ELISA VALIDATION GUIDE

CYTOKINE ASSAYS FOR USE IN DRUG DISCOVERY RESEARCH, BIOPHARMA AND CELL & GENE THERAPY APPLICATIONS

KRISHGEN BioSystems

OUR REAGENTS, YOUR RESEARCH

VALIDATION OF PRECISIONBIND HUMAN TNFa / TNA-alpha / TNFA ELISA KIT (Catalog No KB1145) AS PER FDA/ICH GUIDELINES FOR BIOANALYTICAL METHOD VALIDATION

This validation protocol has been adopted in line with the Methodology and Analytical Procedures Guideline recommended by FDA/ICH.

Document History

First Codification	History	Date
Version#1	**VALIDATION DATA OF PRECISIONBIND HUMAN TNFa / TNA-alpha / TNFA ELISA KIT (Catalog No KB1145)**	31.07.2025

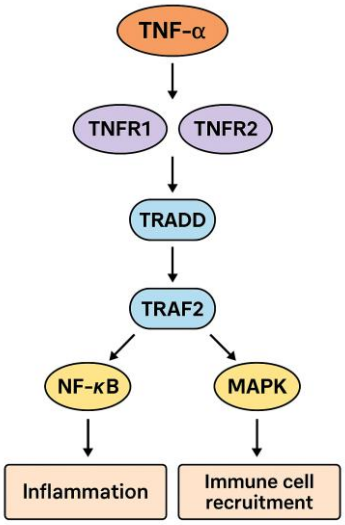
Approved Quality Control	Approved Product Development	Approved Operations Head
Prairna B	Atul G	K Jain

Background

1. Introduction to TNF-α

Tumor Necrosis Factor-alpha (TNF-α) is a pro-inflammatory cytokine predominantly produced by activated macrophages, T lymphocytes, and natural killer (NK) cells. It plays a pivotal role in immune regulation, apoptosis, cell proliferation, and inflammation. Dysregulated TNF-α signaling contributes to autoimmune diseases, chronic inflammation, cancer, and septic shock. Consequently, TNF-α serves as a critical biomarker and therapeutic target in modern drug discovery and development programs.

2. Significance in Drug Discovery Research



2.1 Target Identification and Validation

- TNF-α is central to pathways mediating chronic inflammation (e.g., NF-κB, MAPK).
- Elevated TNF-α levels are observed in rheumatoid arthritis, psoriasis, Crohn's disease, and sepsis.
- Inhibition of TNF-α or its receptor (TNFR1/TNFR2) is a validated therapeutic strategy.

2.2 Assay Development

- TNF-α serves as a model cytokine for screening anti-inflammatory agents via:
    - ELISA (quantification of TNF-α levels)
    - Cell-based neutralization assays (e.g., L929 cytotoxicity assay)
    - Multiplex cytokine panels

2.3 Biomarker for Efficacy and Safety

- TNF-α levels correlate with drug-induced immunomodulation.
- Used to evaluate cytokine release syndrome (CRS) risk during preclinical and clinical trials.

3. Relevance in Biopharmaceutical Development

3.1 Monoclonal Antibodies and Biosimilars

- Anti-TNF therapies (e.g., Infliximab, Adalimumab, Etanercept) are among the top-selling biologics globally.
- Biosimilar development requires:
    - TNF-α binding assays (affinity comparison)
    - Neutralizing potency assays (cell-based and biochemical)
    - Comparability studies for regulatory submissions

3.2 PK/PD Studies

- TNF-α levels are monitored as a pharmacodynamic marker in clinical trials of anti-inflammatory drugs and biologics.

3.3 Safety Evaluation

- Excessive TNF-α suppression increases infection risk (e.g., tuberculosis reactivation), necessitating precise assay quantification for safety assessments.

4. Importance in Cell and Gene Therapy (CGT)

4.1 Cytokine Release Syndrome (CRS) Monitoring

- TNF-α is a key mediator of CRS in CAR-T, TCR therapies, and gene-modified hematopoietic stem cell therapies.
- Real-time TNF-α measurement guides tocilizumab/steroid interventions in clinical settings.

4.2 Immune Modulation Biomarker

- TNF-α quantification is critical in profiling immune responses during **CGT clinical trials** to evaluate efficacy and safety.

4.3 Genetic Modulation

- Gene-editing approaches target **TNF-α suppression** to treat inflammatory conditions or reduce immune rejection in cell-based therapies.

Scope of Validation

The PrecisionBind Human TNFa / TNA-alpha / TNFA ELISA (Catalog No KB1145) kit is considered by us during the validation of this kit in accordance with ICH Q2 (R1) guidelines. The document is prepared based on tests run in our laboratory and does not necessarily seek to cover the testing that may be required at user's end for registration in, or regulatory submissions. The objective of this validation is to demonstrate that it is suitable for its intended purpose - detection of TNF alpha.

Validation characteristics considered by us in accordance with the guidelines are listed below:

- Specificity and Selectivity.
- Sensitivity (LOD & LOQ).
- Linearity and Range.
- Accuracy and Precision (Intra/Inter-Assay).
- Matrix Effect (serum, plasma and CSF).
- Sample Handling and Storage Conditions.

- References (TNF-α Cmax Values and Recommended ELISA Range).

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay to assure quality results.

For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

For any queries or support on the data and its performance, please contact us at sales1@krishgen.com

Intended Use of the ELISA

The PrecisionBind Human TNFa ELISA kit is intended to measure the TNFalpha (Tumor Necrosis Factor alpha) in serum, plasma, cell culture supernatant and other biological fluids.

Principle of the Assay

This ELISA is a sandwich immunoassay. Antibodies are coated on 96 well plates. The antigen protein present in sample and standard respectively bind to the coated wells. The wells are washed and an antibody:HRP Conjugate is added which binds to the bound complex in the well. Washing is performed to remove any unbound material. TMB substrate is added and the enzyme reaction is stopped by dispensing of stop solution into the wells. The optical density (OD) of the solution at 450 nm is directly proportional to the amount of antigen protein present in the standard or samples.

Validation Parameters and Acceptance Criteria

1. TNF-α Cmax Values and Recommended ELISA Range

This table summarizes TNF-α Cmax levels across diseases and suggests corresponding ELISA working ranges.

Application	Expected TNF-α Range (pg/ml)	Recommended ELISA Range (pg/ml)
Healthy Baseline	<10	0 - 50
Chronic Inflammatory Disease (RA, IBD, Psoriasis)	20 - 200 (peaks up to 500)	0 - 1,000
Sepsis / Cytokine Storm	200 - 3,000 (rare >10,000)	0 - 5,000 (extendable to 10,000)
Cell & Gene Therapy Cytokine Release Monitoring	50 - 1,000	0–2,000

Note: Assay sensitivity < 5 pg/mL recommended for baseline detection; upper limit ≥ 5,000 pg/ml advised for CRS monitoring.

The PrecisionBind Human TNFalpha ELISA kit is developed using an assay range of 7.8 - 500 pg/ml with the dilutional linearity accuracy to measure responses as per the application table above on patient Cmax values. The kit has also been validated upto 6400 fold dilution and the values are within the acceptable range.

2. Specificity and Selectivity

2.1 Specificity

The capture antibody and detection antibody are both specific to TNFa and are monoclonal antibodies. They show a high affinity to bind to native as well as recombinant TNFa.

2.2 Selectivity

The ELISA has no or low cross reactivity to IL-1β (IL-1beta), IL-6, or TNF-β (TNFbeta).

2.3 NIBSC validation

The standard used in the kit is calibrated against an international standard from the National Institute of Biological Standards and Control (NIBSC), Potters Bar, Hertfordshire EN6 3QG, UK. 1 ng of supplied standard equals 46 U of 12/154 NIBSC-standard.
Therefore 1000 pg/ml is equivalent to 46 U of TNFα as per NIBSC.

2.4 LOD, LOQ and IC50

LOD (Limit of Detection)
The lowest analyte concentration that can be reliably distinguished from blank/background noise but not necessarily quantified precisely.

Statistically:
LOD = Mean of Blank + 3X SD of Blank
(3σ criterion is most common).

LOD for PrecisionBind Human TNFalpha ELISA = 3.1 pg/ml

LOQ (Limit of Quantitation)
The lowest analyte concentration that can be quantified with acceptable accuracy and precision.

Statistically:
LOQ = Mean of Blank + 10X SD of Blank
(10σ criterion is most common).

LOQ for PrecisionBind Human TNFalpha ELISA - 9.3 pg/ml

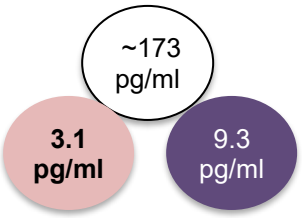
IC50 in ELISA (Half Maximal Inhibitory Concentration)
IC50 = The concentration of an inhibitor (drug, antibody, compound) required to reduce the signal (e.g., binding, enzymatic activity) by 50% compared to the maximum signal in the assay.

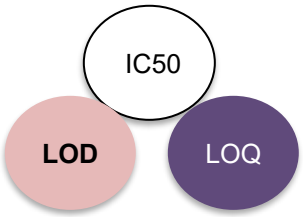
In ELISA, this is commonly used for:
Neutralization ELISA: Quantifies potency of antibodies inhibiting target–ligand interaction.
Drug Potency Testing: Measures concentration at which drug inhibits 50% of target activity.

IC50 for PrecisionBind Human TNFalpha ELISA = ~173 pg/ml

Summary:

Parameter	Value (pg/mL)
LOD	3.1 pg/ml
LOQ	9.3 pg/ml
IC50	~ 173 pg/ml

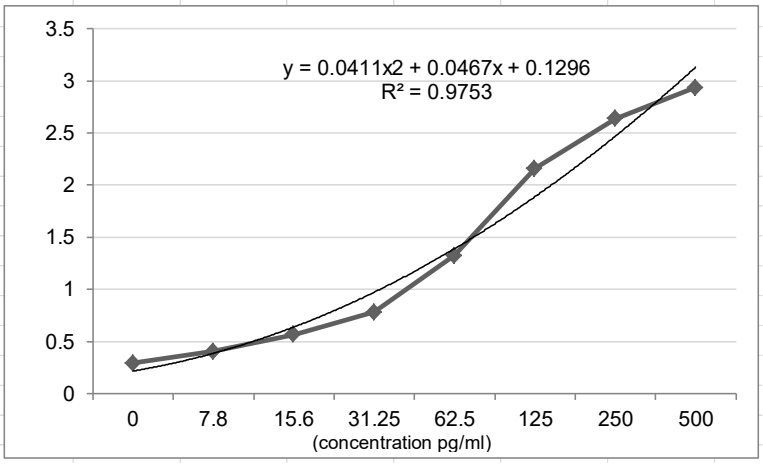


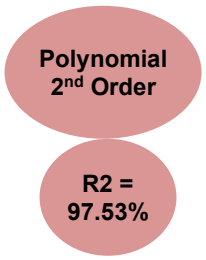


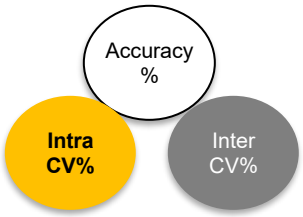
Regulatory Note:
LOD *S/N ≥ 3:1, LOQ ≥ 10:1, %CV ≤ 20% **S/N = Signal / Noise Ratio*

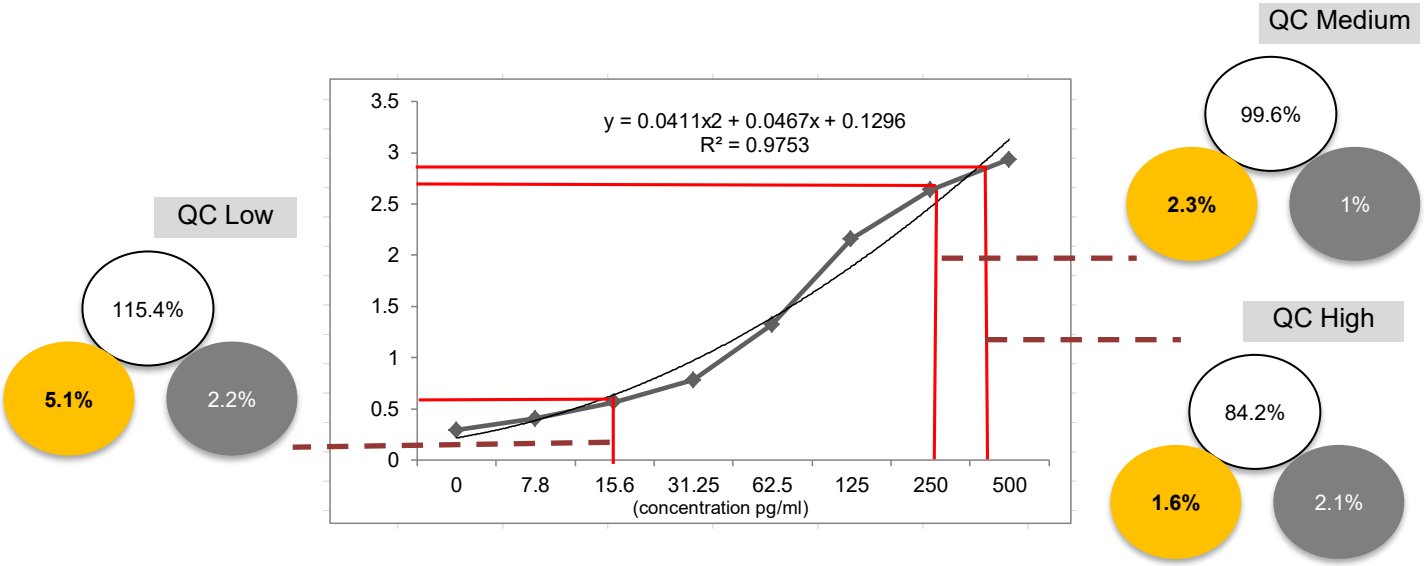
2. Linearity and Range

Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery
0	0.291		--
7.8	0.404	8.3	106.1
15.6	0.567	18.9	121.4
31.25	0.784	30.2	96.5
62.5	1.324	60.7	97.0
125	2.158	128.8	103.0
250	2.639	247.9	99.2
500	2.934	492.5	98.5
Low QC (15.6 pg/ml)	0.506	18.0	115.4
Mid QC (250 pg/ml)	2.619	249.1	99.6
High QC (400 pg/ml)	2.795	336.9	84.2









3. Accuracy and Precision (Intra / Inter-Assay)

A) Intra-Assay:

Standard Concentration (pg/ml)	Mean OD450	SD	%CV
7.8	0.302	1.25	5.1
125	1.061	2.03	2.3
500	2.114	2.68	1.6

B) Inter Assay:

Standard Concentration (pg/ml)	Mean OD450	SD	%CV
15.6	0.309	0.68	2.2
250	1.067	1.11	1.0
1000	2.086	4.47	2.1

4. Parallelism and Matrix Effect

Sample Dilution factor – Human Serum, Human Plasma and Human CSF samples have been tested. Sample dilution Factor for all three matrices is 1:50 dilution.

Neat Human Serum, Human Plasma and Human CSF were spiked with 250 pg/ml Human TNF alpha and ELISA assay was run.

Sample	Mean OD450	Interpolated Concentration	% Recovery
Neat Human CSF	2.655	282.1	112.8
Neat Human Plasma	2.657	283.1	113.2
Neat Human Serum	2.789	372.4	149.0

A) Serum:

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:100	500	2.936	444.0	88.8	112.6
1:200	250	2.645	241.8	96.7	103.4
1:400	125	2.099	126.5	101.2	98.8
1:800	62.5	1.309	59.4	95.1	105.2
1:1600	31.25	0.758	29.1	93.1	107.4
1:3200	15.6	0.529	16.7	107.3	93.4
1:6400	7.8	0.381	7.0	90.3	111.0

B) Plasma:

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:100	500	2.844	405.3	81.1	123.4
1:200	250	2.537	222.5	89.0	112.4
1:400	125	1.944	120.3	96.3	103.9
1:800	62.5	1.482	79.9	127.9	78.2
1:1600	31.25	0.783	35.9	114.8	87.1
1:3200	15.6	0.457	14.2	90.7	110.4
1:6400	7.8	0.373	5.5	70.3	142.4

C) Cerebrospinal Fluid (CSF):

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:100	500	2.917	456.7	91.3	109.5
1:200	250	2.502	212.8	85.1	117.5
1:400	125	1.902	115.9	92.7	107.9
1:800	62.5	1.427	76.0	121.6	82.2
1:1600	31.25	0.627	26.3	84.1	118.9
1:3200	15.6	0.494	17.1	109.6	91.4
1:6400	7.8	0.400	8.8	112.9	88.7

Results:

i) Parallelism is maintained across the 1:100 to 1:6400 dilutions.

ii) % Recovery for most dilutions falls within the acceptable range of 80%–120%.

iii) No significant matrix effect observed at higher dilutions.

iv) The PrecisionBind Human TNFalpha ELISA kit was tested for matrix effect on human serum, plasma, CSF and physiological buffer 7.4 to mimic tear fluid samples.

5. Sample Handling and Storage Conditions

A.) Sample collection and handling:

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

Cell Culture Supernatant*:* If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at temperature <-20°C. Avoid repeated freeze/thaw cycles.

Serum: Use a serum separator tube and allow clotting for 30 minutes, then centrifuge for 10 minutes at 1000 x *g*. Remove serum layer and assay immediately or store serum samples at temperature <-20°C. Avoid repeated freeze/thaw cycles.

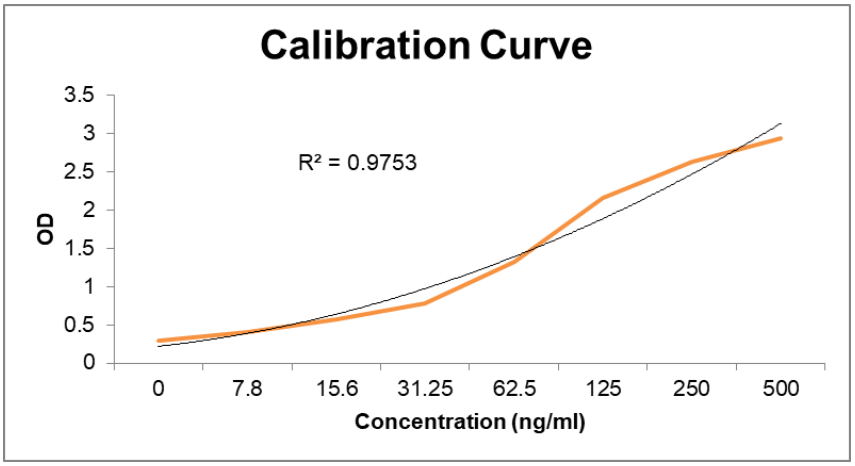
Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1000 x *g* within 30 minutes of collection. Assay immediately or store plasma samples at temperature <-20°C. Avoid repeated freeze/thaw cycles.

B) Storage conditions:
Store main kit components at 2-8°C.

Store recombinant lyophilized standard at 2-8°C. Upon reconstitution aliquot standards into polypropylene vials and store at -20°C as per assay requirements. Do not freeze thaw for more than two times.

Before using, bring all components to room temperature (18-25°C). Upon assay completion return all components to appropriate storage conditions.

Graphs, Maps and Appendices:



Calibration of Standard Used in the KIT

- NIBSC Standard 12/154 TNFalpha
- PrecisionBind Human TNFalpha Kit Standard

Matrix Effect Heat Map

	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
Serum							
Plasma							
Cell Culture Supernatant							
Tissue Lysate							
Cell Culture Supernatant							
Tear Fluid / PBS7.4							

Determined Limits for Acceptance according to EMA/FDA and CLSI regulations

	Limits for Acceptance (EMA/FDA)	Determined Limits for Acceptance (CLSI)
Intra Precision	CV < 20% (25% at LLOQ)	-
Inter Precision	CV < 20 % (25% at LLOQ)	-
Accuracy at LLOQ	Recovery 100 ± 20% (100 ±25%	-
Total Error (TE)	TE < 30% (40% at LLOQ and ULOQ)	-
Specificity/Interference	Recovery 100 ± 25%2	H (null hypothesis) = 100 ± 25 %
Parallelism/Linearity	CV < 30%2	Deviation from linearity < 20%
LLOQ / LoQ	Recovery 100 ± 25%	TE % < 32.9%

References

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