

Protein A ELISA Assay

Validation Summary Catalog # F400

Summary and Explanation

The data summarized below was generated by Cygnus Technologies to establish the performance parameters and validity of this kit to measure Protein A leachates. This data is intended to supplement and not replace user generated validation data. The data is representative of what a laboratory can expect to achieve when following the kit insert recommended protocols. Significant differences in these performance parameters may be indicative of problems with reagents, laboratory equipment, or technique and should be investigated before reporting results.

It is recommended that a user validation study include at least the following experiments to validate this kit for use with their product: (1) Each user should perform intra and inter assay precision experiments to establish their procedural proficiency. (2) Each user should perform recovery experiments using their test sample matrices. Such a study can be performed by adding known amounts of the 10ng/mL standard provided with this kit to the final product or any intermediate samples, which are to be tested. Ideally these test sample matrices should be devoid of Protein A or have very low levels (< 1ng/mL) determined prior to adding the 10ng/mL standard. Such an experiment will establish the degree of sample matrix interference in the recovery of HCPs. (3) Laboratories should also perform dilutional recovery experiments on their actual samples. This experiment assumes that at least some of the test samples from the purification process will have detectable levels of Protein A. Such samples are to be serially diluted with Sample Diluent Cat# I028, or some appropriate diluent previously shown to give acceptable recovery. When diluted, samples should give essentially the same value at each dilution when multiplied by the appropriate dilution factor. This experiment establishes the condition of antibody excess for accurate quantitation and determines that typical process samples do not have HCPs in the "Hook Region" of the concentration response curve.

Materials & Methods Used

Materials	
Anti-Protein A:HRP Conjugate, Lots #77-97, 77-87	Cat #F401
Anti-Protein A Microtiter strips, Lots #2687 & 2377	Cat #F402
Protein A Standards, Lots #2087 and 2177	Cat #F403
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.	#1-Protein A HRP Pages 1-44

Assay Summary

Cygnus now offers 3 ELISA kits for detection of Protein A. Our first two kits, Cat # F050 and F050H have been sold for several years and have proven to be valid for use in detecting both natural and structurally conserved recombinant Protein A. This newest kit Cat #F400, will better detect unnatural recombinant constructs of Protein A such as GE Healthcare's MabSelect SuRe™ ligand. Prior to the availability of this unique construct, all Protein A used for antibody purification was either natural Protein A derived from *Staphylococcus aureus* or recombinant Protein A (*E.coli* expressed) as patented and sold by Repligen. Repligen recombinant Protein A is very similar to natural Protein A with over 99% homology. Both Protein A preparations contain essentially the same 5 subunit binding domains (A, B, C, D, & E) and both have molecular weights of ~ 44.7kDa. The new GE Healthcare MabSelect SuRe™ construct is a structurally unique molecule. MabSelect SuRe™ is derived only from the B binding domain of natural Protein A. It is a multimerized, tetramer of the B domain with a C-terminal cysteine introduced for site-directed immobilization. The molecular weight of MabSelect SuRe™ is 26.7kDa.

The Cygnus Protein A Kits, Cat #s F050 and F050H, use antibodies made to natural Protein A with validation studies performed against only the natural and the highly conserved recombinant Protein A. With the introduction of MabSelect SuRe™ we have recently had the opportunity to evaluate its reactivity in these kits. Our preliminary data has shown that it has only ~20% weight to weight reactivity relative to the Protein A standards in these kits. This 20% reactivity may be greater or less depending upon other factors such as the product antibody being purified, which may contribute to further interference or non-specificity.

Protein A ELISA kit, Cat. #F400, addresses the low reactivity to MabSelect SuRe™ seen in our other kits while incorporating several other improvements in the methodology. This new assay has replaced the biotinylated antibody used in our original kits with a different antibody directly labeled with HRP. Improvements include more sensitivity, simplified assay protocol, more standards, and less probability of product matrix interference. This kit detects natural, conserved recombinant, and MabSelect SuRe™ Protein A ligands essentially 1:1. Similar to the F050H assay, the new F400 assay uses a sample denaturation step to overcome sample/product antibody interferences. Because so many laboratories have successfully validated and are routinely using our earlier kits #F050 & #F050H, we will continue to offer these kits indefinitely. First time evaluators of Protein A detection methods are encouraged to evaluate the new #F400 kit regardless of which type of Protein A they use.

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 50 μ L of #F054 into a microfuge tube containing 200 μ L 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 $-6000 \times g$ 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 as indicated in the product insert.

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
0ng/mL	0.053 0.061	0.057
0.1ng/mL	0.084 0.081	0.083
0.25ng/mL	0.142 0.139	0.141
0.6ng/mL	0.239 0.249	0.244
1.5ng/mL	0.486 0.473	0.480
4ng/mL	1.285 1.327	1.306
10ng/mL	2.696 2.642	2.669

Precision

Precision is defined as the percent coefficient of variation (%CV). This is calculated by dividing the standard deviation by the mean value for a number of replicate determinations of three different control samples in the low, mid and high concentration range of the assay. While actual precision may vary from laboratory to laboratory and technician to technician, it is recommended that all operators performing this test are

encouraged to establish precision using a similar study before reporting results.

Intra-assay:

# of tests	Mean ng/mL	%CV
12	0.258	8.3
12	1.546	2.0
12	4.373	5.1

Inter-assay:

# of assays	Mean ng/mL	%CV
10	0.253	10.0
10	1.496	4.6
10	4.046	6.1

Sensitivity

Protein A concentration corresponding to a signal 2 standard deviations above the mean of the zero standard is defined as the limit of detection (LOD). This was determined by averaging the standard curve results over 6 assays. The mean signal of the zero standard plus 2 SD yielded a LOD of ~ 100 pg/mL.

Specificity

There are 3 types of Protein A used for chromatographic purification of immunoglobulins: 1) Natural Protein A derived from *S. aureus*, 2) structurally conserved recombinant Protein A, and 3) the unnatural construct sold by GE Healthcare as "MabSelect Sure™". The antibodies in this kit have been shown to react essentially 1:1, and thus this kit can be used to detect all three types. If there is doubt about the type or origin of your Protein A, you are encouraged to evaluate your Protein A for reactivity relative to the standards used in this kit. If there is a significant difference on a weight-to-weight or molar basis, you should be able to apply an appropriate correction factor to the test results using the standards in this kit or, alternatively replace the standards provided with your own standards.

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 $\pm 20\%$ of the added Protein A. The table below 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 yield accurate recovery of Protein A levels, and thus each user is cautioned here and in the kit directions insert to evaluate their sample matrices for recovery in a similar experiment before reporting results with this assay.

Sample Matrix	Protein A Added ng/mL	% Recovery (assayed/ added x100)
#1. 2mg/mL normal hlgG	16	98
#2. 4mg/mL normal hlgG	16	83
#3. Customer Ab @ 4mg/mL	8	105
#4. Customer Ab @ 4mg/mL	4	90
#5. Customer Ab @ 1.6mg/mL	4	110
#6. Customer Ab @ 1.6mg/mL	2	95

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Company Information

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