Protein A-h ELISA Assay
Sample Denaturation
Modified Procedure with a Boiling Step

Validation Summary
Catalog # F050H

Summary and Explanation

The original F050H kit used only an acid treatment step to dissociate the Protein A bound to the product antibody so that the kit anti-Protein A antibodies could detect it accurately. This procedure was shown to be accurate for all antibodies at concentrations less than or equal to 1mg/mL. At higher product antibody concentrations, some customers reported under-recovery of Protein A spiked into their antibody. These customers did not want to dilute their antibody further believing it would significantly compromise the sensitivity of detection for Protein A. In response to this concern, we investigated other ways to overcome product antibody interference that would not require as much dilution of the sample. The addition of a boiling step after the acid dissociation, was shown to remove the product antibody without affecting the subsequent recovery of Protein A. This procedure better insures the accuracy and robustness of the assay. The following study was performed to validate this protocol change. The only change required to the previous F050H kit is a new kit directions insert to reflect the addition of the boiling step. All other reagents and manufacturing processes remained the same and thus the validation study is abbreviated from what would be required if kit components were changed. Customers may continue with the old non-boiling protocol if they do not wish to validate the new protocol.

Materials & Methods Used

Materials	
Anti-Protein A:Biotinylated Conjugate, Lot 5010B	Cat #F051H
Streptavidin:Alkaline Phosphotase, Lot 5010	Cat #F009
Anti-Protein A Microtiter coated plate, Lot 13030	Cat #F052
Protein A Standards	Cat #F053
The protocol as defined in the kit insert was used in this validation.	
Data References: Raw data for these experiments are recorded in Cygnus Notebook.	#2-Protein A /B12 Pages 75-89

Sample Treatment Procedure

1. Dilute all samples including standards and control by adding 1 part Sample Denaturing Buffer, Catalog #F054 to 4 parts of sample. (For example: Pipet 50µL of #F054 into a microfuge tube containing 200µL of sample.)
2. Mix thoroughly by vortexing.
3. Make a small pin or needle hole in the cap of each microfuge tube to allow for venting of heated, expanded air inside the tube.
4. Place tubes in a preheated 100°C heat block or boiling water bath for 5 minutes. This heating step will typically result in a denatured protein precipitate containing the product antibody.
5. Remove tubes, allow to cool for 5 minutes and then centrifuge at ~6000 x g for 5 minutes in a microfuge or other adapted centrifuge. Carefully remove the microfuge tube from the centrifuge. The Protein A is in the supernatant.
6. Proceed to assay protocol.

Assay Procedure

1. Pipette 100µL of biotinylated anti-Protein A (#F051H) into all wells.
2. Pipette 50µL of the supernatant from the denatured standards, controls, and samples into wells.
3. Transfer to rotator and incubate at room temperature, 24°C ± 4°, for 1 hour at 180 rpm.
4. Dump the contents into waste or gently aspirate using a multi-channel pipettor. Blot and vigorously bang out residual liquid over low lint absorbance paper. Wash generously with diluted wash solution by flooding the wells with solution from a squirt bottle or by pipetting in ~350µL. Repeat for a total of 4 times. Wipe off any liquid from the bottom outside of the wells as any residue can interfere in the reading step.
5. Pipette 100µL of Streptavidin:Alkaline Phosphatase (#F009) into each well.
6. Transfer to rotator and incubate at room temperature for 1 hour at 180 rpm.
7. Aspirate. Wash a total of 4 times as in step 4 above.
8. Pipette 100µL of PNPP substrate.
9. Incubate for 30 minutes.
10. Read absorbance at 405/492nm blanking on the 0 standard.

Standard Curve

Typical standard curve data from an actual assay run using a point to point fit is shown below. Actual OD values may change from lab to lab, run to run, or lot to lot. For this reason, we do not recommend use of OD levels as absolute QC parameters. The most important QC parameter involves the use of real analyte controls assayed in each run across the relevant analytical range of the assay. Do not rely on your curve fit algorithm parameters to quality control this assay. Those parameters such as R^2, slope, intercept, upper and lower asymptotes etc. are too indirect and insensitive to provide critical analytical control.

Standard	Duplicate OD Values	Mean OD	%CV
0ng/mL	0.070 0.073	0.072	2.9
0.25ng/mL	0.123 0.120	0.122	1.7
1ng/mL	0.210 0.219	0.215	3.0
4ng/mL	0.596 0.601	0.599	0.6
16ng/mL	1.691 1.761	1.726	2.9

Sensitivity

Limit of Detection (LOD) - The Protein A concentration corresponding to an OD signal 2 standard deviations above the mean of the zero standard is defined as the LOD. This was determined by averaging the standard curve results over 6 assays. The LOD was ~100 pg/mL.

Precision

Because there was no change to the assay protocol a complete precision study profile was not performed. Precision is defined in this study as the average % coefficient of variation on duplicate sample OD values. The average %CV on duplicate samples over 6 assays was 2.3%. This is excellent precision and consistent with the results obtained with the non-boil sample preparation. Because actual precision may vary from laboratory to laboratory, and technician to technician it is recommended that all operators demonstrate acceptable precision before reporting results.

Recovery/Matrix Interference

Defined as the ability of the assay method to correctly quantitate known concentrations of Protein A in a given sample matrix, accuracy was evaluated by spiking Protein A into various human antibody preparations. Recommended acceptable limits of recovery are +\- 20% of the added Protein A. The data shows the recovery in spikes ranging from 2 to 16ng/mL of Protein A into 3 different hIgG samples: a normal hIgG preparation isolated from human serum, and 2 previously problematic customer antibodies at 4 and 1.6mg/mL, that did not yield acceptable recovery with the old non-boiling protocol. This data demonstrates that the boiling protocol gives acceptable recovery in all 3 preparations. This does not ensure that all antibody preparations will be yield accurate recovery of Protein A levels, and thus each user is cautioned here and in the kit insert to evaluate their sample matrices for recovery in a similar experiment before reporting results with this assay.

Recovery in Various Sample Matrices

Sample Matrix	Protein A Spike	% Recovery
Normal hIgG, 2mg/mL	16ng/mL	98
Normal hIgG, 4mg/mL	16ng/mL	83
Customer Ab @ 4mg/mL	8ng/mL	105
Customer Ab @ 4mgnmL	4ng/mL	90
Customer Ab @ 1.6mg/mL	4ng/mL	110
Customer Ab @ 1.6mg/mL	2ng/mL	95

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Company Information

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