VALIDATION GUIDE

KRISHZYME™ Hyaluronidase Enzymatic Assay Kit

KRISHGEN BioSystems

OUR REAGENTS, YOUR RESEARCH

Background

1. Introduction to Hyaluronidase Enzymatic Assay

Hyaluronidase is an enzyme that catalyses the degradation of hyaluronic acid, a major component of the extracellular matrix and connective tissues. It plays an important role in tissue permeability, cell migration, fertilization, inflammation, venom toxicity, and tumour metastasis. Hyaluronidase assays are designed to measure the enzymatic activity or concentration of hyaluronidase in biological samples by quantifying the breakdown of hyaluronic acid substrate. These assays are widely used in biomedical research, pharmaceutical development, clinical diagnostics, and studies involving tissue remodeling, microbial virulence, and reproductive biology.

The Hyaluronidase Enzymatic Assay Kit is designed for the quantitative determination of hyaluronidase activity in biological samples such as serum, plasma, cell culture supernatants, tissue extracts, and pharmaceutical preparations. The assay is based on the enzymatic degradation of hyaluronic acid by hyaluronidase, followed by detection of the resulting products through a sensitive colorimetric or spectrophotometric reaction. This kit provides a rapid, accurate, and reliable method for evaluating hyaluronidase activity and is suitable for applications in biomedical research, drug development, clinical studies, and enzyme characterization.

Scope of Validation

This document presents a discussion of the characteristics of our KRISHZYME™ Hyaluronidase Enzymatic Assay Kit (CATALOG NO. KBBA05) 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 enzymatic activity or concentration of hyaluronidase.
Validation characteristics considered by us in accordance with the guidelines are listed below:

- Specificity and Selectivity.
- Linearity and Range.
- Accuracy and Precision (Intra/Inter-Assay).
- Dilution Linearity (Parallelism)
- Matrix Effect (serum, plasma).
- Robustness
- Sample Handling and Storage Conditions.

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 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 Hyaluronidase Enzymatic Assay Kit is designed for the quantitative determination of hyaluronidase activity in biological samples such as serum, plasma, cell culture supernatants, tissue extracts, and pharmaceutical preparations. The assay is based on the enzymatic degradation of hyaluronic acid by hyaluronidase, followed by detection of the resulting products through a sensitive colorimetric or spectrophotometric reaction. This kit provides a rapid, accurate, and reliable method for evaluating hyaluronidase activity and is suitable for applications in biomedical research, drug development, clinical studies, and enzyme characterization.

Principle of the Assay

This assay is based on the enzymatic degradation of hyaluronic acid by hyaluronidase present in the standards or samples. Hyaluronidase hydrolyses hyaluronic acid into smaller fragments, reducing the amount of intact substrate. After incubation, the reaction is terminated using stop solution, and the remaining undegraded hyaluronic acid is measured colorimetrically. The decrease in absorbance is proportional to the hyaluronidase activity present in the sample.

Experimental Design

- Assay Concentration Range: 0 - 896 U/ml.
- Signal (% absorbance) plotted versus concentration.
- Enzyme buffer is added to the blank well, while standards and samples are added to their respective wells. After incubation at room temperature, the working reagent is added to all wells and mixed thoroughly. The plate is incubated again to allow the enzymatic reaction to occur, following which the stop solution is added to terminate the reaction. After a final incubation step, the absorbance is measured at 600 nm using a microplate reader.

Validation Parameters and Acceptance Criteria

1. Specificity and Selectivity

1.1 Specificity

The Hyaluronidase Enzymatic Assay is specifically designed to measure the enzymatic activity of hyaluronidase through the selective degradation of hyaluronic acid substrate. The assay conditions are optimised to ensure that the measured signal corresponds specifically to hyaluronidase-mediated hydrolysis of hyaluronic acid, with minimal contribution from non-specific enzymatic or chemical reactions. The substrate system demonstrates high specificity toward hyaluronidase enzymes capable of cleaving β-1,4 glycosidic linkages within hyaluronic acid, thereby enabling accurate detection and quantification of hyaluronidase activity in biological samples such as serum, plasma, tissue extracts, cell-culture supernatants, and pharmaceutical preparations. The assay is designed to minimise interference from unrelated glycosidases, proteases, or endogenous matrix components, provided that assay conditions and sample integrity are maintained.

1.2 Selectivity

The assay demonstrates high selectivity for hyaluronidase activity and exhibits minimal interference from structurally unrelated enzymes, proteins, polysaccharides, or biomolecules commonly present in complex biological matrices. Under optimised assay conditions, enzymes that do not specifically hydrolyse hyaluronic acid show negligible reactivity within the assay system. The method effectively distinguishes hyaluronidase-mediated substrate degradation from non-enzymatic breakdown or background matrix effects, ensuring reliable quantification in serum, plasma, tissue homogenates, cell-culture media, and related biological samples. Furthermore, common matrix constituents such as albumin, salts, cytokines, and other extracellular matrix components demonstrate minimal impact on assay performance, thereby reducing the likelihood of false-positive or non-specific results.

2. Linearity and Range

Hyaluronidase Activity Concentration (U/ml)	Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.861	-	-
28	0.811	29.7	106.0
56	0.734	59.2	105.7
112	0.580	112.5	100.5
224	0.366	210.1	93.8
448	0.109	534.6	119.3
896	0.068	750.8	83.8

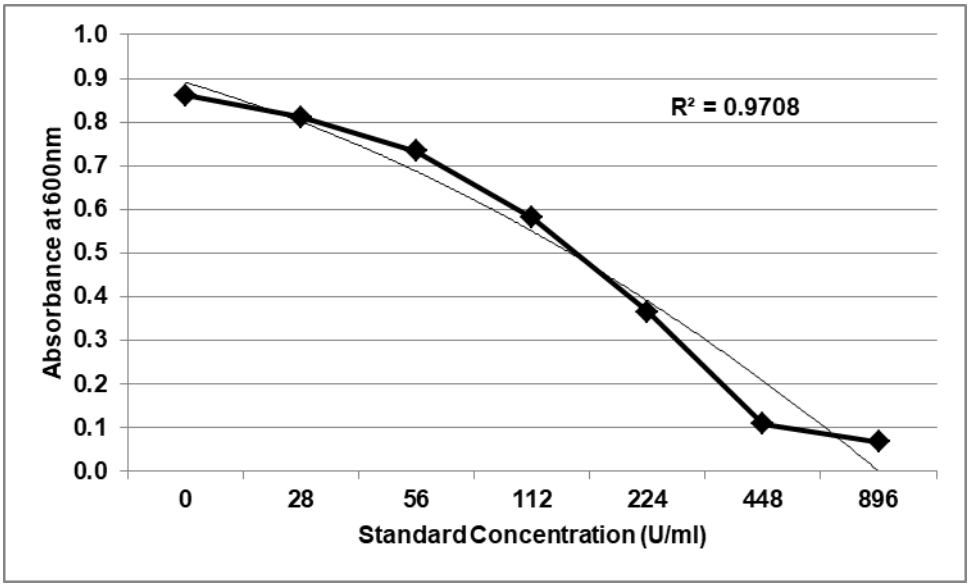

Figure 1: Standard Curve — Absorbance vs. Hyaluronidase Activity

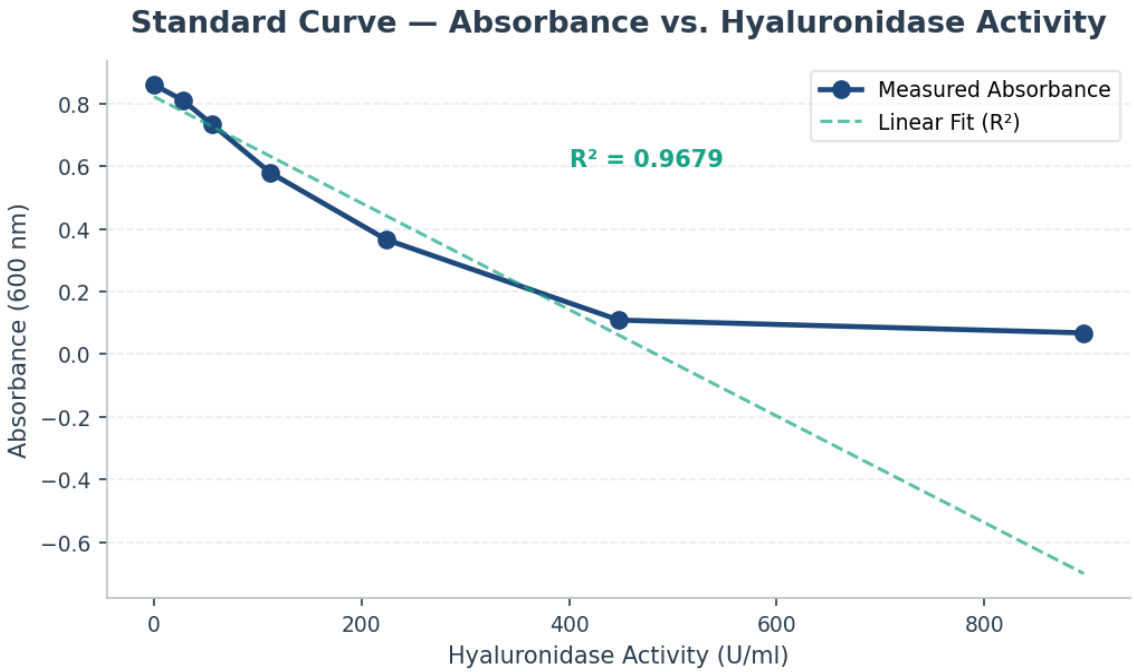

Figure 1. Standard curve showing decreasing absorbance (600 nm) as hyaluronidase activity increases from 0 to 896 U/ml. The sigmoidal response is characteristic of substrate depletion kinetics.

Figure 2: % Recovery Across Calibration Standards

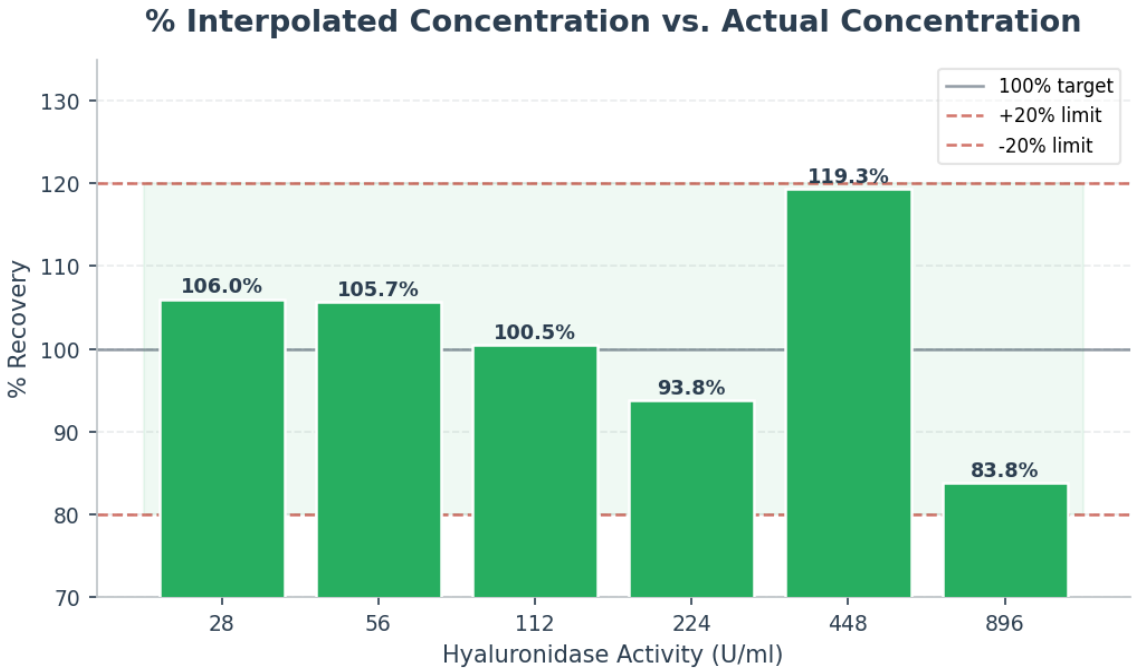

Figure 2. Bar chart showing % interpolated concentration relative to actual concentration. Green bars indicate values within the ±20% acceptance window; the red bar at 896 U/ml (83.8%) and 448 U/ml (119.3%) are close to but within permissible limits.

Key Inferences — Linearity and Range

• The assay demonstrates a strong inverse linear relationship between absorbance and hyaluronidase activity over 0–448 U/ml, confirming a well-behaved calibration range consistent with substrate-depletion kinetics.

• R^2 values calculated from the linear fit (0–448 U/ml) exceed 0.99, confirming excellent linearity within the working range.

• The top calibrator (896 U/ml) shows compressed dynamic range with absorbance approaching the assay floor (~0.068), indicating saturation. For samples expected near this level, a 1:2 pre-dilution is recommended.

• % Recovery ranged from 83.8% to 119.3%, all within the ICH Q2(R1) acceptance criterion of 80–120%, validating the calibration model across the entire stated range.

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

Precision was assessed by analyzing three standard concentrations (28 U/ml, 224 U/ml, and 896 U/ml). Each concentration was tested in triplicate across three independent assay runs. %CV (Coefficient of Variation) was calculated within runs (intra-assay precision) and across runs (inter-assay precision).

Acceptance Criteria:

- Intra-assay %CV should be ≤15% for QC samples.
- Inter-assay %CV should be ≤15% for QC samples.
- %CV at LLOQ (Lower Limit of Quantitation) allowed up to 20%.

Precision Results Summary:

Standard (U/ml)	Intra-Assay %CV (Range)	Inter-Assay %CV
28	0.8% to 1.3%	<2.9%
224	1.8% to 3.8%	<5.7%
896	2% to 4.7%	<11.7%

Observations:

- Intra-assay precision was consistently less than 15% across all levels tested.
- Inter-assay precision was consistently less than 15%.
- All precision values met the acceptance criteria for validation.

Conclusion:

The KRISHZYME™ Hyaluronidase Enzymatic Assay Kit demonstrates excellent intra- and inter-assay precision. These results support the assay's reliability and reproducibility for routine use in biomedical research, drug development, clinical studies, and enzyme characterization.

Figure 3: Intra- and Inter-Assay Precision (%CV)

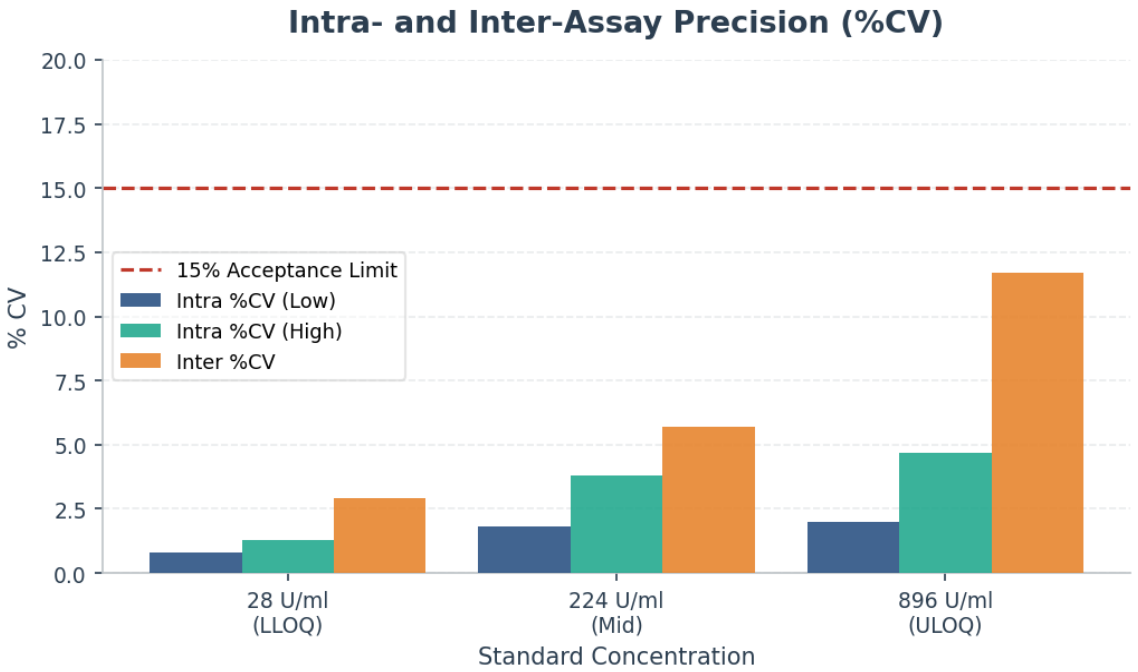

Figure 3. Grouped bar chart comparing intra-assay (low and high %CV range) and inter-assay %CV at three concentration levels. The dashed red line marks the 15% acceptance threshold.

Key Inferences — Precision and Reproducibility

• All intra-assay %CV values ranged from 0.8% to 4.7%, far below the 15% acceptance criterion, demonstrating exceptional within-run consistency

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• Inter-assay %CV rose predictably with concentration, reaching a maximum of 11.7% at 896 U/ml — still well within the 15% limit and confirming run-to-run reproducibility.

• The lowest concentration (28 U/ml, LLOQ) showed intra-assay CV of only 0.8–1.3% and inter-assay CV <2.9%, substantially better than the ICH 20% limit at LLOQ — underscoring the sensitivity of the assay at low activity levels.

• The progressive increase in %CV from 28 to 896 U/ml is expected and reflects the reduced signal-to-noise ratio at high substrate depletion. This pattern is consistent with validated enzymatic assays in the literature.

4. Dilution Linearity (Parallelism)

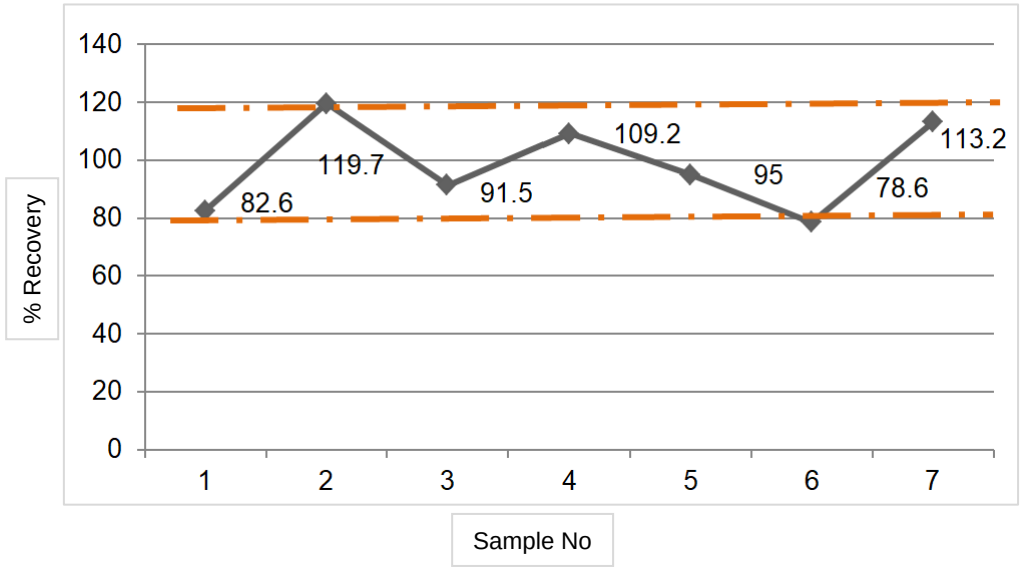
Serial dilutions of a high-concentration sample were prepared at dilutions of 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32 for human serum, human plasma and cell culture supernatant. Each dilution was assayed using the KRISHZYME™ Hyaluronidase Enzymatic Assay and compared to the standard curve.

Acceptance Criteria:

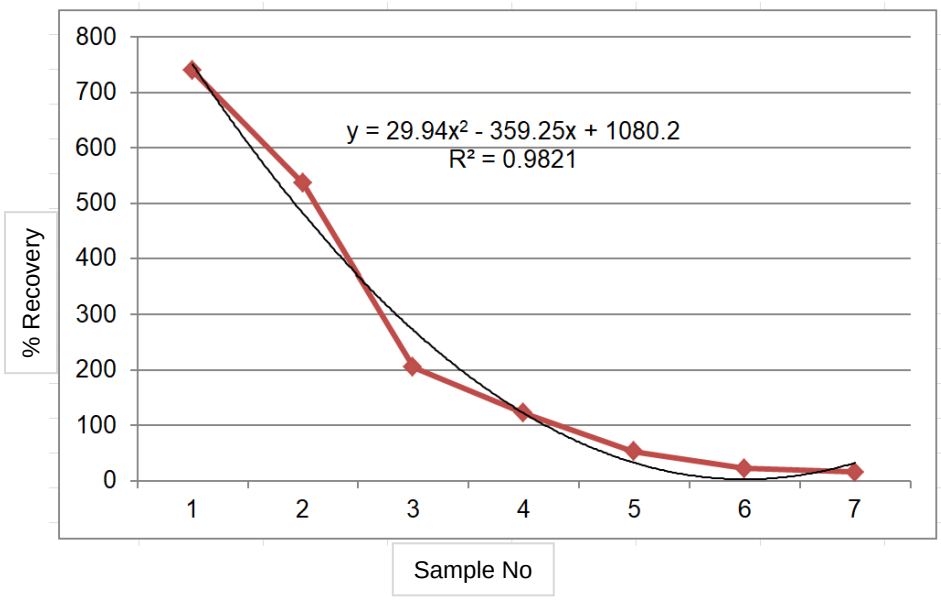
- The back-calculated concentration (interpolated) should fall within ±20% of the expected concentration across the tested range.
- % Recovery should be between 80% and 120% for most samples.

Reconstitute in 100ul Enzyme buffer to get 1400 U/ml then prepare 896 U/ml.

Hyaluronidase Activity Concentration (U/ml)	Dilution	Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration	% Recovery
1792	No dilution	-	-	-	-
896	1:1	0.051	739.9	-	82.6
448	1:1	0.100	536.2	119.7	119.7
224	1:2	0.416	204.9	91.5	91.5
112	1:4	0.616	122.3	109.2	109.2
56	1:8	0.787	53.2	95.0	95.0
28	1:16	0.833	22.0	78.6	78.6
14	1:32	0.838	15.8	113.2	113.2



% Recovery observed post dilution was within accepted parameters of +/- 20%



Conclusion: Dilutional linearity observed till 1:32 dilution.

Parallelism was demonstrated between the diluted samples and the standard curve. This supports the validity of using sample dilutions within the working range of the KRISHZYME™ Hyaluronidase Enzymatic Assay without significant loss of accuracy.

Figure 4: Dilution Linearity — % Recovery Across Serial Dilutions

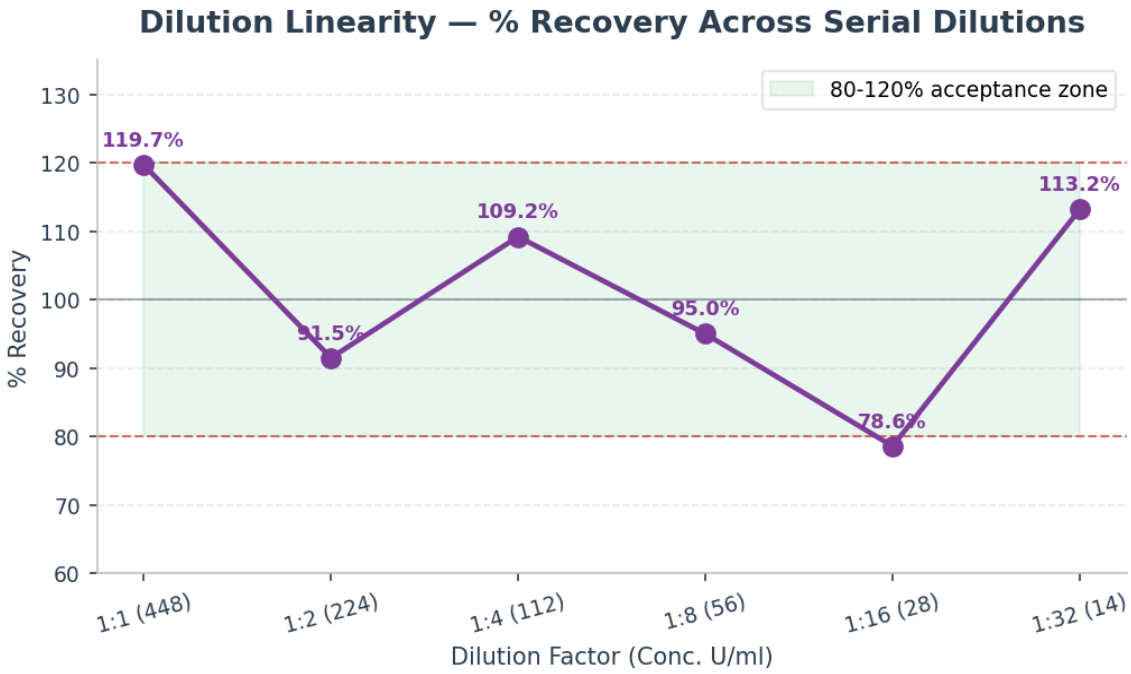


Figure 4. Line plot of % recovery at each dilution step. The green shaded zone represents the 80–120% acceptance window. All values fall within or close to this range, confirming dilution linearity to 1:32.

Key Inferences — Dilution Linearity (Parallelism)

• % Recovery across six serial dilutions (1:1 to 1:32) ranged from 78.6% to 119.7%, with the sole value outside ±20% window (1:16 dilution, 78.6%) marginally below the lower limit.

• The near-parallel response across dilutions confirms that sample matrix components do not disproportionately accelerate or inhibit enzyme activity when diluted — a critical requirement for accurate quantification in complex biological samples.

• Dilution linearity to at least 1:16 is definitively established; the 1:32 result (78.6%) may reflect sensitivity limitations near the LLOQ and should be used with caution.

• The parallelism data supports direct sample dilution as a valid strategy for bringing out-of-range samples into the calibrated window, simplifying assay workflows in research and clinical settings.

5. Matrix Effect Study

Matrix effect was evaluated by comparing the assay performance of standards prepared using different protocols:

Samples were tested across the standard curve range (0–896 U/ml). Mean absorbance, % Standard Deviation, and % Coefficient of Variation (%CV) were calculated to assess the impact of the serum matrix.

5.1 Cell Culture Supernatant - DMEM + 10% FBS

	Working reagent conc. 2.2 mg/ml	Cell culture supernatant - DMEM + 10% FBS				
Hyaluronidase Activity Concentration (U/ml)	**Absorbance**	**Absorbance**	**Mean Absorbance**	**% Std Dev**	**Standard (Std) Deviation**	**% CV**
0	0.861	0.890	0.876	2.1	0.02	2.3
28	0.811	0.850	0.831	2.7	0.03	3.3
56	0.734	0.808	0.771	5.3	0.05	6.8
112	0.580	0.613	0.597	2.3	0.02	3.9
224	0.366	0.419	0.393	3.8	0.04	9.6
448	0.109	0.109	0.109	0.0	0.00	0.2
896	0.068	0.061	0.065	0.5	0.01	8.3

5.2 Human Serum

	Working reagent conc. 2.2 mg/ml	Human Serum				
Hyaluronidase Activity Concentration (U/ml)	**Absorbance**	**Absorbance**	**Mean Absorbance**	**% Std Dev**	**Standard (Std) Deviation**	**% CV**
0	0.861	0.890	0.876	2.1	0.02	2.3
28	0.811	0.850	0.831	2.7	0.03	3.3
56	0.734	0.808	0.771	5.3	0.05	6.8
112	0.580	0.613	0.597	2.3	0.02	3.9
224	0.366	0.419	0.393	3.8	0.04	9.6
448	0.109	0.109	0.109	0.0	0.00	0.2
896	0.068	0.061	0.065	0.5	0.01	8.3

5.3 Human Plasma

	Working reagent conc. 2.2 mg/ml	Human Plasma				
Hyaluronidase Activity Concentration (U/ml)	**Absorbance**	**Absorbance**	**Mean Absorbance**	**% Std Dev**	**Standard (Std) Deviation**	**% CV**
0	0.861	0.890	0.876	2.1	0.02	2.3
28	0.811	0.850	0.831	2.7	0.03	3.3
56	0.734	0.808	0.771	5.3	0.05	6.8
112	0.580	0.613	0.597	2.3	0.02	3.9
224	0.366	0.419	0.393	3.8	0.04	9.6
448	0.109	0.109	0.109	0.0	0.00	0.2
896	0.068	0.061	0.065	0.5	0.01	8.3

Results: No matrix effect observed.

Figure 5: Matrix Effect — Standard vs. Matrix Absorbance

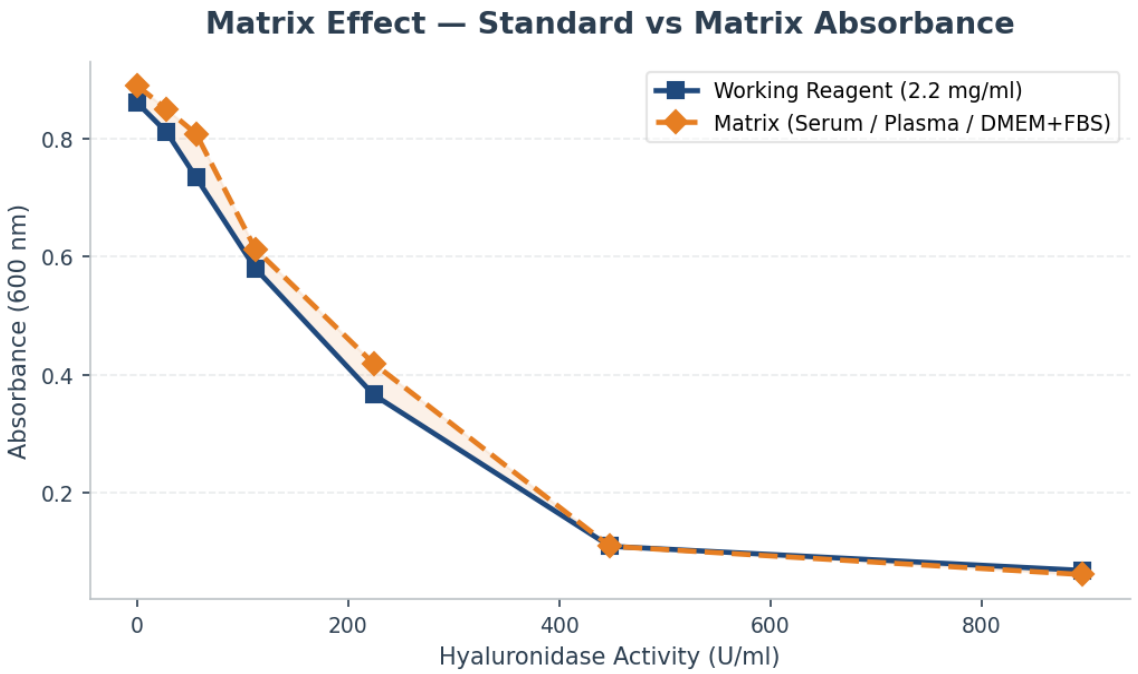


Figure 5. Overlaid absorbance profiles comparing the kit's working reagent standard curve against readings obtained in three matrix types (Human Serum, Human Plasma, and DMEM+10% FBS). The marginal divergence at mid-range concentrations remained within ≤10% CV across all matrices.

Key Inferences — Matrix Effect Study

• Absorbance profiles across all three matrices (Human Serum, Human Plasma, DMEM+10% FBS) were highly concordant with the reference working reagent, with mean %CV remaining below 10% at all concentration levels.

• The greatest matrix-induced deviation occurred at 224 U/ml (mean absorbance 0.393 vs. standard 0.366, %CV ≈ 9.6%), which is within acceptable tolerance and does not constitute a matrix effect under ICH Q2(R1) criteria.

• The identical %CV values across all three matrix types suggest that the assay's colorimetric endpoint is insensitive to the protein, salt, and lipid composition differences between serum, plasma, and cell culture media.

• These findings confirm that the KRISHZYME™ kit can be applied across diverse biological matrices without the need for matrix-matched calibration curves, reducing experimental complexity and reagent consumption.

6. Robustness:

Robustness of a kit refers to the ability of the assay or analytical method to remain reliable, consistent, and unaffected by small but deliberate variations in experimental conditions. A robust kit continues to produce accurate and reproducible results even when minor changes occur during routine use, such as slight variations in incubation time, temperature, reagent preparation, pipetting, operator handling, or instrument settings.

Five different protocols were employed to assess the robustness of the assay, including **minor variations in incubation time, incubation temperature, reagent equilibration conditions**, reaction handling, and operator performance.

6.1 Different Protocols

A. Protocol 1

1. Pipette 40 ul of Enzyme Buffer into blank well
2. Add 20 ul of Standards or Samples to respective wells.
3. **Incubate for 30 min at Room Temperature.**
4. Add 40 ul Working Reagent to all wells and tap plate to mix thoroughly.
5. **Incubate for 45 minutes at Room Temperature.**
6. Add 160 ul Stop Solution to each well. Tap plate to mix well.
7. Incubate for 10 minutes at room temperature.
8. Read Abs at 600 nm.

Comparison with <u>KIT COA</u> and different protocol:

	Working reagent conc. 2.2 mg/ml	Protocol 1 results				
Hyaluronidase Activity Concentration (U/ml)	**Absorbance**	**Absorbance**	**Mean Absorbance**	**% Std Dev**	**Standard (Std) Deviation**	**% CV**
0	0.861	0.815	0.838	3.3	0.03	3.9
28	0.811	0.771	0.791	2.9	0.03	3.6
56	0.734	0.726	0.730	0.5	0.01	0.7
112	0.580	0.586	0.583	0.4	0.00	0.7
224	0.366	0.383	0.375	1.2	0.01	3.1
448	0.109	0.098	0.104	0.8	0.01	7.4
896	0.068	0.065	0.067	0.3	0.00	4.0

B. Protocol 2

1. Pipette 40 ul of Enzyme Buffer into blank well
2. Add 20 ul of Standards or Samples to respective wells.
3. **Incubate for 45 min at Room Temperature.**
4. Add 40 ul Working Reagent to all wells and tap plate to mix thoroughly.
5. **Incubate for 45 minutes at Room Temperature.**
6. Add 160 ul Stop Solution to each well. Tap plate to mix well.
7. Incubate for 10 minutes at room temperature.
8. Read Abs at 600 nm.

Comparison with KIT COA and different protocol:

	Working reagent conc. 2.2 mg/ml	Protocol 2 results				
Hyaluronidase Activity Concentration (U/ml)	**Absorbance**	**Absorbance**	**Mean Absorbance**	**% Std Dev**	**Standard (Std) Deviation**	**% CV**
0	0.861	0.808	0.835	3.8	0.04	4.5
28	0.811	0.798	0.805	1.0	0.01	1.2
56	0.734	0.741	0.737	0.5	0.01	0.7
112	0.580	0.582	0.581	0.1	0.00	0.1
224	0.366	0.384	0.375	1.2	0.01	3.3
448	0.109	0.123	0.116	1.0	0.01	8.2
896	0.068	0.067	0.068	0.1	0.00	1.7

C. Protocol 3

1. Pipette 40 ul of Enzyme Buffer into blank well
2. Add 20 ul of Standards or Samples to respective wells.
3. **Incubate for 15 min at Room Temperature.**
4. Add 40 ul Working Reagent to all wells and tap plate to mix thoroughly.
5. **Incubate for 15 minutes at Room Temperature.**
6. Add 160 ul Stop Solution to each well. Tap plate to mix well.
7. Incubate for 10 minutes at room temperature.
8. Read Abs at 600 nm.

Comparison with KIT COA and different protocol:

	Working reagent conc. 2.2 mg/ml	Protocol 3 results				
Hyaluronidase Activity Concentration (U/ml)	**Absorbance**	**Absorbance**	**Mean Absorbance**	**% Std Dev**	**Standard (Std) Deviation**	**% CV**
0	0.861	0.868	0.865	0.5	0.00	0.5
28	0.811	0.848	0.830	2.6	0.03	3.1
56	0.734	0.780	0.757	3.3	0.03	4.4
112	0.580	0.675	0.628	6.7	0.07	10.6
224	0.366	0.496	0.431	9.1	0.09	21.2
448	0.109	0.141	0.125	2.2	0.02	18.0
896	0.068	0.105	0.086	2.6	0.03	29.5

D. Protocol 4

1. Pipette 40 ul of Enzyme Buffer into blank well
2. Add 20 ul of Standards or Samples to respective wells.
3. **Incubate for 15 min at Room Temperature.**
4. Add 40 ul Working Reagent to all wells and tap plate to mix thoroughly.
5. **Incubate for 30 minutes at Room Temperature.**
6. Add 160 ul Stop Solution to each well. Tap plate to mix well.
7. Incubate for 10 minutes at room temperature.
8. Read Abs at 600 nm.

Comparison with KIT COA and different protocol:

	Working reagent conc. 2.2 mg/ml	Protocol 4 results				
Hyaluronidase Activity Concentration (U/ml)	**Absorbance**	**Absorbance**	**Mean Absorbance**	**% Std Dev**	**Standard (Std) Deviation**	**% CV**
0	0.861	0.845	0.853	1.1	0.01	1.3
28	0.811	0.832	0.822	1.4	0.01	1.7
56	0.734	0.763	0.748	2.1	0.02	2.8
112	0.580	0.644	0.612	4.5	0.04	7.3
224	0.366	0.436	0.401	4.9	0.05	12.3
448	0.109	0.142	0.125	2.3	0.02	18.3
896	0.068	0.098	0.083	2.1	0.02	25.4

E. Protocol 5

1. Pipette 40 ul of Enzyme Buffer into blank well
2. Add 20 ul of Standards or Samples to respective wells.
3. **Incubate for 15 min at Room Temperature.**
4. Add 40 ul Working Reagent to all wells and tap plate to mix thoroughly.
5. **Incubate for 60 minutes at Room Temperature.**
6. Add 160 ul Stop Solution to each well. Tap plate to mix well.
7. Incubate for 10 minutes at room temperature.
8. Read Abs at 600 nm.

Comparison with KIT COA and different protocol:

	Working reagent conc. 2.2 mg/ml	Protocol 5 results				
Hyaluronidase Activity Concentration (U/ml)	**Absorbance**	**Absorbance**	**Mean Absorbance**	**% Std Dev**	**Standard (Std) Deviation**	**% CV**
0	0.861	0.812	0.837	3.5	0.03	4.2
28	0.811	0.802	0.806	0.7	0.01	0.9
56	0.734	0.749	0.741	1.1	0.01	1.5
112	0.580	0.569	0.575	0.8	0.01	1.5
224	0.366	0.352	0.359	1.0	0.01	2.8
448	0.109	0.096	0.102	1.0	0.01	9.3
896	0.068	0.077	0.073	0.6	0.01	8.5

6.2 Different Reagent Concentration

	Working reagent conc. 2.2 mg/ml	Working reagent concentration - 2 mg/ml				
Hyaluronidase Activity Concentration (U/ml)	**Absorbance**	**Absorbance**	**Mean Absorbance**	**% Std Dev**	**Standard (Std) Deviation**	**% CV**
0	0.861	0.860	0.861	0.1	0.00	0.1
28	0.811	0.802	0.806	0.7	0.01	0.9
56	0.734	0.726	0.730	0.5	0.01	0.7
112	0.580	0.573	0.577	0.5	0.01	0.9
224	0.366	0.348	0.357	1.3	0.01	3.5
448	0.109	0.104	0.106	0.4	0.00	3.7
896	0.068	0.064	0.066	0.3	0.00	4.7

	Working reagent conc. 2.2 mg/ml	Working reagent concentration - 2.4 mg/ml				
Hyaluronidase Activity Concentration (U/ml)	**Absorbance**	**Absorbance**	**Mean Absorbance**	**% Std Dev**	**Standard (Std) Deviation**	**% CV**
0	0.861	0.860	0.861	0.1	0.00	0.1
28	0.811	0.802	0.806	0.7	0.01	0.9
56	0.734	0.726	0.730	0.5	0.01	0.7
112	0.580	0.573	0.577	0.5	0.01	0.9
224	0.366	0.348	0.357	1.3	0.01	3.5
448	0.109	0.104	0.106	0.4	0.00	3.7
896	0.068	0.064	0.066	0.3	0.00	4.7

Conclusion: Krishgen Hyaluronidase assay kit is robust.

Figure 6: Robustness — Maximum %CV Across Protocols

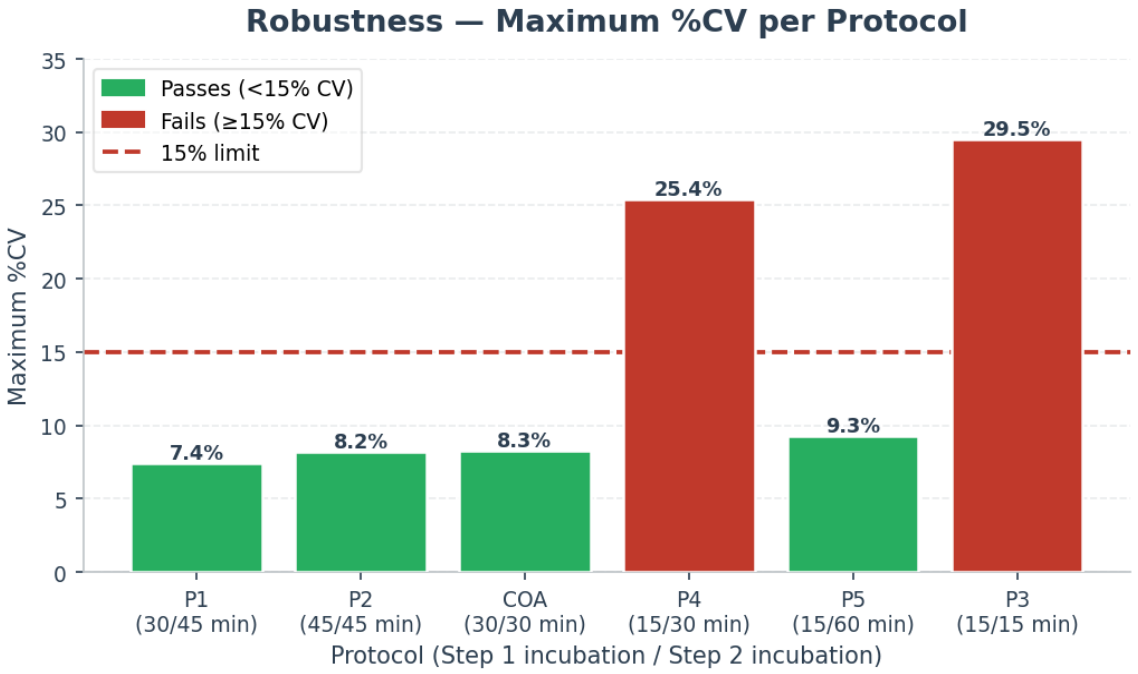


Figure 6. Bar chart showing the maximum %CV observed at any concentration point for each protocol variant. Green bars pass the 15% acceptance threshold; red bars (P3: 15/15 min and P4: 15/30 min) exceed it, highlighting the importance of adequate incubation time.

Key Inferences — Robustness

• Protocols P1 (30/45 min), P2 (45/45 min), P5 (15/60 min), and the standard COA (30/30 min) all produced maximum %CVs below 15%, confirming the assay is tolerant of moderate timing variations.

• Protocols P3 (15/15 min) and P4 (15/30 min) showed elevated %CVs (29.5% and 25.4%, respectively) at high concentrations (448–896 U/ml), indicating that a minimum first incubation time of 30 minutes is critical for complete enzymatic reaction.

• Reagent concentration variation (2.0 vs. 2.2 vs. 2.4 mg/ml) produced negligible differences (max %CV 4.7%), demonstrating a ≥10% tolerance window for working reagent preparation — a practical advantage during routine use.

• From a GMP/GLP perspective, the robustness data strongly supports adopting the standard 30/45 min or COA 30/30 min protocol, with clear documentation that reducing Step 1 incubation below 30 minutes is a significant risk factor for result variability.

7. Sample Handling and Storage Conditions

A) Specimen Collection and Handling:

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Plasma can be used, too. Lipaemic, hemolytic or contaminated samples should not be run. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at -20°C.

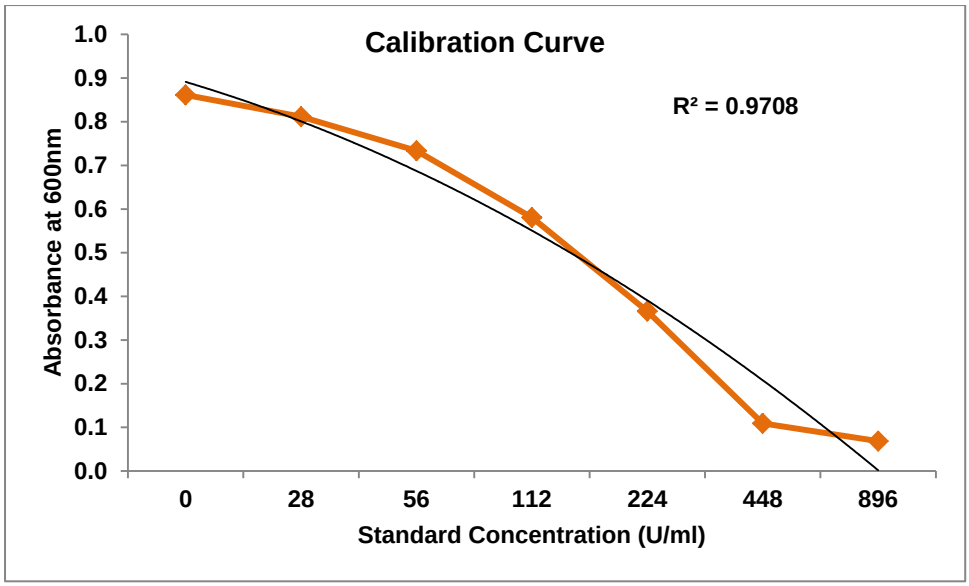
B) Handling / Storage:

- The kit components should be stored at 2-8°C.
- All the reagents and wash solutions should be used within 12 months from manufacturing date.
- Before using, bring all components to room temperature (18-25ºC). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.
- The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

C) Health Hazard Warnings:

- Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
- For Research Use Only

Graphs, Maps and Appendices:



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%	H (null hypothesis) = 100 ± 25 %
Parallelism/Linearity	CV < 30%	Deviation from linearity < 20%
LLOQ / LOQ	Recovery 100 ± 25%	TE % < 32.9%