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 IL-2 ELISA KIT (Catalog No KB1064) 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 IL-2 ELISA KIT (Catalog No KB1064)**	31.07.2025

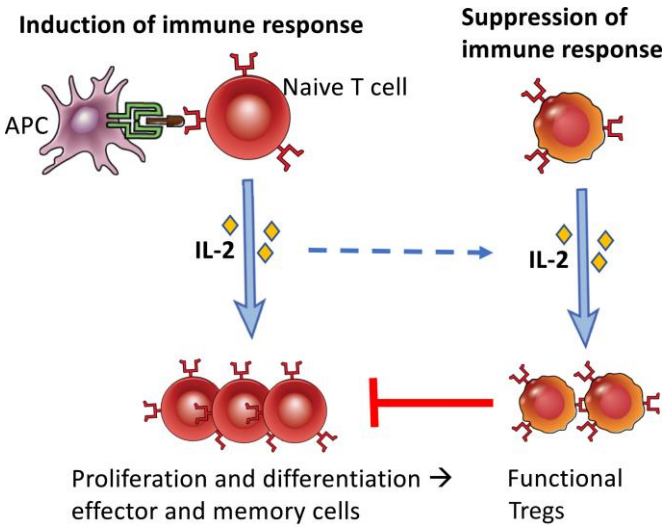
Approved Quality Control	Approved Product Development	Approved Operations Head
Prairna B	Atul G	K Jain

Background

1. Introduction to IL-2

Interleukin 2 (IL-2) is a cytokine, a type of protein produced by the white blood cells that plays a crucial role in the immune system. It primarily acts as a growth factor for T lymphocytes (T cells), stimulating their proliferation and differentiation. IL-2 also affects other immune cells like B lymphocytes and natural killer cells influencing their activity and development. Subsequently, IL-2 significantly takes part in the development of regulatory T cells that helps in suppressing excessive immune responses and maintains immune homeostasis.

2. Significance in Drug Discovery Research



2.1 Target Identification and Validation

- IL-2 is a primary regulator for T cell proliferation, differentiation and survival.
- It has a dual role promoting immune activation by the help of NK cells and T cells followed by immune tolerance via T cells, Tregs.
- Dysregulation or inhibition of IL-2 signalling cascade is associated with cancer, certain autoimmune disorders and immune deficiencies.
- The modulation of IL-2 or its receptor subunits is considered a validated therapy in autoimmunity and immune-oncology.

2.2 Assay Development

- IL-2 is widely used in bioassays for screening immunomodulatory drugs and biologics via:
  - ELISA (For quantification of IL-2 secretion)
  - Proliferation assays using IL-2 dependent CTLL-2 or NK cell lines
  - Reporter assays for downstream STAT5 phosphorylation or transcriptional activity.
  - Flow cytometry based assays to interpret IL-2 receptor expression and treg Expansion.

2.3 Biomarker for Efficacy and Safety

- IL-2 levels serve as pharmacodynamics biomarkers in T cell expansion therapies and cancer immunotherapy.
- Plays a critical role in evaluation of risk of cytokine release syndrome (CRS), especially in therapies involving T cell engagers or CAR-T cells.
- Monitoring production of IL-2 helps in assessment of drug induced T-cell activation, immunotoxicity and cytokine release.

3. Relevance in Biopharmaceutical Development

3.1 Monoclonal Antibodies and Biosimilars

- IL-2 based therapies (e.g., Aldesleukin, Bempegaldesleukin) and IL-2-targetting antibodies are in clinical use and development for cancer, autoimmune diseases and immune deficiencies.
- Engineering of IL-2 variants aims in selectively stimulating effector T cells or regulatory T cells
- Biopharmaceutical development involves
    - IL-2 receptor binding assays (affinity and selectivity forIL-2Rα, β, and γ chains)
    - Bioactivity assays (e.g., CTLL-2 proliferation or STAT 5 phosphorylation)
    - Comparability studies for engineered IL-2 analogs or fusion proteins.

3.2 PK/PD Studies

- The levels of IL-2 and downstream biomarkers (e.g., pSTAT5, CD25 expression) are considered as pharmacodynamics endpoints in clinical trials.
- It is used for model dose–response relationships and guide dosing regimens for immunotherapies.

3.3 Safety Evaluation

- IL-2 therapies are associated with side effects like systemic immune activation and cytokine release syndrome (CRS).
- Quantitative IL-2 assays are important to monitor cytokine surges, immune-related adverse events, and off-target immune responses.
- Safety assessments with IL-2 assays involve cytokine profiling and Treg/effector T cell balance analysis.

4. Importance in Cell and Gene Therapy (CGT)

4.1 Cytokine Release Syndrome (CRS) Monitoring

- IL-2 is a central cytokine involved in T cell activation and influences in the development of CRS in CAR-T and TCR-engineered therapies.
- Elevated levels of IL-2, alongside IL-6 and IFN-γ, are targeted as early indicators of severe immune activation.
- Real-time IL-2 monitoring helps in decision-making for immunosuppressive interventions (e.g., corticosteroids, IL-2R blockade).

4.2 Immune Modulation Biomarker

- Measurement of IL-2 levels is important for profiling T cell kinetics, effector vs. regulatory T cell ratios, and immune persistence in CGT trials.
- It is used as a pharmacodynamics marker for efficacy in cancer immunotherapies and immune tolerance in autoimmunity-targeting CGTs.

4.3 Genetic Modulation

- Gene-editing strategies (e.g., CRISPR/Cas9) aim to:
- Increase IL-2 production in T cells to boost anti-tumor immunity.
- Modify IL-2 or IL-2R genes to bias signalling toward effector or regulatory functions.

- Knock-in/knock-out approaches for regulating IL-2 expression for safer and more durable T cell therapies.

Scope of Validation

Human IL-2 (Catalog No KB1064) 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 IL-2.

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 (IL-2 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

PrecisionBind Human Interleukin 2 (IL2 / IL-2) ELISA kit is intended to measure the IL-2 (Interleukin 2) 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. IL-2 Cmax Values and Recommended ELISA Range

This table summarizes IL-2 Cmax levels across diseases and suggests corresponding ELISA working ranges.

Application	Expected Il-2 Range (pg/mL)	Recommended ELISA Range (pg/mL)
Healthy baseline	<5	0–50
Chronic inflammatory disease (RA, IBD, psoriasis)	10–100 (peaks up to 500)	0–500
Sepsis / cytokine storm	100–1000 (rare <2000)	0-2000 (extendable to 5000)
Cell & gene therapy cytokine release monitoring	50–500	0–1,000

Note: Assay sensitivity <2 pg/mL recommended for baseline detection; upper limit ≥2,000 pg/mL advised for CRS monitoring.

The PrecisionBind Human IL-2 ELISA kit is developed using an assay range of 15.6 - 1000 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 1280 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 IL-2 and are monoclonal antibodies. They show a high affinity to bind to native as well as recombinant IL-2.

2.2 Selectivity
The ELISA has no or low cross reactivity to IL-2 Rα IL-2 Rβ IL-2 Rγ.

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 15 U of 86/504 NIBSC-standard. ***Therefore 1000 pg/ml is equivalent to 15 U of IL-2 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 IL-2 ELISA = 2.01 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 IL-2 ELISA – 6.1 pg/ml

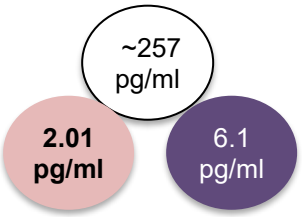
IC50 in ELISA (Half Maximal Inhibitory Concentration)
IC50 = The concentration of an inhibitor (drug, antibody, compound) required to reduce the signal (e.g., binding, enzymatic activity) by 50% compared to the maximum signal in the assay.

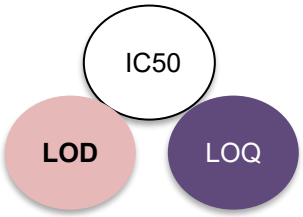
In ELISA, this is commonly used for:
Neutralization ELISA: Quantifies potency of antibodies inhibiting target–ligand interaction.
Drug Potency Testing: Measures concentration at which drug inhibits 50% of target activity.

IC50 for PrecisionBind Human IL-2 ELISA = ~257 pg/ml

Summary:

Parameter	Value (pg/mL)
LOD	2.01 pg/ml
LOQ	6.1 pg/ml
IC50	~ 257 pg/ml

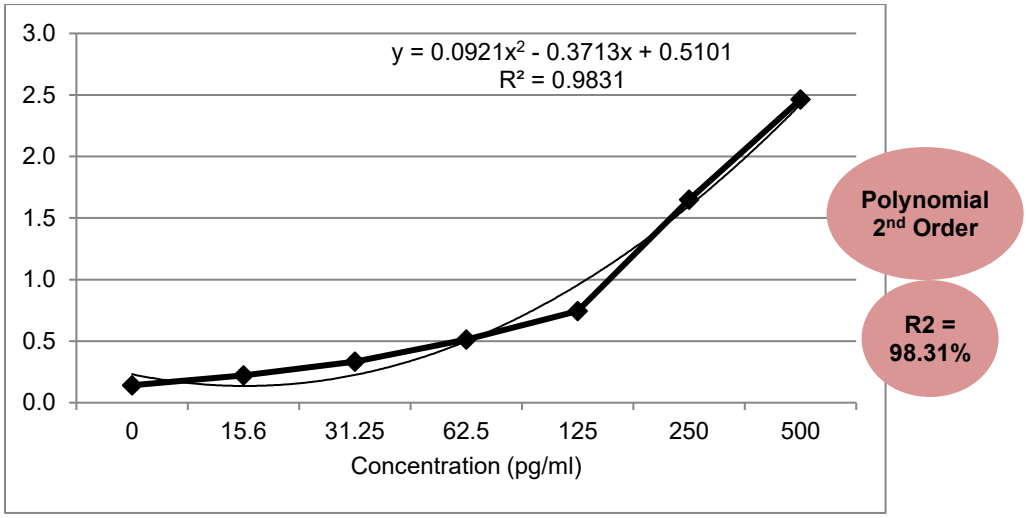


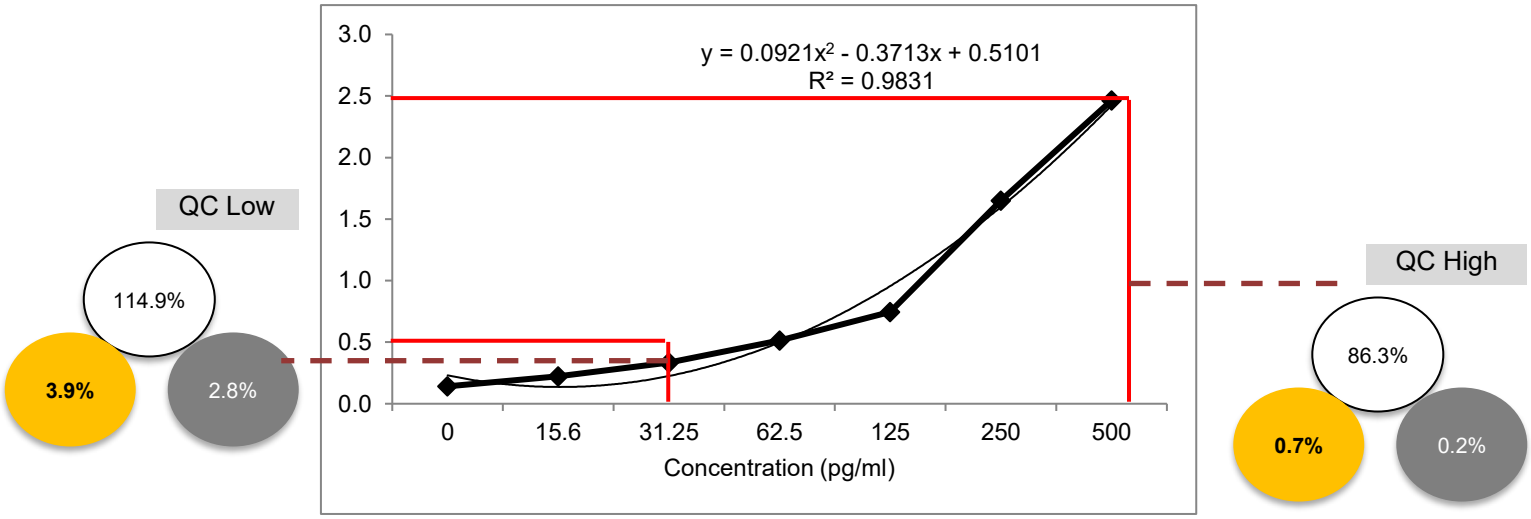


Regulatory Note:
LOD S/N ≥ 3:1, LOQ ≥ 10:1, %CV ≤ 20% **S/N = Signal / Noise Ratio*

3. Linearity and Range

Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery
0	0.142	--	--
15.6	0.222	12.5	80.1
31.25	0.334	37.2	119.0
62.5	0.514	78.2	125.1
125	0.745	112.8	90.2
250	1.650	254.0	101.6
500	2.464	509.0	101.8
1000	2.871	965.8	96.6
Positive Control (750 pg/ml)	2.651	642.0	85.6
Low QC control (31.25 pg/ml)	0.337	35.9	114.9
High QC control (800 pg/ml)	2.700	690.8	86.3





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

A) Intra-assay:

Standard (pg/ml)	Mean OD	SD	%CV
15.6	0.234	0.74	3.9
250	1.629	0.55	0.4
1000	2.876	1.74	0.7

B) Inter assay:

Standard (pg/ml)	Mean OD450	SD	%CV
15.6	0.234	0.65	2.8
250	1.638	2.19	103
1000	2.871	0.46	0.2

5. 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 1:10 dilution.

Neat Human Serum, Human Plasma and Human CSF were spiked with 500 pg/ml Human IL-2 and ELISA assay was run.

Sample	Mean OD450	Interpolated Concentration	% Recovery
Neat CSF samples	2.813	845.9	169.2
Neat Plasma	2.750	750.4	150.1
Neat Human Serum	2.883	996.3	199.3

A) Serum:

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:20 dilution	1000	2.871	965.8	96.6	103.5
1:40 dilution	500	2.260	415.4	83.1	120.4
1:80 dilution	250	1.646	253.3	101.3	98.7
1:160 dilution	125	0.747	113.1	90.4	110.6
1:320 dilution	62.5	0.506	76.9	123.0	81.3
1:640 dilution	31.25	0.296	36.2	115.9	86.3
1:1280 dilution	15.6	0.231	13.2	84.6	118.4

B) Plasma:

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:20 dilution	1000	2.842	900.9	90.1	111.0
1:40 dilution	500	2.427	489.2	97.8	102.2
1:80 dilution	250	1.623	248.9	99.6	100.5
1:160 dilution	125	0.729	110.5	88.4	113.2
1:320 dilution	62.5	0.494	74.9	119.9	83.4
1:640 dilution	31.25	0.304	38.2	122.4	81.7
1:1280 dilution	15.6	0.231	14.0	89.4	112.0

C) Cerebrospinal Fluid (CSF):

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:20 dilution	1000	2.870	963.4	96.3	103.8
1:40 dilution	500	2.400	475.6	95.1	105.1
1:80 dilution	250	1.646	253.3	101.3	98.7
1:160 dilution	125	0.694	105.4	84.3	118.6
1:320 dilution	62.5	0.543	82.8	132.4	75.5
1:640 dilution	31.25	0.312	40.2	128.6	77.8
1:1280 dilution	15.6	0.229	12.8	82.3	121.7

Results:

i) Parallelism is maintained across the 1:20 to 1:1280 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 IL-2 ELISA kit was tested for matrix effect on human serum, plasma, CSF and physiological buffer 7.4 to mimic tear fluid samples.

6. 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.

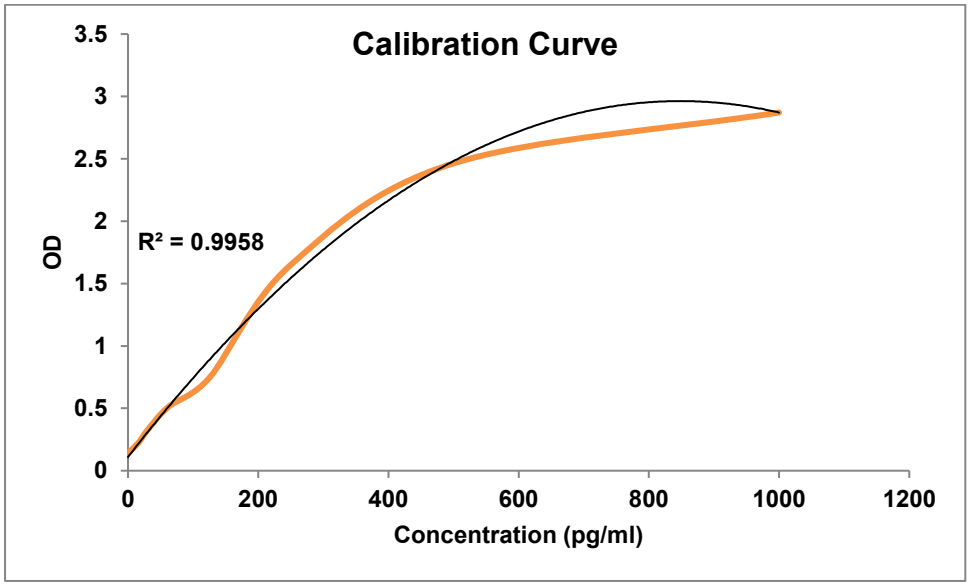
Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1000 x *g* within 30 minutes of collection. Assay immediately or store plasma samples at temperature <-20°C. Avoid repeated freeze/thaw cycles.

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 86/504 IL-2

PrecisionBind Human IL-2 Kit Standard

Matrix Effect Heat Map

	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Serum							
Plasma							
Cerebrospinal Fluid (CSF)							

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%

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Version No. 1.0, dated 31.07.2025