously described³. Briefly, serum and control samples were subject to acid dissociation by addition of 600 mM acetic acid for 60 minutes at RT w/shaking in a 96-well polypropylene microplate. Samples were then transferred to 384-well assay plate for the neutralization and capture step. The addition of 2X drug-acceptor bead (final concentration of 20 μ g/mL)-biotin-drug (final concentration of 1 nM) mix was added followed by incubation at RT for 60 minutes w/shaking. SA Donor beads were added during the detection step to a final concentration of 20 μ g/mL. For spiked sample during CCP determination experiments an additional 2 μ L of drug/PNHS or PNHS was added to serum and control samples prior to acidification steps using the MultiFlo.

Solution ELISA Assay Setup

The solution ELISA assay was performed as previously described³. Briefly, serum and control samples were subject to acid dissociation by addition of 80 mM acetic acid for 30 minutes at RT w/shaking in a 96-well polypropylene microplate. Samples were then transferred to 96-well assay plate for the neutralization and labeling step. The addition of 3X labeling mix (1 M Tris-HCl, pH=8.0, biotin-drug @ 3 μ g/mL and 3 μ g/mL DIG-drug) was followed by incubation overnight at 4°C. The samples were then transferred for capture to streptavidin coated assay plates prewashed 3 x 200 μ L PBS/0.1% Tween20 followed by incubation at RT for 60 min. The plate was washed 4 x 300 μ L PBS/0.1% Tween20 followed by addition of anti-DIG HRP conj. diluted 1:40,000 in PBS-casein buffer and incubated at RT for 2 hours. The final wash was 4 x 300 μ L PBS/0.1% Tween20 followed by addition of 100 μ L ECL luminescent reagent. For spiked sample during CCP determination experiments an additional 2 μ L of drug/PNHS or PNHS was added to serum and control samples prior to acidification steps using the MultiFlo.

Materials and Methods (Continued)

AlphaLISA Automated Assay Workflow

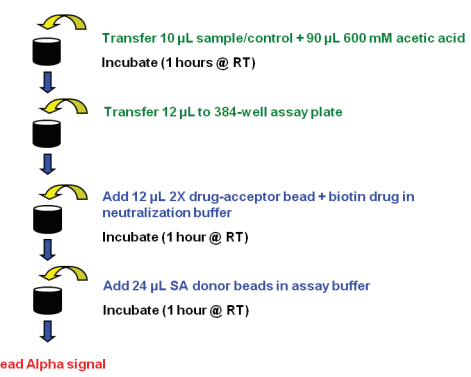


Figure 3 – AlphaLISA Automated Assay Procedure. Transfer of sample/controls accomplished by Precision. Assay specific reagents dispensed using MultiFlo. Detection of Alpha signal accomplished using the Synergy NEO.

Solution ELISA Automated Assay Workflow



Figure 4 – Solution ELISA Automated Assay Procedure. Transfer of sample/controls accomplished by Precision. Assay specific reagents dispensed using MultiFlo. Plate washing was accomplished by ELX50. Detection of Alpha signal accomplished using the Synergy NEO.

Z'-Factor

- Twenty-four replicates of either HPC or PNHS were assayed to determine the Z'-factor for both assay formats.
- The calculated Z'-factor were 0.74 and 0.57 for the AlphaLISA and solution ELISA assay formats, respectively. A Z'-factor >0.5 indicates a robust assay.
- The AlphaLISA assay format appears to provide significantly less variability when compared to the solution ELISA format.

Assay Sensitivity and Prozone Effect

- Assay sensitivity evaluates the characteristics of the PC to determine the lowest concentration meeting the acceptance criteria for inter- and intra-assay precision as well as the ability to detect ~5% false positives among samples.
- The prozone effect evaluates if the assay is deficient at very high ADA concentrations.
- A 11-point 1:2 serial titration of the PC at 10X the HPC (10 μ g/mL) and zero point in PNHS were prepared to generate an ADA standard curve.
- The acceptance criteria requires that the mean relative counts of each prozone dilution that is \geq PSCP be \geq subsequent dilution.

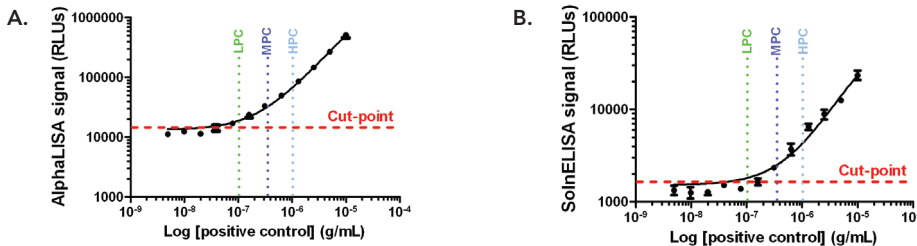


Figure 5 – Assay Sensitivity and Prozone. Assay sensitivity and prozone were assessed by performing a 11-point 1:2 serial dilution of the positive control starting a concentration of 10X the HPC plus a zero concentration point. **A.** AlphaLISA titration assay data; **B.** Solution ELISA titration assay data.

- Each dilution greater than the PSCP for each assay met the acceptance criteria defined above.
- No prozone effect was detected when the PC was spiked in the PNHS at 10X the HPC and serially diluted as noted by a nearly linear response at very high concentrations.

Inter- and Intra-Assay Precision

- The data from inter- and intra-assay precision testing is used in conjunction with the above Assay Sensitivity data for determination of assay sensitivity.
- Three PC concentrations were selected at 100, 350, and 1000 ng/mL (LPC, MPC and HPC) for sensitivity testing to determine the concentration meeting the inter- and intra-assay acceptance criteria and then used to set the LPC.
- For the AlphaLISA assay format each PC was tested in quadruplicate on 6 occasions.
- For the solution ELISA assay format, each PC was tested in quadruplicate on two occasions.
- The group mean, SD and %CV were calculated for each experiment and used to determine intra-assay precision (Table 3A).
- The group mean, SD and %CV of all experiments was calculated and represent inter-assay precision (Table 3B).
- Acceptance criteria requires that the RC of PCs of 2 or more replicates \geq blank mean RCs and PSCP (calculated retrospectively), %CV \leq 25% for all PCs, and global mean RCs of LPC \leq MPC and HPC.

A. Intra- and Inter-assay Precision (AlphaLISA)				B. Inter- and Intra-assay Precision (Solution ELISA)			
Samples		Mean counts	Mean intra-assay precision %CV	Samples		Mean counts	Mean intra-assay precision %CV
PNHS		10502	6.1	PNHS		7579	3.2
LPC		20507	6.2	LPC		14473	10.3
MPC		47073	3.8	MPC		45114	9.0
HPC		269873	6.3	HPC		197304	10.4

Table 3 – Assay Precision. **A.** The inter- and intra-assay variability was analyzed using quadruplicate determinants on six occasions using the AlphaLISA assay format. **B.** Solution ELISA inter- and intra-assay variability was examined using quadruplicate data points on two occasions.

- The assay precision results for all PCs were within the acceptable limits as defined above.
- The PSCPs were calculated for each method at 12,202 counts and 11,258 RLUs for AlphaLISA and Solution ELISA, respectively.

Screening Cut-Point

- Screening cut-point (CP) is used for determination of threshold for identification of sample as negative (<CP) or potentially positive (\geq CP) for presence of ADAs.
- Analysis of 50 lots of normal human serum (NHS, 25 male, 25 female) was performed using each method.
- Blanks were prepared with pooled normal human serum (PNHS).
- Use average of replicates, assume normal distribution.
- CP is calculated:

$$CP = \text{mean} + 1.645 \times SD \text{ (95}^{\text{th}} \text{ percentile)}$$

- Correction factor is calculated:

$$CF = CP / \text{Mean Blank (counts)}$$

- Serum lots with values higher than CP on greater than 50% of CP determination occasions were removed from calculation and analyzed in confirmatory assay as true or false positives.
- Final CP calculated using remaining lots.

A. Screening Cut-Point (AlphaLISA)			B. Screening Cut-Point (Solution ELISA)		
1st Interaction Screening		2nd Interaction Screening	1st Interaction Screening		2nd Interaction Screening
Cut-Point		Cut-Point	Cut-Point		Cut-Point
Mean Counts		11891	Mean Counts		9306
n		50	n		50
SD		921	SD		15624
Cut-Point (counts)		13406	Cut-Point (counts)		35009
Mean Blank (counts)		11149	Mean Blank (counts)		5797
Correction Factor		1.20	Correction Factor		6.0

Table 4 – Screening Cut-Point Determination. **A.** AlphaLISA determination of CP using 50 individual lots of NHS, analyzed in quadruplicate on a total of two occasions. **B.** Solution ELISA determination of CP using the same 50 individual lots of NHS, analyzed on a total of two occasions. Four samples were removed for the second iteration.

- Determination of second iteration CPs resulted in CF determinants of 1.16 and 1.3 for AlphaLISA and solution ELISA, respectively.
- The plate specific cut-point (PSCP) is determined in all subsequent experiment:

$$PSCP = \text{Mean Blank Relative Counts (RC)} / (\text{after removal of maximum 2 outliers}) \times CP$$

Confirmatory Cut-Point

- Determination of the CCP (% signal inhibition) is used to confirm positives or negatives of samples identified as potentially reactive in CP screening.
- The same 50 individual lots were spiked with the drug and analyzed.
- CCP blanks and positive controls (PCs), spiked and unspiked, were present on each plate.
- Two PCs, low PC (LPC) and high PC (HPC), and individuals lots were spiked with 25 μ g/mL drug.
- All samples were run concurrently with unspiked samples used in the PC determination.
- Calculate overall mean of sample, PC and Blanks for both spiked and unspiked.
- Calculate the % inhibition:

$$\% \text{ Signal Inhibition} = [1 - (\text{spiked sample or control} / \text{unspiked sample or control})] \times 100$$

- Mean and SD were calculated using the % inhibition of all the lots in each experiment.
- The CCP is then calculated:

$$CCP = \text{mean} + 2.33 \times SD \text{ (99}^{\text{th}} \text{ percentile)}$$

- Lots with % inhibition greater than CCP were removed for 2nd iteration.
- Final CCP used for final drug competition test cut-point (Table 4).
- Acceptance criteria requires % inhibition of HPC>LPC>CCP>Blanks (PNHS) (Table 5).

A. Confirmatory Cut-Point (AlphaLISA)				B. Confirmatory Cut-Point (Solution ELISA)			
1st interaction		2nd interaction		1st interaction		2nd interaction	
Confirmatory Cut-Point		Confirmatory Cut-Point		Confirmatory Cut-Point		Confirmatory Cut-Point	
Mean		-0.9		14		10	
n		50		50		47	
SD		6.7		21		12	
Cut-Point (counts)		14.7		62		39	

Table 5 – Confirmatory Cut-Point. **A.** AlphaLISA determination of CCP using 50 individual lots of NHS, both unspiked and spiked with drug, analyzed in quadruplicate on a total of two occasions. No samples were removed for the second iteration. **B.** Solution ELISA determination of CP using the same 50 individual lots of NHS, both unspiked and spiked with drug, analyzed on total of two occasions. Three samples were removed for the second iteration.

A. Control results for the CCP (AlphaLISA)				B. Control Results for the CCP (Solution ELISA)			
Samples		Mean Counts	Status with regards to CCP	Samples		Mean Counts	Status with regards to CCP
LPC		17094	\geq CCP	LPC		7965	\geq CCP
HPC		38727	\geq CCP	HPC		30162	\geq CCP
PNHS		11052	< CCP	PNHS		4703	< CCP

Table 6 – Results of Controls for CCP. **A.** AlphaLISA controls for CCP determination. **B.** Solution ELISA controls for determination of CCP.

- Four human serum lots were identified as outliers during CP analysis for each assay method.
- The four lots identified in the AlphaLISA assay produced % inhibition signal values lower than the 99th percentile and were therefore included in the CCP calculation.
- Three of the four lots identified in the Solution ELISA assay produced % inhibition signal values higher than the 99th percentile and were therefore excluded in the CCP calculation.
- False positives due to non-specific binding (NSB) were identified at rates of 20% and 2%, for AlphaLISA and solution ELISA methods, respectively.
- The CCPs calculated were 14.7% and 39%, for AlphaLISA and Solution ELISA, respectively.
- The LPC, HPC and PNHS spiked with 25 μ g/mL drug meet the acceptance criteria described above.

Drug Tolerance

- Drug tolerance determines the extent to which circulating drug interferes with assay performance due to competition between circulating drug and the assay antigen for ADAs.
- Each PC was prepared at a 2X concentration and spiked with a 2:1 serial dil. of drug at 2X the final assay concentrations of 0-200 μ g/mL in PNHS.
- Unspiked samples were also prepared and all were incubated for 1 hr @ RT.
- For the AlphaLISA assay format each PC was tested in quadruplicate.
- For the solution ELISA assay format, each PC was tested in duplicate.
- The % difference was calculated for PC w/w/o drug as follows:

$$\% \text{ Difference} = ((\text{Mean relative counts of spiked} - \text{Mean relative counts unspiked sample}) / \text{Mean relative counts unspiked sample}) \times 100$$

A. Control results for the CCP (AlphaLISA)				B. Control Results for the CCP (Solution ELISA)			
Samples		Mean Counts	Status with regards to CCP	Samples		Mean Counts	Status with regards to CCP
LPC		17094	\geq CCP	LPC		7965	\geq CCP
HPC		38727	\geq CCP	HPC		30162	\geq CCP
PNHS		11052	< CCP	PNHS		4703	< CCP

Table 7 – Drug Tolerance. **A.** Drug tolerance was determined using quadruplicate determinants on one occasion using the AlphaLISA assay format. **B.** Solution ELISA drug tolerance was examined using duplicate data points on one occasion.

- Drug tolerance can be defined by the lowest concentration of drug inhibiting detection of the PC and has mean relative counts below the PSCP.
- For the AlphaLISA assay, the drug concentration of 25 μ g/mL at the LPC and 100 μ g/mL at the HPC did not show interference.
- For the solution ELISA assay, the drug concentration of 12.5 μ g/mL at the LPC and 50 μ g/mL at the HPC did not show interference.

Thus, for both assays the LPC was set at 100 ng/mL and drug tolerance was set at > 25 μ g/mL and 12.5 μ g/mL for AlphaLISA and solution ELISA, respectively.

Specificity and Selectivity

- Specificity and sensitivity was determined by analyzing 10 individual normal human serum lots (5 male, 5 female).
- For specificity testing each individual lot was tested unspiked (Table 8).
- For sensitivity testing each individual lot was spiked with PC in PNHS at LPC (100 ng/mL), and HPC (1000 ng/mL) concentrations (Table 9).
- Each lot was tested in quadruplicate or in duplicate, on one occasion for the AlphaLISA or solution ELISA assay formats, respectively.
- Controls were prepared by spiking LPC and HPC in PNHS.
- For selectivity the individual lots were compared to reference samples by calculating % difference as follows:

$$\% \text{ Difference (recovery)} = ((\text{Mean RC of PC} - \text{Mean RC of PC PNHS}) / \text{Mean RC of PC PNHS}) \times 100$$

A.

Specificity				Specificity			
Serum Lot #		Mean counts	Status with regards to the PSCP	Serum Lot #		Mean counts	Status with regards to the PSCP
PHNHS	8013	-	< PSCP	Serum 6	9131	-	< PSCP
Serum 1	8672	< PSCP		Serum 7	8912	< PSCP	
Serum 2	12206	>PSCP		Serum 8	8627	< PSCP	
Serum 3	9086	< PSCP		Serum 9	8882	>PSCP	
Serum 4	9121	< PSCP		Serum 10	8601	< PSCP	
Serum 5	8509	< PSCP					

B.

Specificity				Specificity			
Serum Lot #		Mean counts	Status with regards to the PSCP	Serum 6		Mean counts	Status with regards to the PSCP
PHNHS	14384	-	< PSCP	Serum 6	24692	>PSCP	
Serum 1	7408	< PSCP		Serum 7	89818	>PSCP	
Serum 2	7564	< PSCP		Serum 8	8132	< PSCP	
Serum 3	20750	< PSCP		Serum 9	9940	< PSCP	
Serum 4	12017	< PSCP		Serum 10	9732	< PSCP	
Serum 5	7222	< PSCP					