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**Protein A ELISA kit  
Catalog # F400  
Validation Report  
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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 some appropriate diluent previously shown to give acceptable recovery. We strongly recommend use of our approved diluent for this assay, Cat # I028. 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 (hook effect) and lack of significant product antibody interference.

**Summary:** *Cygnus Technologies* 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 was released in September of 2007. 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 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 about a 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.

The Cat. #F400 assay 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. The #F400 kit detects natural, conserved recombinant, and MabSelect SuRe™ Protein A ligands essentially 1:1. Like 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.

- Materials:**
- Protein A Standards, Cat # F403, lots 2087 and 2177
  - HRP labeled chicken anti-Protein A (Product # F401) lots 77-97 and 77-87
  
  - Anti-Protein A coated Strips, Lots 2687 and 2377

**Methods:      Sample Treatment Procedure:**

1. Dilute all samples including the standards and control by adding 1 part of sample denaturing buffer (Cat #F054) to 2 parts of sample. Mix by vortexing. (For example: pipet 100µL of F054 into a microfuge tube containing 200µL of sample.)
2. Make a small pin or needle hole in the cap of each microfuge tube to allow for venting of heated air inside the tube.
3. Place tubes in a 100°C heat block or boiling water bath for 5 minutes.
4. 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.
5. Proceed to assay protocol.

**Assay Procedure:**

1. Pipette 100µL of HRP labeled anti-Protein A (F051H) into all wells.
2. Pipette 50µL of supernatant from the denatured samples, standards, and controls into wells indicated on the work list.
3. Transfer to microtiter plate shaker at 180rpm and incubate at room temperature for 2 hours.

4. Dump contents to waste. Blot and vigorously bang out residual liquid over low lint absorbent paper. Wash generously with diluted wash solution by flooding wells with solution from a squirt bottle. 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 TMB substrate (F004) into each well.
6. Incubate for 30 minutes.
7. Read absorbance at 450/650nm and calculate results.

**Data References:** Raw data for these experiments are recorded in Notebook #1-Protein A HRP pages 1-44.

**ELISA Validation:** Typical absorbance data for the kit standards tested in duplicate and using the are shown below in Table 1.

**Table 1**  
**Typical Protein A ELISA Standard Curve**

Standard Concentration	Absorbance 1 <sup>st</sup> well	Absorbance 2 <sup>nd</sup> well	Mean absorbance
0 (ng/mL)	0.053	0.061	0.057
0.1	0.084	0.081	0.083
0.25	0.142	0.139	0.141
0.6	0.239	0.249	0.244
1.5	0.486	0.473	0.480
4.0	1.285	1.327	1.306
10.0	2.696	2.642	2.669

**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. Table 2 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 directions insert to evaluate their sample matrices for recovery in a similar experiment before reporting results with this assay.

**Table 2**  
**Recovery in Various Sample Matrices**

Sample Matrix	Protein A Spike	% Recovery
#1 2mg/mL normal hIgG	16ng/mL	98
#2 4mg/mL normal hIgG	16ng/mL	83
#3 Customer Ab @ 4mg/mL	8ng/mL	105
#4 Customer Ab @ 4mg/mL	4ng/mL	90
#5 Customer Ab @ 1.6mg/mL	4ng/mL	110
#6 Customer Ab @ 1.6mg/mL	2ng/mL	95
		<b>Average recovery = 96.8</b>

**Precision:** Both intra-assay (n=12 replicates) and inter-assay (n=10 assay) precision studies were conducted using 3 control samples in the low, middle, and upper ranges of the standard curve. The table below shows the mean values and %CVs. Each laboratory and technician performing this test are encouraged to establish precision using a similar study.

Intra-assay			Inter-assay		
# of tests	Mean ng/mL	%CV	# of assays	Mean ng/mL	%CV
12	0.258	8.3	10	0.253	10.0
12	1.546	2.0	10	1.496	4.6
12	4.373	5.1	10	4.046	6.1

**Specificity:** There are 3 types of Protein A used for chromatographic purification of immunoglobulins: 1) Natural Protein A derived from *Staph. 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.

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