Development and Validation of a Sensitive Anti-PEG IgG Assay

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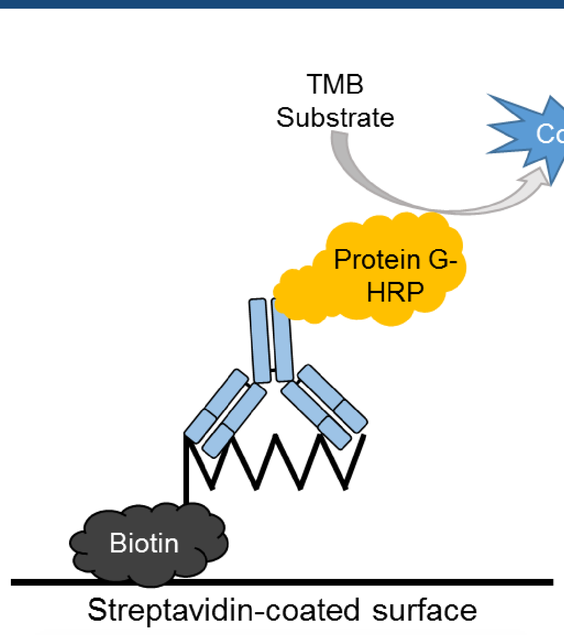
ABSTRACT

PEGylated biotherapeutics administered to patients can elicit immune responses and formation of anti-drug antibodies, which may lead to reduction of drug efficacy, and adverse safety consequences. Anti-drug antibodies (ADA) generated against PEGylated biotherapeutics include anti-protein and anti-PEG antibodies. It is recommended by the FDA Guidance for Industry on Immunogenicity Assessment for Therapeutic Protein Products that ADA assays for PEGylated therapeutic proteins should be able to detect both anti-protein and anti-PEG antibodies. So far, efforts to develop methods for detection of anti-PEG IgG and IgM antibodies in human serum failed to demonstrate sufficient assay sensitivity and specificity. Here we describe development and validation of a sensitive, specific, and robust anti-PEG IgG assay in human serum with the LOD of 234 ng/mL and the LLOQ of 469 ng/mL based on a mouse monoclonal anti-PEG IgG antibody used as a positive control. This assay is applicable to human and non-human sera.

INTRODUCTION

The conjugation of polyethylene glycol (PEG) to therapeutics is commonly used to increase a drug's half-life in circulation. It has been generally accepted that PEGylated molecules as well as PEG alone are of low immunogenicity. However, emerging studies have reported that PEGylated therapeutics could be immunogenic (Garay and Labaune, 2011, Yang and Lai, 2015) and that anti-PEG antibodies can cause reduction in drug efficacy (Armstrong et al., 2007) and even anaphylactic reactions (Poindinger et al., 2016). It is, therefore, critical to develop and implement specific methods for anti-PEG antibodies detection in conjunction with monitoring of ADA responses directed against PEGylated biopharmaceuticals. Despite reported efforts, development of a sensitive, robust, and specific anti-PEG IgG assay in human serum remains to be challenging (Schellekens et al., 2013). Amongst the published papers, the highest assay sensitivity achieved for an anti-PEG IgG assay was 800-1,000 ng/mL in human serum using an acoustic membrane microparticle platform (Dong et al., 2015). Here we present a validated anti-PEG IgG ELISA assay with an unparalleled LOD of 234 ng/mL and the LLOQ of 469 ng/mL.

ASSAY DESIGN

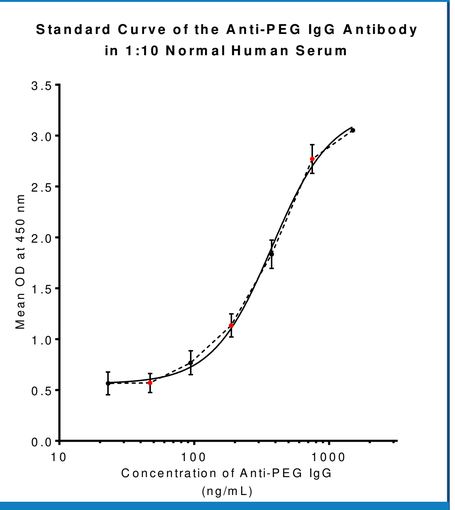


- Capture with biotin-conjugated methoxy PEG (mPEG, 5 kDa MW) in a streptavidin 96-well assay plate
- Universal IgG detection for different species with Protein G-HRP detection reagent
- Wash Buffer and Assay Diluent supplemented with an alternative detergent which does not interfere PEG-anti-PEG binding
- Assay Diluent and wash protocol optimized to lower assay background
- Positive Control Reagent (PC) used is a commercial mouse anti-PEG monoclonal IgG antibody

RESULTS – ASSAY VALIDATION

STANDARD CURVE

- The standard curve of anti-PEG IgG antibody assay was plotted using a commercial mouse anti-PEG monoclonal IgG antibody titrated in 1:10 normal human serum. Error bars represent standard deviations for each of the data points.



PRECISION

- The intra-assay and inter-assay precision of all tested concentration of anti-PEG IgG PC and the NC are ≤14.6% and ≤19.7%, which met the industry-wide acceptance criterion of %CV ≤20%.

Intra-assay Precision

Concentration of Anti-PEG IgG PC				
Concentration in 1:10 NHS (ng/mL)	Calculated Concentration in Neat Serum (ng/mL)	*Mean OD at 450 nm	*SD	*%CV
1,500	15,000	3.042	0.010	0.3
750	7,500	2.822	0.162	5.8
375	3,750	1.893	0.128	6.8
187.5	1875	1.065	0.108	10.1
93.8	938	0.698	0.087	12.4
46.9	469	0.560	0.082	14.6
23.4	234	0.528	0.025	4.8
NC (0)	NC (0)	0.431	0.033	7.7

* Mean OS, SD, and %CV were calculated from 6 replicate wells (n=3 in duplicate wells in a single run

Inter-assay Precision

Concentration of Anti-PEG IgG PC				
Concentration in 1:10 NHS (ng/mL)	Calculated Concentration in Neat Serum (ng/mL)	**Mean OD at 450 nm	**SD	**%CV
1,500	15,000	**3.052**	0.016	0.5%
750	7,500	**2.771**	0.141	5.1%
375	3,750	**1.834**	0.139	7.6%
187.5	1875	**1.135**	0.114	10.1%
93.8	938	**0.767**	0.117	15.3%
46.9	469	**0.570**	0.094	16.5%
23.4	234	**0.565**	0.111	19.7%
NC (0)	NC (0)	**0.365**	0.047	12.8%

** Mean OS, SD, and %CV were calculated from 24 replicate wells (n=12 in duplicate wells in three runs by two analysts

SENSITIVITY

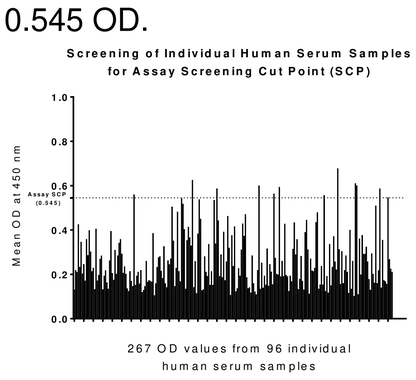
- The assay sensitivity was determined on the basis of reproducibility of the titration of the anti-PEG IgG PC.
- The limit of detection (LOD) is 234 ng/mL.
- The lower limit of quantification (LLOQ) is 469 ng/mL.

Concentration of Anti-PEG IgG PC						t-test (OD signal of each PC concentration compared to NC)	
Concentration in 1:10 NHS (ng/mL)	Calculated Concentration in Neat Serum (ng/mL)	**Mean OD at 450 nm	**SD	**%CV	**s/n Ratio	P-value	Significantly different
1,500	15,000	**3.052**	0.016	0.5%	8.36	< 0.05	Yes
750	7,500	**2.771**	0.141	5.1%	7.59	< 0.05	Yes
375	3,750	**1.834**	0.139	7.6%	5.02	< 0.05	Yes
187.5	1875	**1.135**	0.114	10.1%	3.11	< 0.05	Yes
93.8	938	**0.767**	0.117	15.3%	2.10	< 0.05	Yes
46.9	**469**	**0.570**	0.094	16.5%	1.56	< 0.05	**Yes**
23.4	**234**	**0.565**	0.111	19.7%	1.55	< 0.05	**Yes**
NC (0)	NC (0)	**0.365**	0.047	12.8%	1.00	N/A	N/A

** Mean OS, SD, and %CV were calculated from 24 replicate wells (n=12 in duplicate wells in three runs by two analysts

SCREENING CUT POINT

- The assay screening cut point (SCP) was determined by screening 96 normal human donors, assayed three times on two days by two analysts.
- Raw data set were combined from three runs and tested for distribution normality using Shapiro-Wilk test. Neither untransformed nor log-transformed data showed the normal distribution.
- Statistical outliers were removed by inter-quartile range (IQR) analysis.
- A total of 267 OD readings were used to calculate the SCP using non-parametric method (95th percentile).
- The SCP is 0.545 OD.



SPECIFICITY & CONFIRMATION CUT POINT

- The specificity of the assay was confirmed by the ability of an excess mPEG to inhibit the PC antibody-generated signal.
- The confirmation cut point (CCP) was determined by testing the PC in the absence or presence of excess mPEG molecules.
- The percentage signal inhibition (%SI) of each sample was calculated using the formula:

$$Percentage\ Signal\ Inhibition = \left[1 - \left(\frac{\text{Mean OD signal, treated with excess PEG}}{\text{Mean OD signal, untreated}}\right)\right] x\ 100\%$$

Concentration of Anti-PEG IgG PC		% Signal Inhibition (in the presence of 25 µg/well excess PEG MW 5 kDa)
Concentration in 1:10 NHS (ng/mL)	Calculated Concentration in Neat Serum (ng/mL)	
1,500	15,000	**88.9**
750	7,500	**87.2**
375	3,750	**79.2**
187.5	1875	**64.4**
93.8	938	**49.8**
46.9	469	**31.0**
23.4	234	**28.1**
NC (0)	NC (0)	**13.9**
Paired t-test		**NC v.s. 234 ng/mL**
P-value		0.0006
Significantly difference? (P < 0.05)		**Yes**

- A paired t-test was used to compare the %SI of each PC concentration with the %SI of the NC.
- The CCP was determined by the lowest PC concentration which has a significant deference of %SI compared to that of the NC.
- The CCP is 28.1%SI, at the LOD of 234 ng/mL.

SYSTEM SUITABILITY CONTROLS & ASSAY ACCEPTANCE CRTITERIA

- System suitability controls including HQC, MQC, LQC, and NC were selected. Assay acceptance range criteria were defined from the data obtained in inter-assay precision runs, and based on Mean ± 3X SD).

System Suitability Controls	Assay Acceptance Criteria	
	Mean OD at 450 nm	Range (Mean ± 3X SD)
HQC is 7,500 ng/mL in neat serum (or 750 ng/mL in 1:10 serum)	2.771	2.348 – 3.193
MQC is 1,875 ng/mL in neat serum (or 187.5 ng/mL in 1:10 serum)	1.135	0.792 – 1.478
LQC is 469 ng/mL in neat serum (or 46.9 ng/mL in 1:10 serum)	0.570	0.545* – 0.852
Negative Control (NC)	0.365	0.225 – 0.505
HQC, MQC, LQC, NC	≤20% CV	

*Acceptance range of LQC was adjusted to 0.545 – 0.852 since LQC should be above the SCP

CLINICAL SAMPLE ANALYSIS

- The validated anti-PEG IgG antibody assay was used to detect the presence of anti-PEG antibodies in clinical human serum samples from patients dosed with PEGylated therapeutic protein.
- Among the four subjects were analyzed, one of them (subject #3) was screened and confirmed positive at all tested time points.
- An additional serum sample, found positive in the screening and confirmatory assay was used as the human positive control.
- The titer of all tested samples were between 1:10 to 1:80. Subject #3 had the highest antibody titer across all time points (i.e. 1:80), and the titer levels were similar to the human positive control.

Subject	Time Point	Unspiked Sample			Spiked Sample			%SI	Screening assay result	Confirmatory assay result	Titer
		Mean OD	SD	%CV	Mean OD	SD	%CV				
1	A	0.324	0.026	8.1	0.132	0.002	1.2	59.1	Negative	**Positive**	1:10
	B	0.439	0.040	9.0	0.140	0.010	7.1	68.1	Negative	**Positive**	1:40
	C	0.335	0.024	7.0	0.193	0.013	6.6	42.3	Negative	**Positive**	1:10
	D	0.380	0.020	5.2	0.159	0.004	2.7	58.2	Negative	**Positive**	1:20
	E	0.300	0.019	6.4	0.135	0.004	2.6	54.9	Negative	**Positive**	1:10
2	A	0.241	0.030	12.3	0.250	0.017	6.6	-4.0	Negative	Negative	ND
	B	0.235	0.009	3.6	0.202	0.008	3.7	14.2	Negative	Negative	ND
	C	0.221	0.013	6.0	0.193	0.028	14.5	12.8	Negative	Negative	ND
	D	0.169	0.016	9.5	0.145	0.004	2.5	14.4	Negative	Negative	ND
	E	0.177	0.014	7.8	0.179	0.020	11.1	-1.1	Negative	Negative	ND
	F	0.153	0.004	2.7	0.169	0.010	5.6	-10.2	Negative	Negative	ND
	G	0.172	0.008	4.7	0.182	0.026	14.4	-6.0	Negative	Negative	ND
	H	0.171	0.019	11.1	0.187	0.016	8.8	-9.6	Negative	Negative	ND
	I	0.168	0.016	9.8	0.186	0.034	18.5	-10.7	Negative	Negative	ND
	J	0.177	0.007	3.8	0.166	0.005	3.1	6.2	Negative	Negative	ND
	K	0.163	0.008	4.9	0.152	0.009	6.1	6.8	Negative	Negative	ND
	L	0.203	0.020	9.9	0.187	0.021	11.1	8.0	Negative	Negative	ND
	M	0.191	0.001	0.5	0.200	0.010	4.8	-4.5	Negative	Negative	ND
	N	0.177	0.013	7.4	0.197	0.010	4.9	-11.5	Negative	Negative	ND
	O	0.188	0.013	6.9	0.195	0.022	11.4	-3.7	Negative	Negative	ND
	P	0.180	0.017	9.6	0.198	0.010	5.0	-10.0	Negative	Negative	ND
3	A	1.033	0.046	4.5	0.184	0.003	1.6	82.2	**Positive**	**Positive**	1:80
	B	1.327	0.022	1.7	0.167	0.014	8.4	87.4	**Positive**	**Positive**	1:80
	C	0.960	0.099	10.3	0.125	0.004	3.0	87.0	**Positive**	**Positive**	1:80
4	A	0.294	0.061	20.7	0.168	0.018	10.6	42.8	Negative	**Positive**	1:40
	B	0.280	0.033	11.7	0.172	0.001	0.7	38.8	Negative	**Positive**	1:20
	C	0.257	0.025	9.6	0.165	0.013	8.1	35.6	Negative	**Positive**	1:20
	D	0.338	0.025	7.3	0.173	0.024	13.9	49.0	Negative	**Positive**	1:40
	E	0.398	0.030	7.5	0.163	0.017	10.4	58.9	Negative	**Positive**	1:40
Positive control		0.628	0.029	4.6	0.347	0.020	5.6	44.8	**Positive**	**Positive**	1:80

ND: Not determined

CONCLUSION

- An anti-PEG IgG robust assay with unparalleled sensitivity was successfully developed and validated in human serum.
- The LOD and LLOQ were determined to be 234 and 469 ng/mL respectively.
- The assay was successfully used to detect anti-PEG IgG antibodies in clinical human serum samples.
- This assay is applicable to human and non-human sera.

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