ELISA Assay for Determination of Bevacizumab (Pharmacokinetic), VEGF–A (Pharmacodynamic) and Immunogenicity Assessment

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INTRODUCTION: Bevacizumab is a neutralizing anti-vascular endothelial growth factor A (VEGF-A) humanized IgG1 monoclonal antibody (MAb), used to inhibit angiogenesis in cancer. Bevacizumab binds to all VEGF-A isoforms, inhibiting their binding to VEGF receptors. Bevacizumab is effective in the treatment of colorectal, breast, lung, and kidney cancers, in which it acts in synergy with conventional anticancer drugs and interferon.

PHARMACOKINETIC ASSAY

ANALYTICAL METHOD SUMMARY

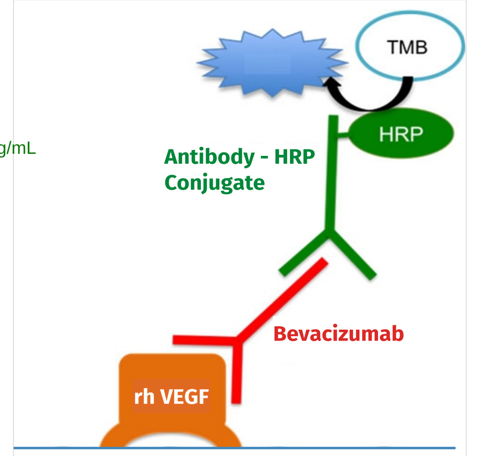
Analyte : Bevacizumab (Avastin Inj. 25mg/mL)* Space : 25 mg/mL

Calibration Curve Range : 2 – 200 µg/mL

Quality Controls (µg/mL) : 2 (LLOQ), 6 (LQC), 85 (MQC), 165 (HQC) & 200 (ULOQ)

Assay Volume : 25 µL

Sample Preparation Method : MRD (1:200) in 1% BSA solution.

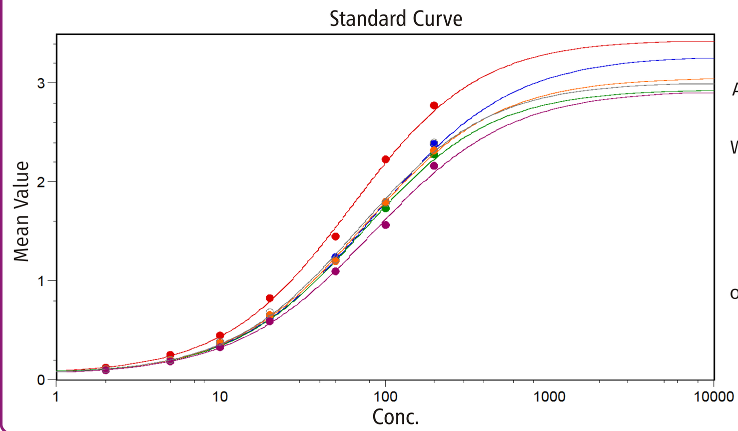


Bevacizumab (Mab)-based indirect ELISA assay was developed using microtiter plates sensitized with a recombinant form of VEGF (rhVEGF) was validated.

Diluted serum samples were pipetted and incubated in the micro plate wells previously coated with purified rhVEGF. The wells were washed to remove any unbound sample components and then incubated with HRP labeled protein A. Unbound labeled protein A was removed and a chromogenic substrate added, resulting in development of the colored reaction product which was directly proportional to the amount of bevacizumab present in the serum sample. The Microplate was analyzed using a colorimetric micro plate reader.

CALIBRATION CURVE

Representative Calibration Curves for Becacizumab in Human Serum, [Concentration vs MeanValue (OD)]



Absorbance readings were performed at 450nm. Wavelength correction was performed at 620nm for optical imperfections. Calibration curves obtained on separate occasions using 4-PL curve and r^2 value was calculated.

* Title of Table

Calibration std. conc. (µg/mL)	Mean calculated conc. (µg/mL)	Std. deviation	% CV	% Bias	Mean Accuracy	n
200	206.2	17.39	8.4	3.1	103	6
100	101.7	8.613	8.5	1.7	102	6
50	49.58	3.935	7.9	0.8	99.2	6
20	19.64	1.523	7.8	1.8	98.2	6
10	10.34	0.29	2.8	3.4	103	6
5	5.119	0.219	4.3	2.4	102	6
2	1.921	0.041	2.1	4	96.1	6

ACCURACY AND PRECISION

Representative QC statistics

Intra-day

QC conc. (µg/mL)	Mean calculated conc. (µg/mL)	Std. deviation	% CV	% Bias	Mean Accuracy	n
200.0	188.447	28.874	15.3	-5.8	94.2	1
165.0	161.708	19.497	12.1	-2.0	98.0	1
85.00	77.297	9.641	12.5	-9.1	90.9	1
6.000	5.623	0.520	9.2	-6.3	93.7	1
2.000	1.897	0.136	7.2	-5.2	94.9	1

Inter-day

QC conc. (µg/mL)	Mean calculated conc. (µg/mL)	Std. deviation	% CV	% Bias	Mean Accuracy	n
200.0	186.265	23.928	12.8	-6.9	93.1	6
165.0	153.954	15.115	9.8	-6.7	93.3	6
85.00	79.869	9.779	12.2	-6.0	94.0	6
6.000	6.039	0.673	11.1	0.6	101.0	6
2.000	1.994	0.287	14.4	-0.3	99.7	6

Results: Bevacizumab results were evaluated using 4 parameter logistic regression weighted 1/Y2 over the analytical range 2.0 to 200 µg/mL. The concentrations of bevacizumab standards were back calculated using regression equation and the coefficient of variation (CV) was less than or equal to 8.5 percent. Inter batch precision (%CV) and inter batch accuracy (% bias) of bevacizumab QC samples was less than 14.5 and -6.9 respectively

STABILITY

Theoretical Bevacizumab Conc. (µg/mL)	Bench Top Stability at RT after 26 Hrs.	Freeze & Thaw cycles (six)	Long Term Stability (after 90 days)
6	-12.2	-3.5	-11.1
165	-6.2	-4.3	-3.2

The absolute recoveries (LQC & HQC, each six sets) of Bevacizumab reported as mean %Bias were determined by comparing with calibration curve and QC samples (freshly prepared).

SPECIFICITY

	RA sera 1		RA sera 2		Trastuzumab	
	2.0 µg/mL (LLOQ)	200.0 µg/mL (ULOQ)	2.0 µg/mL (LLOQ)	200.0 µg/mL (ULOQ)	2.0 µg/mL (LLOQ)	200.0 µg/mL (ULOQ)
Reported Mean (n=6) conc. (µg/mL)	1.930	166.200	1.847	177.600	2.086	226.726
% DEV	1.1	1.7	2.4	3.1	1.9	4.9
% Bias	-3.5	-16.9	-7.7	-11.2	4.3	13.4

The specificity was established by spiking 250.0µg/mL of structurally related drug (Trastuzumab) in to respective concentrations of bevacizumab QC level (LLOQ & ULOQ) in normal sera and compared with calibration curve and QC controls (freshly prepared).The experiment was also performed in diseased sera (rheumatoid factors) for any matrix interference.

SELECTIVITY

Theoretical Concentration 2.00 µg/mL (LLOQ)

Serum type / ID	Reported Mean (n=3) Conc. (µg/mL)	% DEV	% Bias
Lipemic	1.926	5.2	-3.7
Haemolysed (5%)	2.362	6.2	18.1
743 *	2.153	6.1	7.7
744 *	1.792	8.2	-10.4
746 *	2.219	8.1	11.0
747 *	2.306	3.2	15.3
689 *	1.968	3.3	-1.7
690 *	1.927	5.2	-3.7
691 *	1.959	12.1	-2.1

* Normal Human Serum

The selectivity of Bevacizumab was determined by calculating the %DEV of three replicates for each serum spiked at the LLOQ level. Out of ten source of individual serum (Haemolysed serum, Lipemic serum & 8 normal serums), nine were within acceptable criteria however only one serum was failed in %bias (54.9%).

DILUTION INTEGRITY

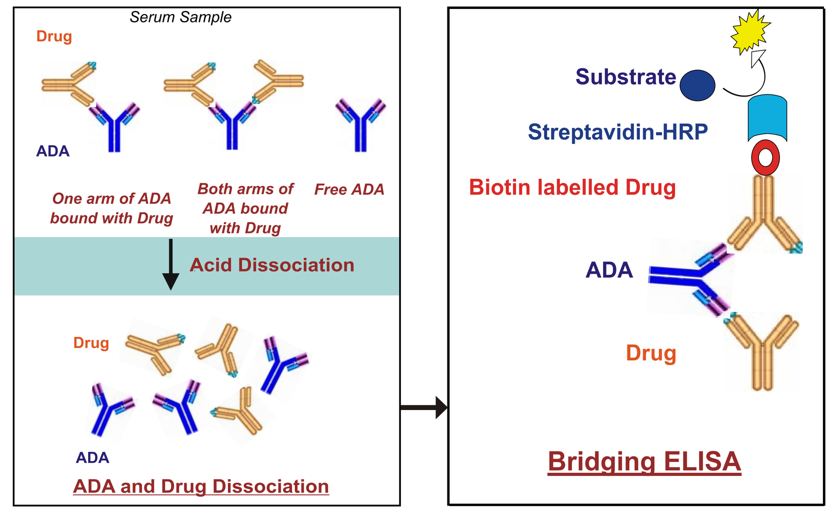
	DHQC (2 fold dilution)	DIQC (2 fold dilution)
Theoretical Conc. (µg/mL)	165.000	380.000
Reported Mean (n=6) conc. (µg/mL)	150.855	376.261
% dev	5.1	11.8
% Bias	-8.6	-1.0

Six replicates of each HQC sample (165 µg/mL) and over-the- curve QC sample (380 µg/mL) were diluted 2 times, referred as DHQC (Diluted High QC) and DIQC (Dilution Integrity QC) respectively. The obtained each result multiplied by 2 and reported as mean concentration

IMMUNOGENICITY ASSAY

An acid dissociation bridging ELISA method for detecting antibodies against bevacizumab was developed and optimized in line with the tiered approach (screening, confirmation by drug displacement and titration).

Diluted serum samples and biotin labeled bevacizumab were both pipetted onto the micro plate wells previously coated with bevacizumab. The plate was then incubated to allow the ADA to form bridge between the labeled and unlabeled drug. HRP labeled streptavidin was then added to the washed microplate. Unbound streptavidin-HRP was removed and a chromogenic substrate added, resulting in development of the colored reaction product, which was proportional to the amount of ADA present in serum.



ANALYTICAL METHOD SUMMARY

Analyte : Anti-Bevacizumab monoclonal antibody

Method : Acid dissociation bridging ELISA

Quality controls (ng/ml) : 5500 (HQC), 450 (LQC)

Assay volume : 50 ul

Sample preparation method : 1:10 Dilution with 0.3 M glacial acetic acid for acid dissociation of sample.

Matrix : Human Serum

STABILITY

Theoretical Anti-Bevacizumab Conc. (ng/mL)	Bench Top Stability at RT after 19 Hrs. (% bias)	Freeze & Thaw cycles (four) (% bias)
450.0 (LQC)	6.1	-4.8
5500 (HQC)	-0.5	2.9

Short term stability of anti- bevacizumab antibody in serum was established for 19 hrs. at ambient temperature. Freeze and thaw stability of the antibody in serum was established for four freeze (-20 ± 10 c) and thaw cycles.

PRECISION

QC conc. (ng/mL)	Intra-assay precision (%CV)	Inter-assay precision (%CV)
450.0 (LQC)	12.8	8.8
5500 (HQC)	2.2	6.7
NQC	8.0	20.0

For ADA assay, intra and inter batch precision (%CV) of QC samples was -2.2 to 12.8 % and 6.7 to 8.8 % respectively.

Matrix Interference :
Percent differences in response between buffer and serum QC was -2.1 to -8.3.

DRUG TOLERANCE

Antibody conc. (ng/mL)	Drug conc. (ug/ml)	Mean response (OD)	Antibody conc. (ng/mL)	Drug conc. (ug/ml)	Mean response (OD)
5500	0	2.111	450	0	0.176
5500	1	0.809	450	1	0.082
5500	3	0.428	450	3	0.048
5500	5	0.293	450	5	0.035
5500	10	0.116	450	10	0.021
5500	20	0.050	450	20	0.015

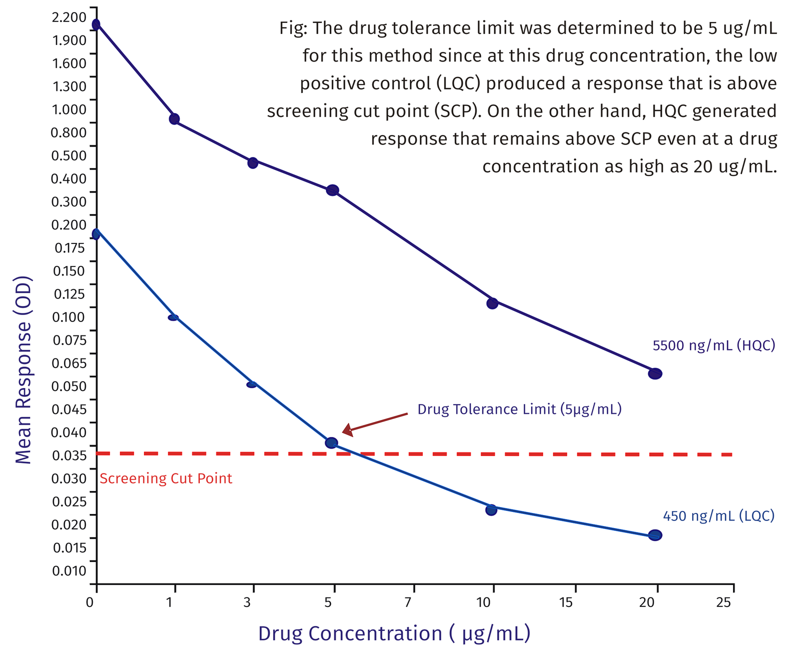


Fig: The drug tolerance limit was determined to be 5 ug/mL for this method since at this drug concentration, the low positive control (LQC) produced a response that is above screening cut point (SCP). On the other hand, HQC generated response that remains above SCP even at a drug concentration as high as 20 ug/mL.

SENSITIVITY

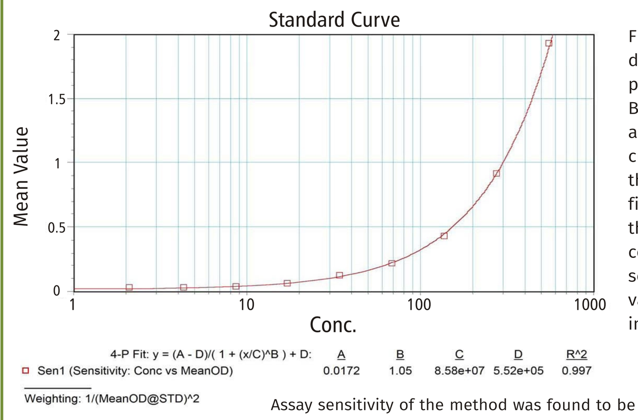


Fig: Representative dilution curve of the positive control anti Bevacizumab antibody. Dilution curves were fitted by the 4 – PL curve fitting method and the corresponding concentration at the screening cut point value were interpolated.

Assay sensitivity of the method was found to be 43 ng/ml

*Sensitivity currently stands at 124 ng/ml.

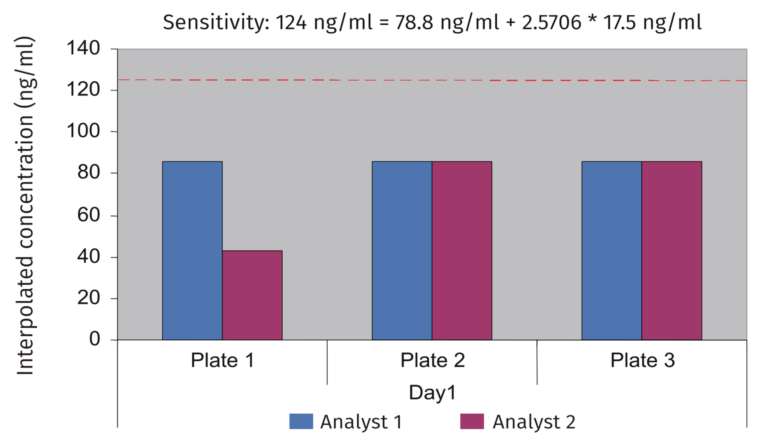


Fig: Interpolated antibody concentration at the screening cut point compared to sensitivity calculated for a 95% consistency level (Sensitivity = Mean interpolated concentration at cut point + t0.05, df * SD)

TITRATION ASSAY

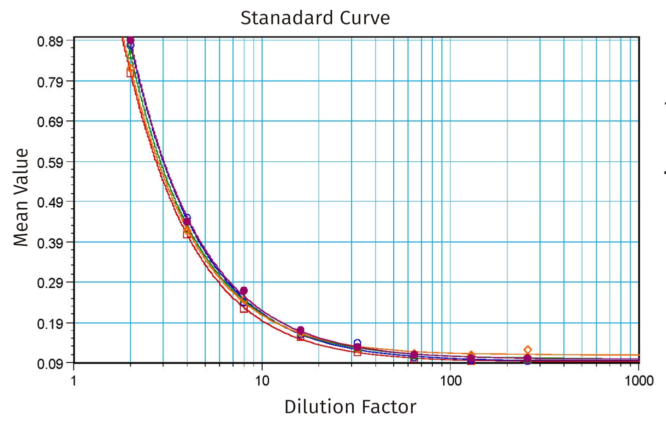


Fig: Representative titration assay curve of five mock ADA positive samples

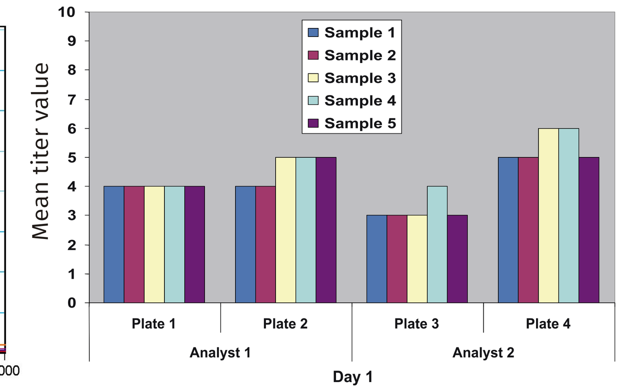
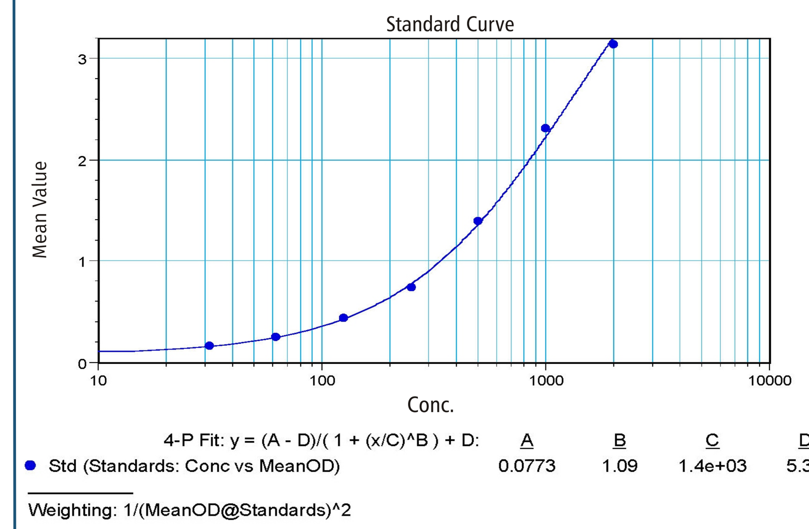


Fig: Mean titer value of five mock positive ADA samples analyzed across four assay plates by two analysts on a single day. Titer is expressed as log of dilution factor.

PHARMACODYNAMIC ASSAY (VEGF-A): COMMERCIAL KIT BASED SANDWICH ELISA ASSAY

Serum samples are pipetted into Anti – VEGF pre-coated microplate wells and VEGF present in sample is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked anti –VEGF polyclonal antibody was added to the wells. A substrate solution then was added to the wells and color developed in proportion to the amount of VEGF present in the serum was measured.



Sample ID	Nominal Conc. (pg/mL)	Mean Calculated Conc. (pg/ml)	% CV	% Bias	n
std1	2000	1957.60	3.0	-2.1	5
std2	1000	1062.27	8.3	6.2	5
std3	500.0	503.494	7.9	0.7	5
std4	250.0	238.802	3.5	-4.5	5
std5	125.0	129.818	7.2	3.9	5
std6	62.50	60.9260	5.8	-2.5	5
std7	31.25	32.9310	6.1	5.4	5
std8	0.000	Range?	17.4	Range?	5

Result: Coefficient of variation (CV) of back calculated concentrations of VEGF calibrators was less than or equal to 8.3 percent.

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CONCLUSION: A rapid, cost-effective, accurate and reproducible assay for analysis of bevacizumab, VEGF–A and quasi-quantitative assay for anti- bevacizumab antibodies in human serum was developed.