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-21 ELISA KIT (Catalog No KB1085) 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-21 ELISA KIT (Catalog No KB1085)**	31.07.2025

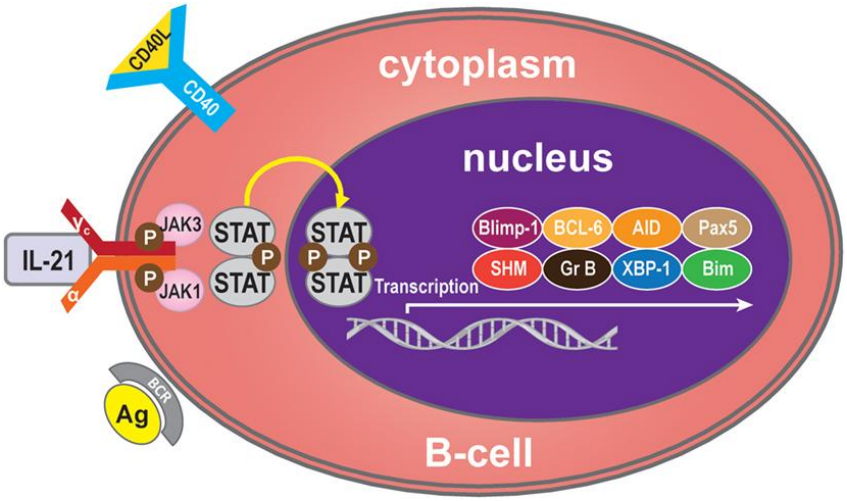
Approved Quality Control	Approved Product Development	Approved Operations Head
Prairna B	Atul G	K Jain

Background

1. Introduction to IL-21

Interleukin-21 (IL-21) is a pleiotropic cytokine majorly secreted by activated $CD4^+$ T cells, particularly T follicular helper (Tfh) cells and Th17 cells. It plays a crucial role in regulation of adaptive immune responses, influencing the function of B cells, T cells, natural killer (NK) cells, and dendritic cells. IL-21 influences the promotion of B cell differentiation, production of antibody, cytotoxic T cell activation, and NK cell-mediated cytotoxicity. The inhibition of IL-21 signalling is associated with autoimmune diseases, chronic viral infections, and cancer. Due to its vast immunomodulatory effects, IL-21 has emerged as a therapeutic target and biomarker in immuno-oncology, vaccine development, and autoimmune disorder research.

2. Significance in Drug Discovery Research



2.1 Target Identification and Validation

- IL-21 is the primary regulator of adaptive immunity, influencing T cell cytotoxicity, B cell maturation, and NK cell function.
- Elevated IL-21 levels are associated with autoimmune diseases (e.g., systemic lupus erythematosus, rheumatoid arthritis), chronic viral infections (e.g., HIV, hepatitis), and cancer (e.g., lymphoma, melanoma).
- Modulation of IL-21 signalling or its receptor (IL-21R) is a validated therapeutic strategy in immuno-oncology, autoimmunity, and vaccine enhancement.

2.2 Assay Development

- IL-21 is used in screening immunomodulatory and anti-proliferative agents through:
  - ELISA or MSD assays for quantification of IL-21 secretion from immune cells
  - Cell-based bioassays (e.g., STAT3 phosphorylation, B cell proliferation, or NK cell activation)
  - Reporter assays for IL-21/IL-21R signaling via JAK/STAT pathway.

2.3 Biomarker for Efficacy and Safety

- IL-21 levels are in co-relation with T and B cell activation, cytotoxic function, and antibody production in response to immunotherapies or vaccines.
- Serves as a biomarker for immune activation, exhaustion, or therapeutic response, particularly in cancer and viral immunotherapy trials.

- Monitoring IL-21 can help assess the risk of immune-related adverse events in therapies involving broad immune stimulation.

3. Relevance in Biopharmaceutical Development

3.1 Monoclonal Antibodies and Biosimilars

- IL-21 and its receptor (IL-21R) are being used in the development of therapeutic antibodies and engineered cytokine variants for cancer immunotherapy, autoimmune diseases, and chronic infections.
- IL-21-based biologics under study include recombinant IL-21, IL-21R-Fc fusion proteins, and IL-21-neutralizing antibodies.
- Biologic development involves:
  o IL-21–IL-21R binding assays (to evaluate affinity and receptor specificity)
  oCell-based bioactivity assays (e.g., STAT3 phosphorylation, T/NK cell proliferation)
- Comparability and stability studies for preclinical and regulatory submissions.

3.2 PK/PD Studies

- IL-21 levels, along with downstream markers such as pSTAT3, $CD8^+$ T cell expansion, or NK cell activation, are used as pharmacodynamics readouts in clinical trials.
- This study focuses on dose optimization and evaluation of immune response durability in IL-21-based therapies.

3.3 Safety Evaluation

- IL-21 can increase cytotoxic immune responses, but excessive stimulation may lead to immune over activation or autoimmune-like toxicities.
- Quantification of IL-21 and monitoring immune biomarkers are critical for assessment of cytokine-mediated toxicity, particularly in combination immunotherapies.
- Safety profiling includes cytokine panel monitoring, immune cell subset analysis, and autoantibody screening.

4. Importance in Cell and Gene Therapy (CGT)

4.1 Cytokine Release Syndrome (CRS) Monitoring

- IL-21 actively takes part in T cell activation and proliferation, and is involved in cytokine cascades during CAR-T and TCR-engineered cell therapies.
- Elevated IL-21 levels may exacerbate CRS symptoms by enhancing cytotoxic immune cell function.
- Real-time monitoring of IL-21, alongside IL-6 and IFN-γ, can support risk stratification and management of immune-related adverse events.

4.2 Immune Modulation Biomarker

- Quantification of IL-21 helps assess immune reconstitution, T cell fitness, and NK cell cytotoxicity in CGT trials.
- It acts as a biomarker of therapeutic efficacy in cancer immunotherapy and as an indicator of immune overactivation in autoimmune-targeted CGTs.

4.3 Genetic Modulation

- Gene-editing strategies explore overexpression of IL-21 in CAR-T cells or NK cells to emphasise their persistence, proliferation, and tumour-killing potential.
- Alternatively, IL-21 inhibition may be used in gene therapies targeting autoimmune diseases or in transplant models to reduce graft-versus-host responses.
- IL-21 pathway engineering is being applied to optimize cytokine signalling in advanced CGT platforms.

Scope of Validation

The PrecisionBind Human IL-21 (Catalog No KB1085) 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 21.
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-21 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-21 ELISA kit is intended to measure the IL-21 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-21 Cmax Values and Recommended ELISA Range

This table summarizes IL-21 Cmax levels across diseases and suggests corresponding ELISA working ranges.

Application	Expected IL-21 Range (pg/ml)	Recommended ELISA Range (pg/ml)
Healthy Baseline	<20	0 - 100
Chronic Inflammatory Disease (RA, IBD, Psoriasis)	50 - 500 (peaks up to 800)	0 - 1,000
Sepsis / Cytokine Storm	200 - 2,000 (rare >5,000)	0 - 5,000
Cell & Gene Therapy Cytokine Release Monitoring	100 - 1,000	0–2,000

Note: Assay sensitivity < 10 pg/mL recommended for baseline detection; upper limit ≥ 5,000 pg/ml advised for CRS monitoring.

The PrecisionBind Human IL-21 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 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-21 and are monoclonal antibodies. They show a high affinity to bind to native as well as recombinant IL-21.

2.2 Selectivity

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

2.3 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 = 2 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 – 5 pg/ml

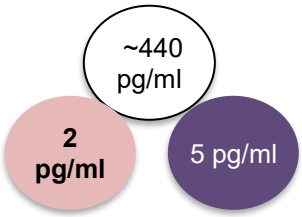
IC50 in ELISA (Half Maximal Inhibitory Concentration)
IC50 = The concentration of an inhibitor (drug, antibody, compound) required to reduce the signal (e.g., binding, enzymatic activity) by 50% compared to the maximum signal in the assay.

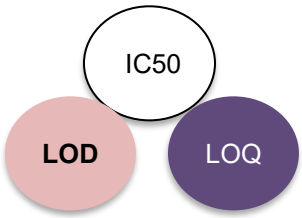
In ELISA, this is commonly used for:
Neutralization ELISA: Quantifies potency of antibodies inhibiting target–ligand interaction.
Drug Potency Testing: Measures concentration at which drug inhibits 50% of target activity.

IC50 for PrecisionBind Human IL-21 ELISA = ~440 pg/ml

Summary:

Parameter	Value (pg/mL)
LOD	2 pg/ml
LOQ	5 pg/ml
IC50	~440 pg/ml

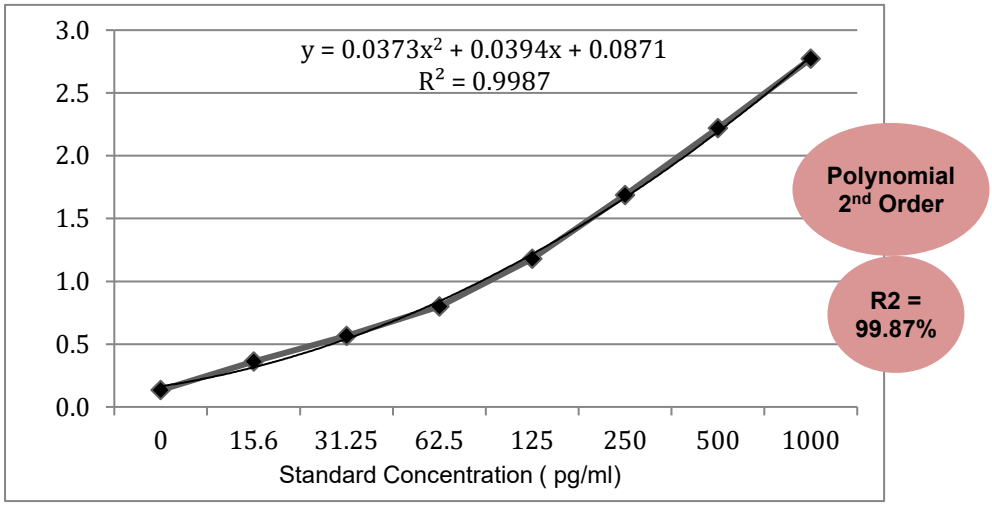


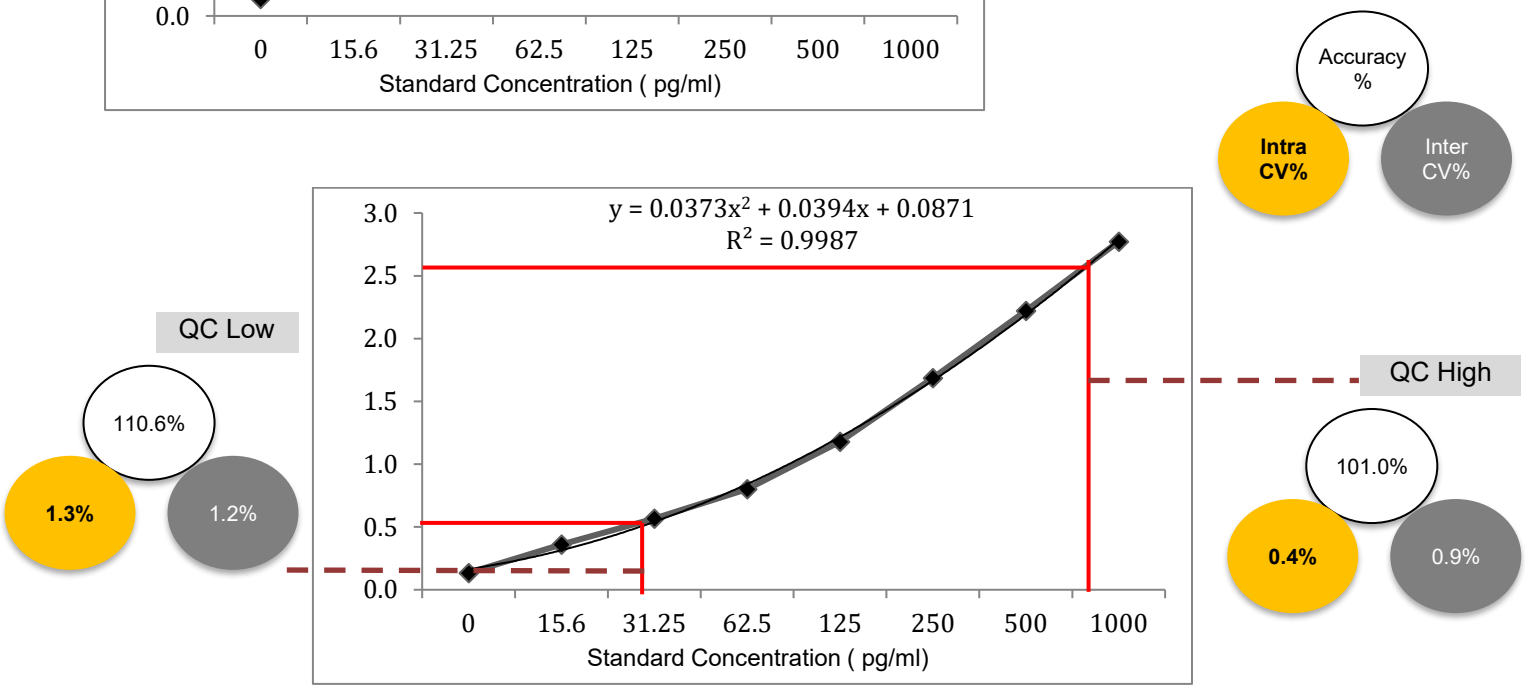


Regulatory Note:
LOD S/N ≥ 3:1, LOQ ≥ 10:1, %CV ≤ 20% *S/N = Signal / Noise Ratio

3. Linearity and Range

Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration	% Recovery
0	0.134	0.1	--
15.6	0.362	14.3	91.7
31.25	0.566	33.3	106.6
62.5	0.801	61.6	98.6
125	1.180	124.0	99.2
250	1.687	253.8	101.5
500	2.221	494.1	98.8
1000	2.773	1004.2	100.4
Positive Control (750 pg/ml)	2.483	712.9	95.1
Low QC control (31.25 pg/ml)	0.568	34.6	110.6
High QC control (800 pg/ml)	2.595	808.2	101.0





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

A) Intra-Assay:

Standard (pg/ml)	Mean OD450	SD	%CV
15.6	0.248	0.33	1.3
250	1.648	1.17	0.7
1000	2.764	1.06	0.4

B) Inter assay:

Standard (pg/ml)	Mean OD450	SD	%CV
15.6	0.245	0.25	1.2
250	1.653	2.36	1.7
1000	2.763	1.92	0.9

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 500 pg/ml Human IL-21 and ELISA assay was run.

Sample	Mean OD450	Interpolated Concentration	% Recovery
Neat Human CSF	2.741	933.7	186.7
Neat Human Plasma	2.631	819.5	163.9
Neat Human Serum	2.883	1109.0	221.8

A) Serum:

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:100 dilution	1000	2.768	1006.9	100.7	100.7
1:200 dilution	500	2.158	472.7	94.5	94.5
1:400 dilution	250	1.701	268.3	107.3	107.3
1:800 dilution	125	1.222	137.0	109.6	109.6
1:1600 dilution	62.5	0.809	64.2	102.7	102.7
1:3200 dilution	31.25	0.401	17.5	56.0	56.0
1:6400 dilution	15.6	0.216	3.8	24.5	24.5

B) Plasma:

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:100 dilution	1000	2.589	763.9	76.4	76.4
1:200 dilution	500	2.092	427.1	85.4	85.4
1:400 dilution	250	1.628	241.4	96.6	96.6
1:800 dilution	125	1.001	93.2	74.6	74.6
1:1600 dilution	62.5	0.811	63.3	101.3	101.3
1:3200 dilution	31.25	0.579	34.0	108.9	108.9
1:6400 dilution	15.6	0.300	8.6	55.1	55.0

C) Cerebrospinal Fluid (CSF):

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:100 dilution	1000	2.617	789.6	79.0	79.0
1:200 dilution	500	2.166	466.0	93.2	93.2
1:400 dilution	250	1.674	256.2	102.5	102.5
1:800 dilution	125	1.219	135.1	108.1	108.1
1:1600 dilution	62.5	0.828	65.8	105.2	105.2
1:3200 dilution	31.25	0.601	36.5	116.7	116.7
1:6400 dilution	15.6	0.321	10.1	64.8	64.7

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-21 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.

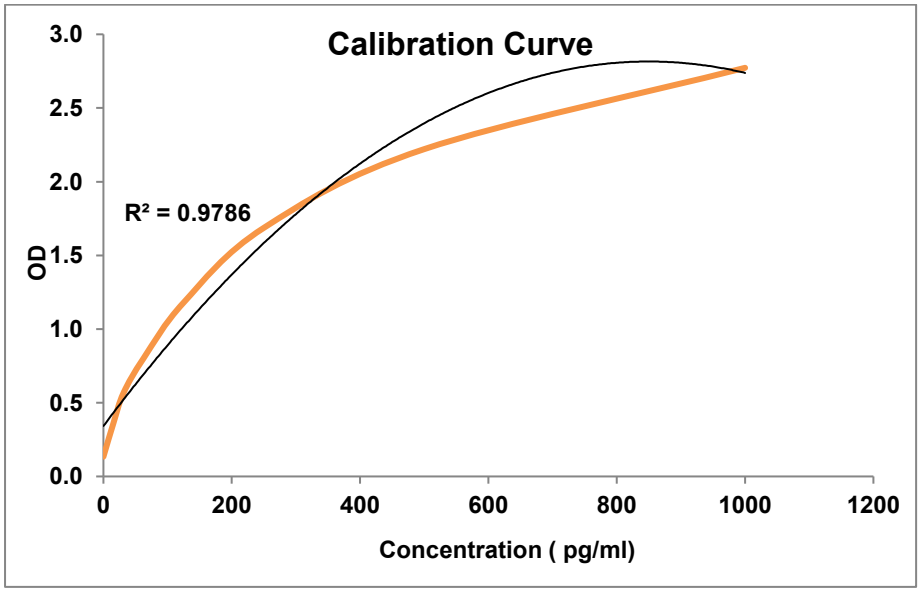
Serum: Use a serum separator tube and allow clotting for 30 minutes, then centrifuge for 10 minutes at 1000 x *g*. Remove serum layer and assay immediately or store serum samples at temperature <-20°C. Avoid repeated freeze/thaw cycles.

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:



Matrix Effect Heat Map

	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
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%

References

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