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 GM-CSF ELISA KIT (Catalog No KB1048) 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

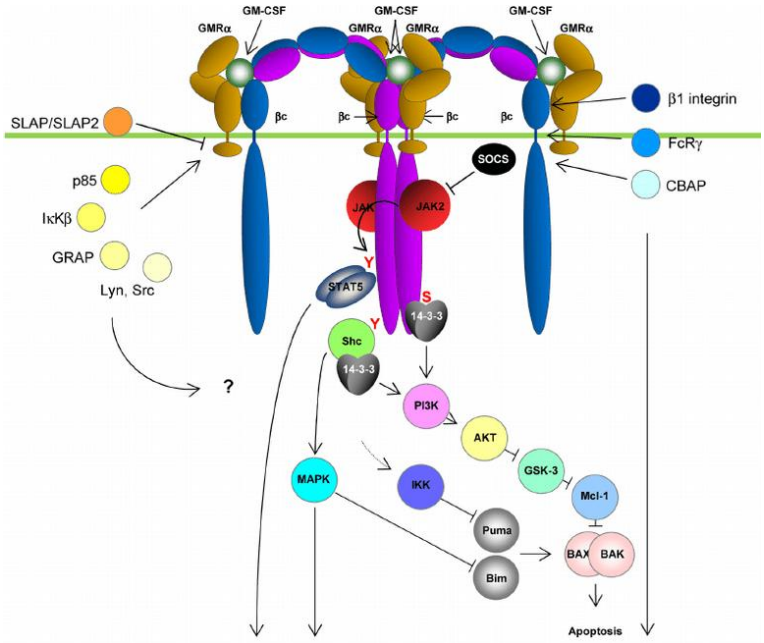
First Codification	History	Date
Version#1	**VALIDATION DATA OF PRECISIONBIND HUMAN GM-CSF ELISA KIT (Catalog No KB1048)**	31.07.2025

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

Background

1. Introduction to Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) is a hematopoietic growth factor and immunomodulatory cytokine that are produced by T cells, macrophages, endothelial cells, fibroblasts, and epithelial cells in response to immune stimuli. It has a significant role in the differentiation, survival, and activation of myeloid lineage cells, including granulocytes, macrophages, and dendritic cells. Beyond haematopoiesis, GM-CSF functions in host defence mechanism, tissue repair, and inflammation. Aberrant GM-CSF expression has been linked to several types of autoimmune diseases (e.g., rheumatoid arthritis, multiple sclerosis), chronic inflammatory conditions, and cytokine release syndrome (CRS). Due to its multifaceted role, GM-CSF is considered as both a therapeutic target and a biomarker in drug development and clinical research.



Cell Signalling Pathways

2. Significance in Drug Discovery Research

2.1 Target Identification and Validation

- GM-CSF is a key mediator of myeloid cell-driven inflammation.
- Elevated GM-CSF levels are observed in autoimmune diseases (e.g., RA, MS), COVID-19-related cytokine storms, and pulmonary disorders.
- GM-CSF neutralization (e.g., via antibodies like mavrilimumab or otilimab) is a validated therapeutic strategy in clinical trials.

2.2 Assay Development

- GM-CSF is used in various assay formats to screen and characterize anti-inflammatory or myeloid-targeting therapies:
  - ELISA for GM-CSF concentration quantification in serum or tissue lysates.
  - Bioassays using TF-1 cell proliferation or STAT5 phosphorylation as a functional readout.
  - Multiplex panels for simultaneous cytokine profiling (with IL-6, TNF-α, IL-1β, etc.).

2.3 Biomarker for Efficacy and Safety

- GM-CSF levels serve as a pharmacodynamic marker to monitor and observe immune modulation during therapeutic interventions.
- It's up regulation in severe disease states makes it a predictive marker for response and toxicity in cytokine-based drug studies.

3. Relevance in Biopharmaceutical Development

3.1 Monoclonal Antibodies and Biosimilars

- Anti-GM-CSF therapies (e.g., lenzilumab, mavrilimumab, otilimab) are in clinical trials for autoimmune and pulmonary disorders.
- Biopharmaceutical development requires:
  - GM-CSF–receptor binding assays to determine affinity and specificity.
  - Neutralization potency assays (cell-based bioassays).
  - Comparability and stability studies for biosimilar and biologic development.

3.2 PK/PD Studies

- GM-CSF is observed and targeted as a biomarker for drug activity in diseases such as rheumatoid arthritis, COVID-19, and GVHD.
- PD assessments include correlating GM-CSF suppression with clinical endpoints and immune cell phenotyping.

3.3 Safety Evaluation

- Over-suppression of GM-CSF can impair host defence and myeloid cell function, increasing infection susceptibility.
- Careful quantitative assessment is necessary to optimize therapeutic window and minimize immunosuppression-related risks.

4. Importance in Cell and Gene Therapy (CGT)

4.1 Cytokine Release Syndrome (CRS) Monitoring

- GM-CSF is a principle upstream driver of CRS, particularly in CAR-T and TCR therapies, by promoting monocyte/macrophage activation and cytokine amplification.
- GM-CSF dysregulation (e.g., with lenzilumab) is being explored to reduce CRS severity without compromising antitumor efficacy.

4.2 Immune Modulation Biomarker

- GM-CSF levels are monitored as part of cytokine panels in CGT trials to evaluate immune activation and inflammatory toxicity.
- Its presence can differentiate between protective immune activity and pathogenic inflammation

4.3 Genetic Modulation

- Gene-edited CAR-T cells with GM-CSF knockout (GM-CSF$^{-/-}$) are being developed to mitigate CRS and neurotoxicity while preserving antitumor potency.
- CRISPR/Cas9 and RNAi strategies targeting GM-CSF are in preclinical development for autoimmune and cell-based immunotherapies.

Scope of Validation

The PrecisionBind Human GM-CSF ELISA (Catalog No KB1048) 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 GM-CSF.

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 (GM CSF 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 GM-CSF ELISA kit is intended to measure the Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) in serum, plasma, cell culture supernatant and other biologicalfluids.

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. GM-CSF Cmax Values and Recommended ELISA Range

This table summarizes GM-CSF Cmax levels across diseases and suggests corresponding ELISA working ranges.

Application	Expected GM-CSF Range (pg/ml)	Recommended ELISA Range (pg/ml)
Healthy Baseline	<5	0 - 50
Chronic Inflammatory Disease (RA, IBD, Psoriasis)	10-150 (peaks up to 300)	0 - 500
Sepsis / Cytokine Storm	100 - 1500 (rare >3,000)	0 - 2,000 (extendable to 5,000)
Cell & Gene Therapy Cytokine Release Monitoring	50 - 1,000	0–2,000

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

The PrecisionBind Human GM-CSF 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 16 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 GM-CSF and are monoclonal antibodies. They show a high affinity to bind to native as well as recombinant GM-CSF.

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 6 U of 88/646 NIBSC-standard.

Therefore 1000 pg/ml is equivalent to 6 U of GM-CSF 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 GM-CSF ELISA = 3.03 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 GM-CSF ELISA - 9.17 pg/ml

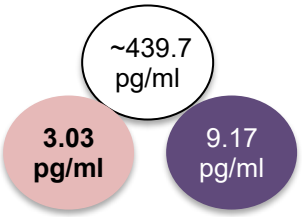
IC50 in ELISA (Half Maximal Inhibitory Concentration)
IC50 = The concentration of an inhibitor (drug, antibody, compound) required to reduce the signal (e.g., binding, enzymatic activity) by 50% compared to the maximum signal in the assay.

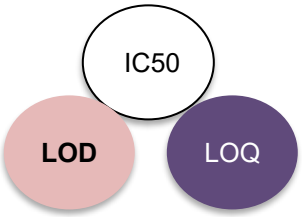
In ELISA, this is commonly used for:
Neutralization ELISA: Quantifies potency of antibodies inhibiting target–ligand interaction.
Drug Potency Testing: Measures concentration at which drug inhibits 50% of target activity.

IC50 for PrecisionBind Human GM-CSF ELISA = ~439.7 pg/ml

Summary:

Parameter	Value (pg/mL)
LOD	3.03 pg/ml
LOQ	9.17 pg/ml
IC50	~ 439.7 pg/ml

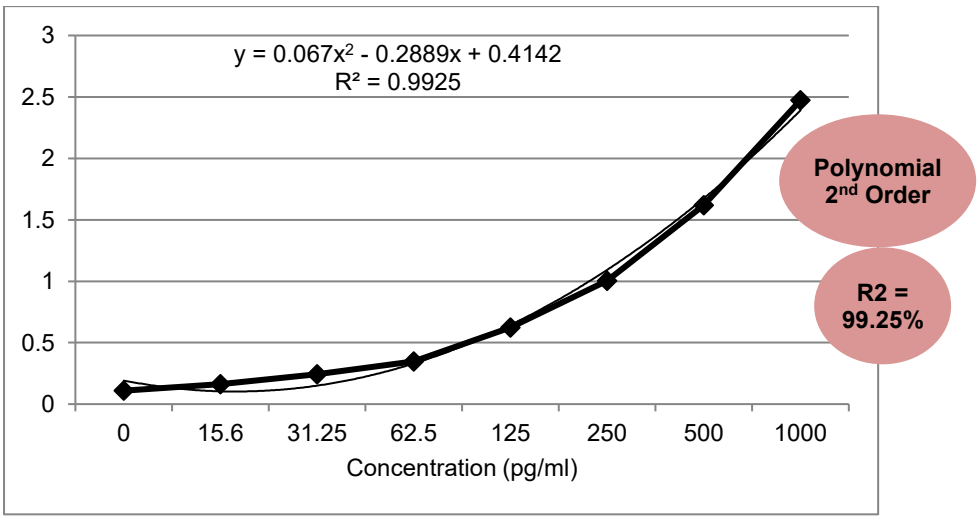


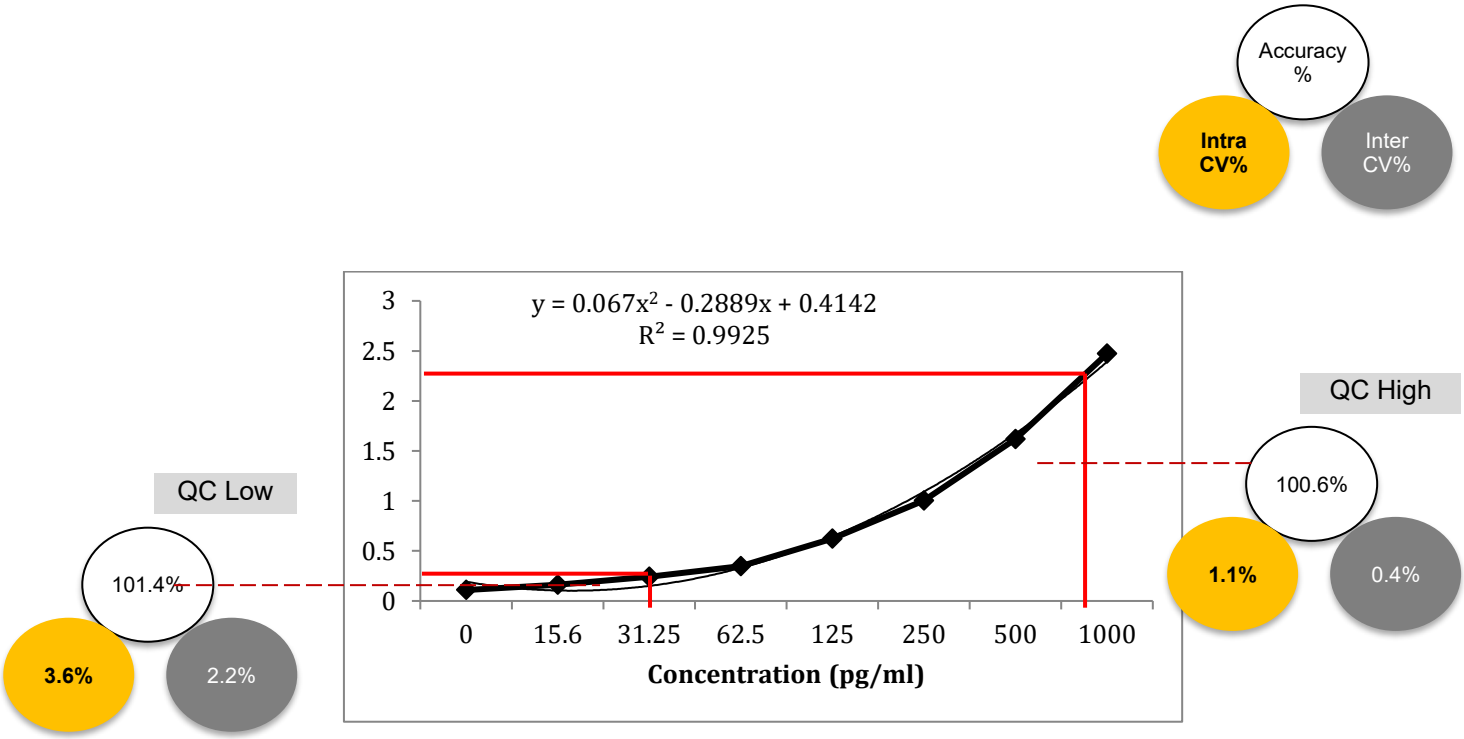


Regulatory Note:
LOD *S/N ≥ 3:1, LOQ ≥ 10:1, %CV ≤ 20% *S/N = Signal / Noise Ratio

3. Linearity and Range

Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery
0	0.109	--	--
15.6	0.162	5.1	33.0
31.25	0.242	24.3	77.8
62.5	0.349	51.4	82.3
125	0.623	127.5	102.0
250	1.004	249.7	99.9
500	1.618	497.3	99.5
1000	2.472	1001.5	100.2
Positive QC (500 pg/ml)	1.720	513.6	102.7
Low QC (31.25 pg/ml)	0.228	31.7	101.4
High QC (800 pg/ml)	2.231	804.6	100.6





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

A) Intra-Assay:

Standard Concentration (pg/ml)	Mean OD450	SD	%CV
15.6	0.329	0.96	3.6
125.0	0.567	1.74	3.8
1000.0	2.493	2.33	1.1

B) Inter Assay:

Standard Concentration (pg/ml)	Mean OD450	SD	%CV
15.6	0.335	0.75	2.2
125.0	0.578	1.42	2.5
1000.0	2.491	1.02	0.4

5. Parallelism and Matrix Effect

Sample Dilution factor – Human Serum, Human Plasma and Human CSF samples have been tested. Neat samples can be run directly.

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

Sample	Mean Absorbance	Interpolated Concentration	% Recovery
Neat CSF samples	2.945	Out of range	Out of range
Neat Plasma	2.807	1344.0	268.8
Neat Human Serum	3.673	Out of range	Out of range

A) Serum:

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:1	1000	2.632	1128.9	112.9	88.6
1:2	500	1.960	669.9	134.0	74.6
1:4	250	1.090	280.9	112.4	89.0
1:8	125	0.762	172.3	137.9	72.5
1:16	62.5	0.466	87.8	140.4	71.2
1:32	31.3	0.367	62.1	198.4	50.3
1:64	15.6	0.254	34.4	220.6	45.4

B) Plasma:

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (pg/ml)	% Recovery	% Deviation
1:1	1000	2.629	1140.7	114.1	87.7
1:2	500	1.900	603.5	120.7	82.8
1:4	250	1.278	333.0	133.2	75.1
1:8	125	0.701	153.0	122.4	81.7
1:16	62.5	0.420	78.9	126.3	79.2
1:32	31.25	0.289	45.7	146.2	68.4
1:64	15.6	0.230	30.6	196.3	51.0

C) Cerebrospinal Fluid (CSF):

Dilution	Expected Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration (ng/ml)	% Recovery	% Deviation
1:1	1000	2.701	1217.8	121.8	82.1
1:2	500	1.942	626.2	125.2	79.8
1:4	250	1.290	337.3	134.9	74.1
1:8	125	0.713	156.3	125.1	80.0
1:16	62.5	0.446	85.6	136.9	73.0
1:32	31.25	0.312	51.5	164.9	60.6
1:64	15.6	0.228	30.1	193.0	51.9

Results:

i) Parallelism is maintained across the 1:1 to 1:16 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 GM-CSF 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.

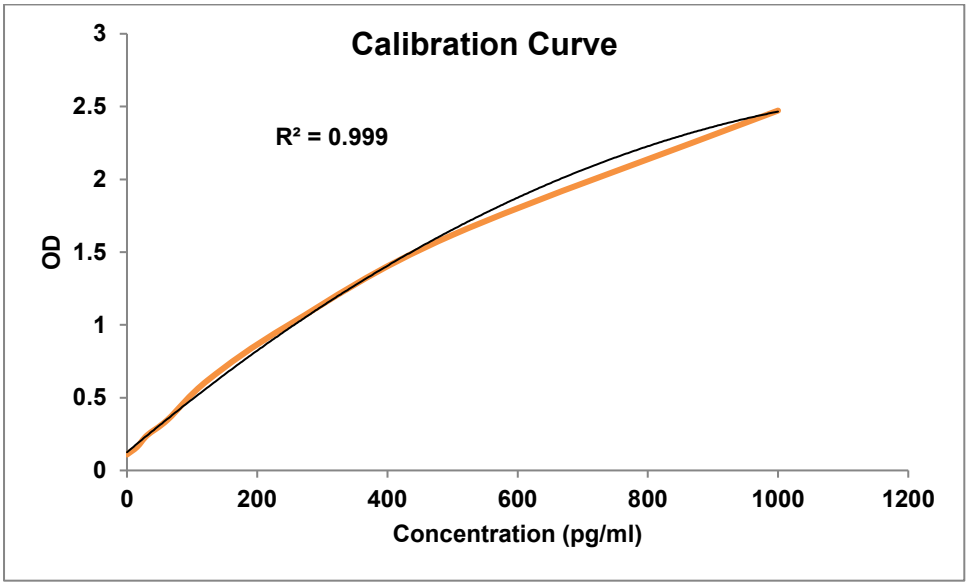
B) Storage conditions:

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 88/646 GM-CSF

PrecisionBind GM-CSF Kit Standard

Matrix Effect Heat Map

	1:1	1:2	1:4	1:8	1:16	1:32	1:64
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

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

EMA Guideline on Biosimilar Monoclonal Antibodies (2012).