Application Note

Detection and quantification of leached Protein A from Eshmuno® A resin using Cygnus F400 Protein A ELISA kit

Project definition

Cygnus Technologies performed the initial feasibility testing of the Eshmuno® A Protein A ligand in the Cygnus Protein A ELISA Kit (Cat# F400). Upon successful completion of the feasibility testing, Cygnus proceeded with quantification of the assay for use in in-process and drug substance samples using the Eshmuno® A Protein A resin.

Feasibility testing

The Eshmuno® Protein A ligand was diluted to 10 ng/mL in Cygnus I028 assay diluent. The sample was then diluted 2-fold an additional six times on the sample treatment plate (100 µL of the previous dilution + 100 µL of I028). The Protein A standards (100 µL) were loaded in columns 1 and 2. Then, 50 µL of sample denaturing solution was added to each of the 96 wells and mixed with a multi-channel pipette. The plate was then sealed and heated for 15 minutes at 80°C. At the completion of the heating step the plate was cooled for 5 minutes and centrifuged at 3,200 x g for 7 minutes. 50 µL of sample and standard were stamped from the sample treatment plate into the assay plate containing 100 µL of conjugate antibody. The plate was sealed and incubated on a plate shaker at 400-600 RPM for 2 hours at room temperature. Following the sample incubation, the plate was washed 4 times with tris buffer saline (TBS) and developed with 3,3',5,5' tetramethylbenzidine (TMB) for 30 minutes. The plate was then read at 450 nm and 650 nm after stopping the development.

Results of feasibility testing

Table 1 shows that the Eshmuno® Protein A ligand had a dilutionally corrected average result of 10.1 ng/mL in the Cygnus Protein A ELISA which translates to a 101% nominal spike recovery using Protein A standard from the kit. The overall coefficient of variation (%CV) of the Eshmuno® Protein A was 6.5%. Based on this result, it is concluded that Cygnus F400 Protein A ELISA kit can detect Eshmuno® A Protein A ligand accurately as is with no modification. The decision was made to proceed to Phase II and qualify the F400 Protein A assay for use with the Merck Millipore Eshmuno® A resin.

Sample	Dilution	Result (ng/mL)	Average Result (ng/mL)	Standard Deviation (ng/mL)	Adjusted Result (ng/mL)	Overall Adjusted Result (ng/mL)	Overall Standard Deviation (ng/mL)	Overall %CV
Eshmuno® A Protein A ligand	1	9.338 10.702	10.02	0.964	10.02	10.07	0.653	6.5%
	2	5.199 5.796	5.5	0.422	11			
	3	2.231 2.492	2.36	0.184	9.45			
	4	1.154 1.324	1.24	0.12	9.91			
	5	0.642 0.724	0.683	0.058	10.92			
	6	0.291 0.32	0.306	0.021	9.78			
	7	0.134 0.159	0.146	0.018	9.38			

Table 1. Quantification of Eshmuno® A Protein A ligand in the Cygnus F400 Protein A ELISA Kit

Qualification of the Cygnus F400 Protein A ELISA for use with the Merck Millipore Eshmuno® A resin

Phase II testing was performed on real world in-process samples to qualify the Cygnus F400 Protein A ELISA for use with the Eshmuno® A resin. For qualification testing the accuracy (spike recovery) and precision (repeatability) of the assay was assessed in 3 real world in-process samples over 2 days. To be successfully qualified, the assay should produce an accuracy of +/- 20% nominal and precision of CV <15% for each sample.

Samples tested

Table 2.
Sample information

Sample	Conditions	Approximate pH	Approximate product concentration (mg/mL)
Eshmuno® A elution	Elution Buffer (0.1M acetic acid, pH 3) neutralized to pH 5 with 2M Tris	5	24.7
Post-CEX	50 mM sodium acetate, pH 5 with gradient to 0.5M NaCl, then neatralized to pH 8 with 2M Tris	8	18.2
Post-AEX	20mM Tris, 25 mM NaCl, pH 7.3	7.3	0.8

Sample testing

Each sample to be analyzed was prepared to an initial dilution in I028 sample diluent according to Row A of the plate map in Table 3. One hundred microliters of I028 sample diluent was added to rows B, C, D, F, G, and H of the sample treatment plate. The pre-diluted samples were loaded (190 µL) into rows A and E of the sample treatment plate according to the plate map. Row E was then spiked with 10 µL/well of spike solution (100 ng/mL) for a total spike of 5 ng/mL. Using a 12-channel pipette, Row A was mixed and 2-fold dilutions (100 µL of the previous dilution + 100 µL of I028) were prepared sequentially down the plate in Rows B, C, and D. One hundred microliters were removed from Row D and discarded. Row E was mixed well and 2-fold dilutions (100 µL of the previous dilution + 100 µL of I028) were prepared sequentially down the plate in Rows F, G, and H. One hundred microliters were removed from Row H and discarded. The Protein A standards (100 µL) were loaded in columns 1 and 2.

Then, 50 µL of sample denaturing solution was added to each of the 96 wells and mixed with a multi-channel pipette. The plate was then sealed and heated for 15 minutes at 80°C. At the completion of the heating step the plate was cooled for 5 minutes and centrifuged at 3,200 x g for 7 minutes. 50 µL of sample and standard were stamped from the sample treatment plate into the assay plate containing 100 µL of conjugate antibody. The plate was sealed and incubated on a plate shaker at 400-600 RPM for 2 hours at room temperature. Following the sample incubation the plate was washed 4 times with TBS and developed with TMB for 30 minutes. The plate then read at 450 nm and 650 nm after stopping the development.

The sample testing was performed on a second day in order to ascertain the repeatability of the F400 Protein A ELISA with these particular samples.

Plate map

Table 3.
Plate map for accuracy and precision test using Cygnus F400 ELISA kit

Protein A standards	Eshmuno® A Elution	Post-CEX	Post-AEX
0 ng/mL	Unspiked 1:800 dilution	Unspiked 1:80 dilution	Unspiked 1:5 dilution
0.1 ng/mL	Unspiked 1:1600 dilution	Unspiked 1:160 dilution	Unspiked 1:10 dilution
0.25 ng/mL	Unspiked 1:3200 dilution	Unspiked 1:320 dilution	Unspiked 1:20 dilution
0.6 ng/mL	Unspiked 1:6400 dilution	Unspiked 1:640 dilution	Unspiked 1:40 dilution
1.5 ng/mL	Spiked 1:800dilution	Spiked 1:80 dilution	Spiked 1:5 dilution
4.0 ng/mL	Spiked 1:1600 dilution	Spiked 1:160 dilution	Spiked 1:10 dilution
10 ng/mL	Spiked 1:3200 dilution	Spiked 1:320 dilution	Spiked 1:20 dilution
blank	Spiked 1:6400 dilution	Spiked 1:640 dilution	Spiked 1:40 dilution

Results

The results of the accuracy and precision testing can be found in tables 4 and 5, respectively. Table 2 shows that the accuracy of the Eshmuno® A elution is 95% nominal and the precision has a %CV of 8.1%. The Post-CEX sample has an accuracy of 90% nominal and the precision has a %CV of 4.5%. The Post-AEX sample has an accuracy of 89% and the precision has a %CV of 14.2%. Based on this testing, all three samples passed the acceptance criteria of +/- 20% nominal for spike recovery and a sample %CV of less than 15%.

	Day	Dilution	Result (ng/mL)	Average Result (ng/mL)	Spike Result (ng/mL)	Average Spike Result (ng/mL)	Average Spike Recovery	Spike Recovery	Dilution Corrected Result (ng/mL)	Reported Result (ng/mL)	Reported Result (ng/mg)
Eshmuno® A Elution	1	800	0.72 0.688	0.704	5.909 5.055	5.48	96%	95%	563.2	573.1	23.2
	2	800	0.725 0.788	0.757	5.057 4.973	5.02	85%		605.2		
	1	1600	0.352 0.344	0.348	2.678 2.673	2.68	93%		556.8		
	2	1600	0.272 0.316	0.294	2.579 2.455	2.52	89%		470.4		
	1	3200	0186 0.182	0.184	1.376 1.339	1.36	94%		588.8		
	2	3200	0.189 0.188	0.189	1.559 1.447	1.50	105%		603.2		
	1	6400	<LOQ <LOQ	<LOQ	0.634 0.614	0.62	100%		<LOQ		
	2	6400	0.101 0.094	0.098	0.713 0.755	0.73	102%		624		
Post-CEX	1	80	0.829 0.842	0.836	5.444 5.4	5.42	92%	90%	66.8	70.3	3.9
	2	80	0.99 0.937	0.964	5.032 4.767	4.90	79%		77.1		
	1	160	0.406 0.362	0.384	2.528 2.615	2.57	88%		61.4		
	2	160	0.434 0.505	0.470	2.578 2.654	2.62	86%		75.1		
	1	320	0.225 0.203	0.214	1.301 1.345	1.32	89%		68.5		
	2	320	0.223 0.187	0.205	1.459 1.516	1.49	103%		65.6		
	1	640	0.106 0.104	0.105	0.697 0.671	0.68	93%		67.2		
	2	640	0.13 0.122	0.126	0.734 0.687	0.71	94%		80.6		
Post-AEX	1	5	0.269 0.284	0.277	5.025 4.675	4.85	91%	89%	1.4	1.7	2.2
	2	5	0.404 0.415	0.410	4.099 4.312	4.21	76%		2.0		
	1	10	0.122 0.123	0.123	2.359 2.126	2.24	85%		1.2		
	2	10	0.184 0.185	0.185	2.161 2.252	2.21	81%		1.8		
	1	20	<LOQ <LOQ	<LOQ	1.112 1.316	1.21	97%		<LOQ		
	2	20	0.098 0.105	0.102	1.334 1.252	1.29	95%		2.0		
	1	40	<LOQ <LOQ	<LOQ	0.592 0.572	0.58	93%		<LOQ		
	2	40	0.048 0.047	0.048	0.622 0.624	0.62	92%		1.9		

Table 4: Accuracy testing of the Eshmuno® A ligand in antibody downstream process using the Cygnus F400 Protein A ELISA kit

	Day	Dilution	Result (ng/mL)	Average Result (ng/mL)	Dilution Corrected Average Result (ng/mL)	Average Unspiked Result (ng/mL)	Standard Deviation	%CV	Overall Average Result (ng/mL)	Overall Standard Deviation (ng/mL)	Overall %CV (Repeatability)
Eshmuno® A Elution	1	800	0.72 0.688	0.704	563.2	584.2	29.70	5.1%	579	46.97	8.1%
	2	800	0.725 0.788	0.757	605.2						
	1	1600	0.352 0.344	0.348	556.8	513.6	61.09	11.9%			
	2	1600	0.272 0.316	0.294	470.4						
	1	3200	0186 0.182	0.184	588.8	596	10.18	1.7%			
	2	3200	0.189 0.188	0.189	603.2						
	1	6400	<LOQ <LOQ	<LOQ	<LOQ	624	NA	NA			
	2	6400	0.101 0.094	0.098	624						
Post-CEX	1	80	0.829 0.842	0.836	66.8	71.96	7.24	10.1%	70	3.19	4.5%
	2	80	0.99 0.937	0.964	77.1						
	1	160	0.406 0.362	0.384	61.4	68.28	9.67	14.2%			
	2	160	0.434 0.505	0.470	75.1						
	1	320	0.225 0.203	0.214	68.5	67.04	2.04	3.0%			
	2	320	0.223 0.187	0.205	65.6						
	1	640	0.106 0.104	0.105	67.2	73.92	9.50	12.9%			
	2	640	0.13 0.122	0.126	80.6						
Post-AEX	1	5	0.269 0.284	0.277	1.4	1.715	0.47	27.4%	2	0.25	14.2%
	2	5	0.404 0.415	0.410	2.0						
	1	10	0.122 0.123	0.123	1.2	1.535	0.44	28.6%			
	2	10	0.184 0.185	0.185	1.8						
	1	20	<LOQ <LOQ	<LOQ	<LOQ	2.03	NA	NA			
	2	20	0.098 0.105	0.102	2.0						
	1	40	<LOQ <LOQ	<LOQ	<LOQ	NA	NA	NA			
	2	40	<LOQ <LOQ	0.048	1.9						

Table 5: Precision testing of the Eshmuno® A Protein A ligand in antibody downstream process using the Cygnus F400 Protein A ELISA Kit

Conclusion

This work has demonstrated the performance of the Cygnus Protein A ELISA Kit (Cat# F400) with samples containing Merck Millipore's Eshmuno® A Protein A ligand. The initial feasibility testing has demonstrated that the Cygnus F400 Protein A ELISA Kit could accurately measure the Eshmuno® A Protein A ligand as is in sample diluent with a spike recovery of 101%. In phase II, the accuracy and precision of the ELISA was assessed using samples from a 3-step antibody purification process. The F400 Protein A ELISA Kit performed within the generally accepted specifications. Based on this testing, the Cygnus F400 Protein A ELISA is qualified for measuring the Eshmuno® A Protein A ligand in a typical downstream process using Eshmuno® A resin. Similar testing should be performed for other purification processes and products prior to instituting this kit as part of routine testing.

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