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-12 ELISA KIT (Catalog No KB1075) 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

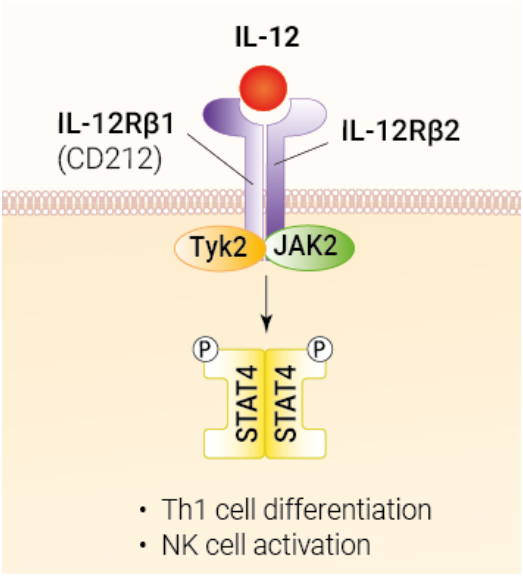
First Codification	History	Date
Version#1	**VALIDATION DATA OF PRECISIONBIND HUMAN IL-12 ELISA KIT (Catalog No KB1075)**	31.07.2025

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

Background

1. Introduction to Interleukin-12

Interleukin-12 (IL-12) is a type of heterodimeric pro-inflammatory cytokine consisting of two subunits (p35 and p40), primarily secreted by antigen-presenting cells such as dendritic cells, macrophages, and B cells. It plays an important role in innate and adaptive immunity by promoting the differentiation of naïve T cells into Th1 cells and stimulating the production of IFN-γ and TNF-α. Dysregulated and inhibited impact of IL-12 expression is implicated in autoimmune diseases, chronic inflammation, infections, and tumor immunity, making it a key biomarker and therapeutic target in immuno-oncology and inflammatory disease research.



2. Significance in Drug Discovery Research

2.1 Target Identification and Validation

- IL-12 plays a pivotal role in Th1 immune responses and activation of NK cell.
- Enhanced levels of IL-12 are involved with autoimmune diseases (e.g., psoriasis, multiple sclerosis), infectious diseases, and inflammatory bowel diseases.
- IL-12 blockade (e.g., anti-p40 antibodies) is a validated strategy in treating psoriasis and Crohn's disease.

2.2 Assay Development

- IL-12 is measured using:
  - ELISA kits (targeting p70 or p40 subunits)
  - Luminex/Mesoscale multiplex assays (for cytokine panels)
  - Cell-based assays to assess IL-12-induced IFN-γ production
  - Reporter gene assays (NF-κB activation)

2.3 Biomarker for Efficacy and Safety

- IL-12 levels are used for:
  - Monitoring the immunomodulatory effects of checkpoint inhibitors or cytokine therapies.
  - Assessing risk and onset of cytokine release syndrome (CRS).
  - Evaluating immune stimulation during vaccination and adjuvant development.

3. Relevance in Biopharmaceutical Development

3.1 Monoclonal Antibodies and Biosimilars

- Ustekinumab (anti-p40 monoclonal antibody) targets both IL-12 and IL-23 that are approved for psoriasis, Crohn's disease, and ulcerative colitis.
- Biosimilar development for IL-12/IL-23 targeting drugs requires:
  - IL-12p40 binding assays
  - Functional neutralization assays
  - Comparability testing for regulatory submissions

3.2 PK/PD Studies

- IL-12 (particularly IL-12p70) acts as a pharmacodynamics biomarker in clinical trials of immunotherapies, vaccines, and anti-inflammatory biologics.

3.3 Safety Evaluation

- Overexpression or excessive activation of IL-12 can lead to the development of autoimmunity and systemic inflammation.
- Accurate measurement is essential for dose optimization and safety profiling, especially in cytokine-based therapies or gene-modified treatments.

4. Importance in Cell and Gene Therapy (CGT)

4.1 Cytokine Release Syndrome (CRS) Monitoring

- IL-12 is a pro-inflammatory driver of CRS in CAR-T, TCR, and gene-modified NK cell therapies.
- Real-time monitoring of IL-12 alongside IFN-γ and IL-6 supports clinical decision-making for tocilizumab or administration of steroid.

4.2 Immune Modulation Biomarker

- IL-12 levels reflects the status of immune activation and are used in:
  - Profiling of tumour immunogenicity
  - Viral vector safety assessment
  - Efficacy evaluation of CGT platforms

4.3 Genetic Modulation

- Novel gene-editing approaches aim to:
  - Increased IL-12 expression in tumor-infiltrating lymphocytes (TILs) for improved anti-tumor response.
  - Limit systemic IL-12 exposure to reduce toxicity while maintaining localized immune activation.

Scope of Validation

The PrecisionBind Human IL-12 ELISA (Catalog No KB1075) 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 Human Interleukin 12.
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 (plasma and CSF).
- Sample Handling and Storage Conditions.
- References.IL-12 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 IL-12 ELISA kit is intended to measure the IL-12 (InterLeukin-12) 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-12 Cmax Values and Recommended ELISA Range

This table summarizes IL-12 Cmax levels across diseases and suggests corresponding ELISA working ranges.

Application	Expected IL-12 Range (pg/ml)	Recommended ELISA Range (pg/ml)
Healthy Baseline	<5	0 - 50
Chronic Inflammatory Disease (RA, IBD, Psoriasis)	10 - 100 (peaks up to 200)	0 - 500
Sepsis / Cytokine Storm	100 - 1,000 (rare >2000)	0 - 2000 (extendable to 5000)
Cell & Gene Therapy Cytokine Release Monitoring	20 - 500	0–1000

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

The PrecisionBind Human IL-12 ELISA kit is developed using an assay range of 31.3 - 2000 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 1600 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-12 and are monoclonal antibodies. They show a high affinity to bind to native as well as recombinant IL-12.

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 8 U of 95/544 NIBSC-standard.
Therefore 1000 pg/ml is equivalent to 8 U of IL-12 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-21 ELISA = 7 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-21 ELISA - 21 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:

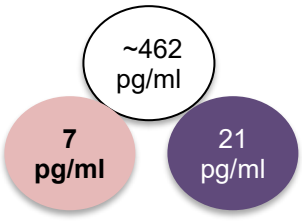
Neutralization ELISA: Quantifies potency of antibodies inhibiting target–ligand interaction.

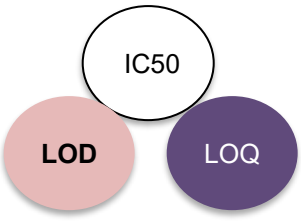
Drug Potency Testing: Measures concentration at which drug inhibits 50% of target activity.

IC50 for PrecisionBind Human IL-21= ~462 pg/ml

Summary:

Parameter	Value (pg/mL)
LOD	7 pg/ml
LOQ	21 pg/ml
IC50	462 pg/ml



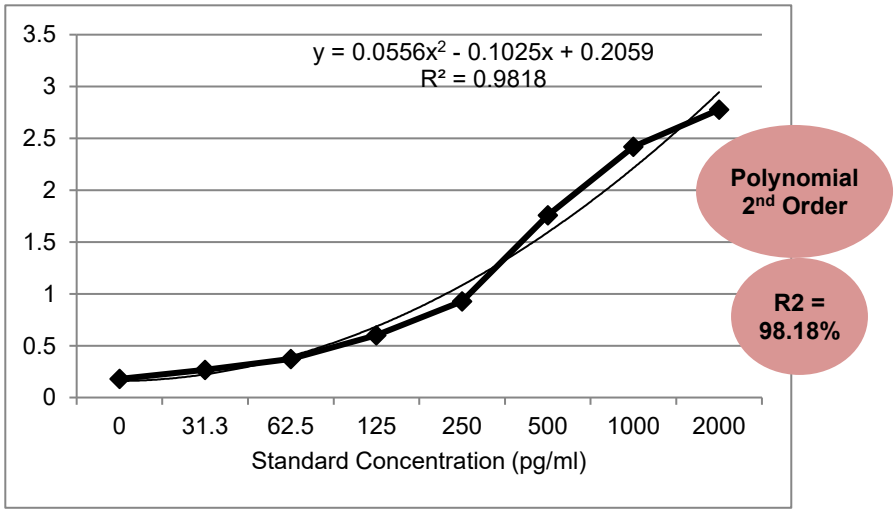


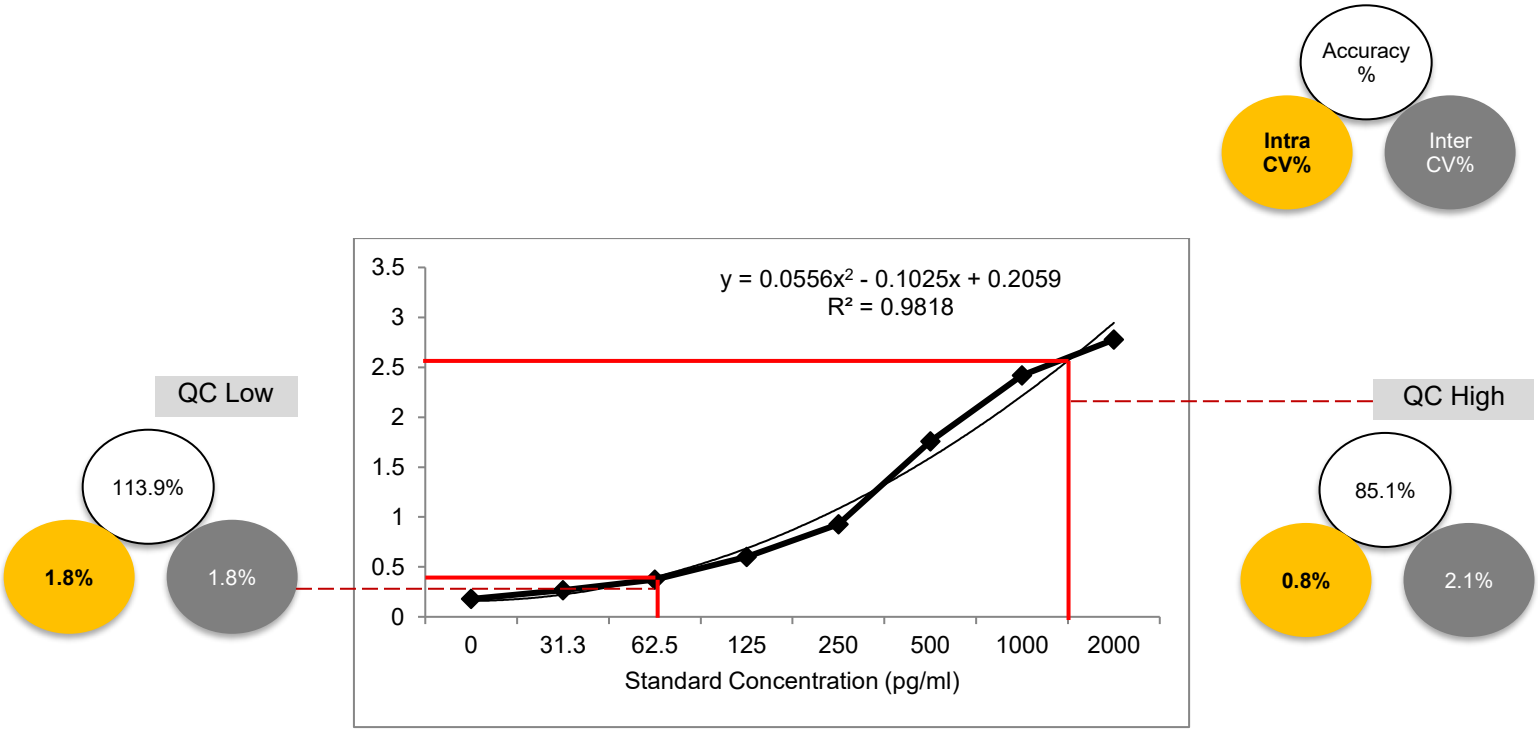
Regulatory Note:

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	% Recovery
0	0.181		--
31.3	0.273	34.8	111.3
62.5	0.377	74.1	118.6
125	0.590	136.4	109.1
250	0.945	232.2	92.9
500	1.756	511.4	102.3
1000	2.420	1014.0	101.4
2000	2.789	1941.9	97.1
Positive Control (1000 pg/ml)	2.499	1029.9	103
Low QC control (62.5 pg/ml)	0.373	71.2	113.9
High QC control (1500 pg/ml)	2.647	1276.8	85.1





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

A) Intra-assay:

Standard (pg/ml)	Mean OD	SD	%CV
31.3	0.319	0.47	1.8
250.0	1.001	1.62	0.2
2000	2.773	4.78	0.8

B) Inter assay:

Standard (pg/ml)	Mean OD	SD	%CV
31.3	0.322	0.57	1.8
250.0	0.999	0.20	2.0
2000	2.788	2.24	2.1

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 1:50 dilution.

Neat Human Serum, Human Plasma and Human CSF were spiked with 1000 pg/ml Human IL-12 and ELISA assay was run.

Sample	Interpolated Concentration	% Recovery
Neat Human CSF	1938.7	193.9
Neat Human Plasma	1774.6	177.5
Neat Human Serum	2169.2	216.9

A) Serum:

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:100	2000	2.701	1610.1	80.5	80.5
1:200	1000	2.203	787.9	78.8	78.8
1:400	500	1.597	443.5	88.7	88.7
1:800	250	1.114	281.2	112.5	112.5
1:1600	125	0.675	160.9	128.7	128.7
1:3200	62.5	0.444	96.5	154.3	154.3
1:6400	31.3	0.282	40.1	128.0	128.2

B) Plasma:

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:100	2000	2.699	1595.1	79.8	125.4
1:200	1000	2.199	832.0	83.2	120.2
1:400	500	1.600	423.5	84.7	118.1
1:800	250	1.200	293.9	117.6	85.1
1:1600	125	0.662	151.4	121.1	82.6
1:3200	62.5	0.455	95.9	153.4	65.2
1:6400	31.3	0.290	41.5	132.5	75.3

C) Cerebrospinal Fluid (CSF):

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:100	2000	2.754	1648.9	82.4	121.3
1:200	1000	2.233	858.0	85.8	116.6
1:400	500	1.636	437.1	87.4	114.4
1:800	250	1.108	268.0	107.2	93.3
1:1600	125	0.700	161.2	128.9	77.6
1:3200	62.5	0.478	102.4	163.8	61.0
1:6400	31.3	0.301	45.9	146.7	68.1

Results:

i) Parallelism is maintained across the 1:100 to 1:1600 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-12 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.

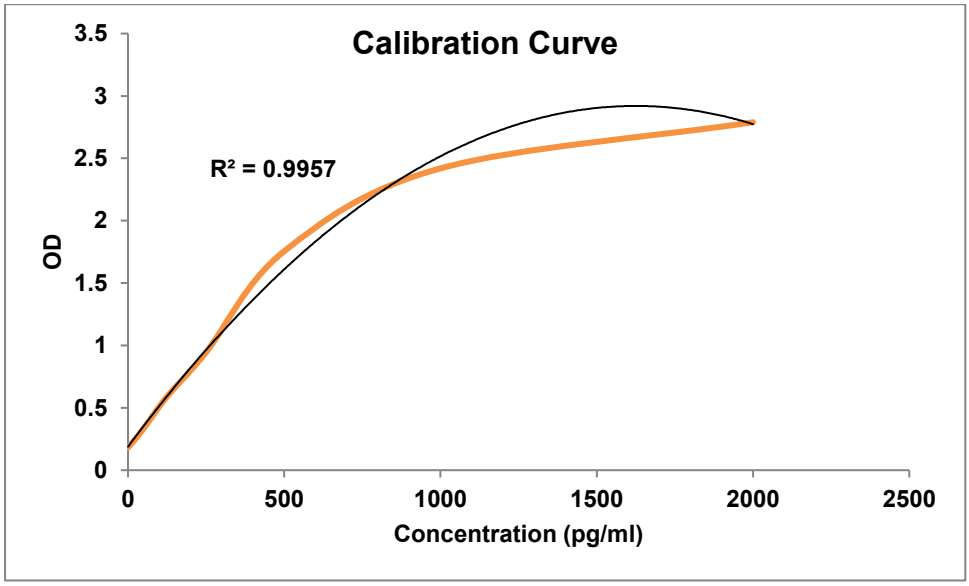
Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1000 x g within 30 minutes of collection. Assay immediately or store plasma samples at temperature < -20°C. Avoid repeated freeze/thaw cycles.

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 95/544 IL-12

PrecisionBind Human IL-12 Kit Standard

Matrix Effect Heat Map

	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
Plasma							
Cerebrospinal Fluid (CSF)							
Serum							

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

Van Wanrooij, R., Zwiers, A., Kraal, G., & Bouma, G. (2012). Genetic variations in interleukin-12 related genes in immune-mediated diseases. *Journal of Autoimmunity*, *39*(4), 359–368. https://doi.org/10.1016/j.jaut.2012.06.002

Xu, Y., Sun, X., & Tong, Y. (2024). Interleukin-12 in multimodal tumor therapies for induction of anti-tumor immunity. *Discover Oncology*, *15*(1). https://doi.org/10.1007/s12672-024-01011-2

FDA Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products (2014).

EMA Guideline on Biosimilar Monoclonal Antibodies (2012).