Development of ELISA Kit for the Measurement of Etanercept and Anti-Etanercept in Serum and Plasma

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Background:

Etanercept is a fusion protein produced by recombinant DNA. It fuses the TNF receptor to the constant end of the IgG1 antibody. First, the developers isolated the DNA sequence that codes the human gene for soluble TNF receptor 2, which is a receptor that binds to tumor necrosis factor-alpha. Second, they isolated the DNA sequence that codes the human gene for the Fc end of immunoglobulin G1 (IgG1). Third, they linked the DNA for TNF receptor 2 to the DNA for IgG1 Fc. Finally, they expressed the linked DNA to produce a protein that links the protein for TNF receptor 2 to the protein for IgG1 Fc. It has U.S. F.D.A. approval to treat rheumatoid, juvenile rheumatoid and psoriatic arthritis, plaque psoriasis and ankylosing spondylitis. TNF-alpha is the "master regulator" of the inflammatory (immune) response in many organ systems.

Objective/Purpose:

The Etanercept ELISA Kit is used to quantify the Etanercept present in serum/plasma in the pharmacokinetic (PK) studies, whereas the Anti-Etanercept ELISA Kit is served as a clinician tool to evaluate the immunogenicity, safety, and efficacy of Etanercept.

Materials & Methods:

Etanercept and Anti-Etanercept Kit method employs the sandwich enzyme immunoassay technique. The assay development was carried out using the commercially available Enbrel® injection. The secondary antibody was conjugated to horse raddish peroxidase (HRP). Microtiter plate (Corning Costar, USA) were coated with monoclonal antibodies and checkerboard titration method was carried out using different permutation and combination of coating antibody, blocking stabilizers (Surmodics Inc, USA), secondary antibody, assay diluent and substrates to achieve the best noise:signal ratio.

The kit was standardized, validated, and optimized for its sensitivity, specificity & cross reactivity, precision, accuracy and reproducibility. The Kit also showed enhanced shelf life, good spiking recovery and dilution linearity.

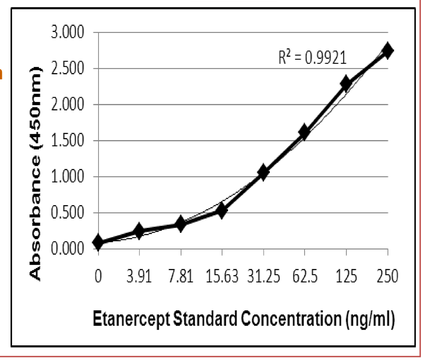
Results:

A] *Etanercept ELISA KIT*:

Quantitative determination of Etanercept in serum and plasma

Standard Curve for the Etanercept ELISA was performed using the required reagent concentrations. Figure 1 shows the standard curve obtained from the Etanercept ELISA. The standards were prepared at various concentrations in assay diluent and protein stabilizers.

Figure1



Etanercept spiked with serum and treated as unknown samples	Mean Abs	%CV	Observed	Expected	% Recovery
0.78	0.198	13.928	2.38	0.78	305.13
1.56	0.1945	3.999	1.99	1.56	127.56
3.125	0.29	2.438	3.73	3.125	119.36
6.25	0.421	0.336	6.09	6.25	97.44
12.5	0.53	8.005	8.1	12.5	64.80
25	1.2325	3.729	24.2	25	96.80
50	1.825	1.937	47	50	94.00
100	2.48	1.140	106	100	106.00
200	2.8575	4.578	177	200	88.50

Etanercept was spiked into serum and was treated as unknown samples and the ELISA was carried out and following characteristics were concluded.

Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. 10 replicates of '0' standards were evaluated and the LOD was found to be 1.56ng/ml.

Limit of Quantitation: It is defined as the lowest concentration for which Coefficient of Variation is <20%. The LOQ is found to be 3.125ng/ml.

Hook Effect: *A high dose hook is indicated in the plotted curve when the assay is saturated by high antigen concentrations.*

Increasing concentrations of Etanercept >250 ng/ml were assayed as unknowns. The hook capacity yielding an absorbance reading less than the 250 ng/ml standard was 6mg/ml.

Recovery by Spiking:

Spiking & Recovery	Mean Abs	%CV	Observed	Expected	% Recovery
Neat	2.925	1.21	530	500	106.00
1:5 dilution	2.476	1.46	529	500	105.80
1:10 dilution	1.895	0.37	508	500	101.60
1:50 dilution	0.725	4.88	596	500	119.20
1:100 dilution	0.388	2.74	548	500	109.60
1:1000 dilution	0.181	16.41	1730	500	346.00

In the above table the data represents, a known amount of analyte is added (spiked) into the serum followed by various fold of dilution. Different serum matrix (Matrix A, Matrix B…) were tried and the best serum matrix was chosen and was the one which gave the best recovery; also on the same basis the dilution factor for serum/plasma was decided.

In the above table, 1:1000dilution factor gave a recovery of 346% which is not acceptable. Also, dilution factor of 1:10 gave the best recovery hence that dilution factor was recommended.

B] *Anti-Etanercept ELISA KIT*:

Qualitative determination of antibodies to Etanercept (Enbrel®) in serum and plasma

In Anti-Etanercept ELISA, the controls (Positive & Negative Controls) were run, the results were as in the below table.

Controls	Abs 1	Abs 2	Mean Abs
Blank	0.070	0.078	0.074
Negative Control	0.077	0.076	0.076
Positive Control	1.899	1.903	1.901

Monoclonal Antibody (Etanercept-Ag) was coated on to the Microtiter plate, the Anti-Etanercept-Ab was spiked into serum for observing the percentage recovery.

Figure 2

Anti-Etanercept Ab (ng/mL)	Mean Abs	%CV	Observed	Expected	% Recovery
0	0.1045	7.44	0	0	0
7.81	0.1395	3.55	10	7.81	128.0
15.63	0.1675	2.96	15.98	15.63	102.2
31.3	0.1865	26.16	35	31.3	111.8
62.5	0.292	5.33	69	62.5	110.4
125	0.4565	3.87	127	125	101.6
250	1.038	0.68	245	250	98.0
500	1.701	6.65	506	500	101.2
1000	2.606	0.87	1050	1000	105.0

Results: The minimum concentration up to which antibodies to Etanercept can be detected in patient samples are 10ng/ml

Conclusion:

- The Krishgen Etanercept ELISA has been developed for fast, reproducible and specific quantification of Etanercept concentrations in plasma and serum in pharmacokinetic studies.
- The Krishgen Anti-Etanercept ELISA Kit can be efficiently used for monitoring antibodies to Etanercept during therapy and offers the clinician a tool for decision on possible preventive measures such as possible addition of immunosuppressive drug to reduce Anti-Etanercept antibodies. With this ELISA test, antibodies to Etanercept can be detected in patients receiving Etanercept (Enbrel®)