BLOG

ISO 20391

Understanding Cell Counting Standards: ISO 20391-2

Experimental Design and Statistical Analysis in Cell Counting

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by ALIGNED GENETICS

Introduction

Why ISO 20391-2 is Needed

Cell counting is not just about tallying numbers; it is the starting point for obtaining reliable data in both research and clinical settings. In processes such as verifying the efficacy of new drugs, monitoring stem cell cultures, and manufacturing cell therapies, cell count data directly determines the reliability and reproducibility of results.

However, in reality, results may differ depending on the researcher, equipment, or laboratory, even when analyzing the same sample. Such inconsistencies reduce research reproducibility and, in clinical practice, may pose risks to patient safety.

The ISO 20391 series was established to address these issues.

- ISO 20391-1 focuses on equipment performance verification through core concepts such as accuracy, precision, and uncertainty, as well as IQ/OQ/PQ procedures.
- ISO 20391-2 takes it a step further by addressing the validation of the method itself, emphasizing experimental design and statistical analysis to ensure the reliability of cell counting methods.

In other words, while ISO 20391-1 asks, “Is the equipment functioning properly?”, ISO 20391-2 asks, “How reliable is this counting method?”—and provides regulations to quantify and prove that reliability.

ISO 20391-1 vs ISO 20391-2

	ISO 20391-1	ISO 20391-2
Focus	Instrument qualification (IQ/OQ/PQ)	Method performance verification (experiment, statistics)
Purpose	Is the instrument operationg properly?	Are the results reliable?
Scope	Instrument installation and operational check	Experimental design, statistical analysis, performance evaluation
Key Metrics	Pass / Fail qualification	Quantitative indicators such as CV, R2, PI

Purpose of ISO 20391-2

The primary purpose of ISO 20391-2 is to verify the performance of cell counting methods using quantitative metrics. It is not merely about “how to count cells,” but about providing numerical evidence to demonstrate how reliable the method truly is.

1. Ensuring research reliability
By quantifying repeatability and reproducibility, it can be demonstrated whether data are consistent.

2. Enhancing international comparability
Using common language and metrics allows results to be compared across laboratories, making the standard suitable for multi-center studies and collaborations.

3. Supporting regulatory and industrial applications
Ensures that QC data can be internationally recognized in processes such as cell therapy and biopharmaceutical manufacturing.

4. Introducing quantitative indicators
Metrics such as CV, R^2, and PI enable objective performance verification, even in situations where no true reference value exists.

Key Concepts of ISO 20391-2

ISO 20391-2 goes beyond simply counting cells and defines the following key concepts to evaluate how reliable the measurement process is :

1. Repeatability

- The degree to which results remain consistent when the same sample is measured multiple times under identical conditions.
- Quantified using the coefficient of variation (CV).
- A lower CV indicates more stable data and higher reliability of the counting method.

2. Reproducibility

- Whether results remain comparable when the same sample is measured on different days, by different operators, or in different laboratories.

3. Inter-laboratory variability

- Evaluation of differences observed when multiple laboratories perform the same experiment using an identical protocol.
- Particularly important for multi-center studies or regulatory submissions.

4. Statistical analysis

- Expressing variability numerically to demonstrate reliability.
- Coefficient of Variation (CV): Assesses precision.
- Coefficient of Determination (R^2): Evaluates linearity and goodness of fit between the dilution series and counting results.
- Proportionality Index (PI): Indicates how much the counting results deviate from the expected proportional relationship with dilution.

Simply put, repeatability is about whether you get the same result in the same setting (same day, same operator, same instrument), while reproducibility is about whether you get consistent results across different settings (different days, operators, or labs).

Procedures of ISO 20391-2 (Experimental Design and Statistical Analysis)

1. Experimental Design

1) Dilution Series

◦ From a single mother cell suspension (or reference material), prepare at least four different dilution fractions (DF).

◦ The DFs should be arranged to evenly cover the operational concentration range (0 < DF ≤ 1), using either linear or logarithmic intervals.

Example: 1, 1/2, 1/4, 1/8

◦ For each DF, prepare at least three independent representative samples, and perform at least three replicate measurements per sample.

◦ (Recommended) Randomize the measurement order, and blind the DF labels.

2) Sample Preparation

◦ Independent representative samples must be prepared at each dilution step (manufacturing and handling should be separated from the same mother sample).

◦ Minimize pipetting errors, cell aggregation, and debris.

◦ Apply the same preparation-to-measurement time across all DFs (e.g., within 15 minutes).

3) Replicate Measurements

◦ Each representative sample at every dilution step should be measured at least three times as a principle.

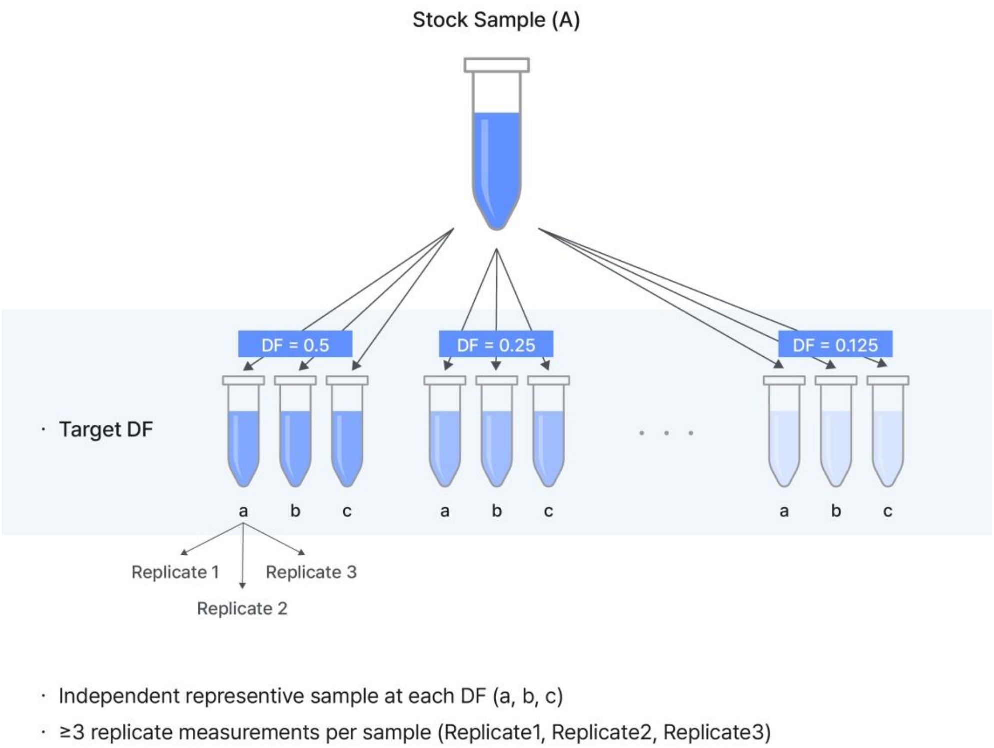

Figure 1. Dilution series experimental design (ISO 20391-2)

A mother (stock) sample is prepared and diluted into multiple fractions (DF). For each dilution fraction, independent representative samples are made, and each representative sample is measured repeatedly (≥3 replicates). This design ensures both repeatability and statistical robustness of the counting method.

2. Statistical Analysis

1) Calculation of mean cell count

◦ Summarize the replicate measurements of each DF (dilution fraction) using the mean (or median).

2) Precision assessment (CV)

◦ CV (%) = 100 × SD / Mean, used to quantify repeatability

◦ Present CV values for each DF to check for concentration-dependent variability.

◦ A lower CV indicates higher precision.

3) Goodness-of-fit evaluation (R^2)

◦ Calculate the linear fit (R^2) between dilution ratio and cell count, but use it only as a reference indicator.

◦ Since R^2 cannot distinguish between random variability and systematic disproportionality, it should not be used alone for pass/fail decisions.

4) Proportionality Index (PI) calculation

◦ Quantifies how much the actual data systematically deviate from the theoretical proportional relationship between dilution and cell count (typically based on smoothed residuals).

◦ Clearly state the calculation formula and interpretation direction (e.g., smaller/larger is better), and provide a 95 % confidence interval whenever possible.

◦ PI is the key metric for assessing proportionality, but it does not represent overall instrument performance by itself.

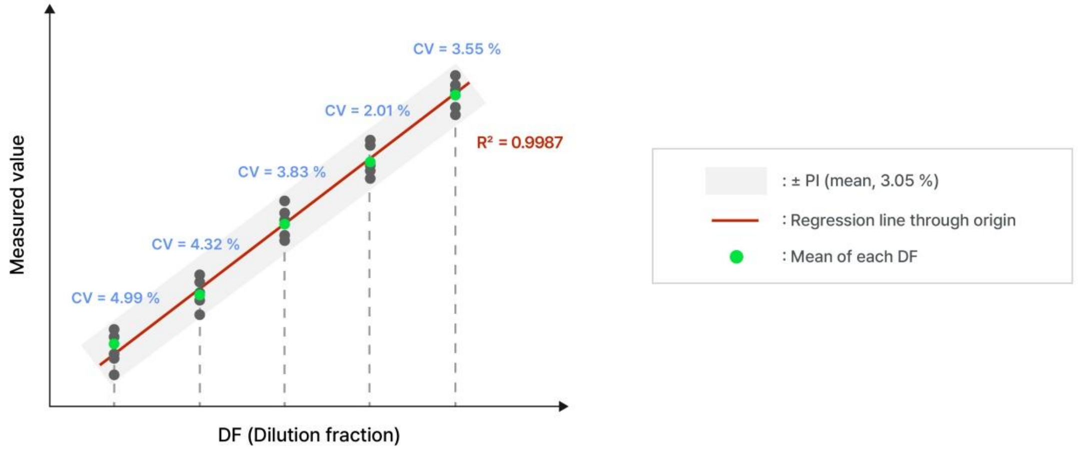

Figure 2. Overview of CV, R^2, and PI (example data)

The red line represents the regression line through the origin, the gray band indicates ± PI(mean), the blue labels show the CV(%) for each DF, and the green dots represent the mean of each DF. The R^2 value on the right indicates the linear fit of the entire dilution series (reference metric). The numbers shown are for illustrative purposes only.

3. Reporting

ISO 20391-2 does not end with the analysis itself; it requires researchers to document their results transparently.

◦ Clearly report quality metrics such as CV, R^2, and PI.

◦ Record details of the experimental design (dilution steps, number of replicates, methods of analysis).

◦ Include sample handling procedures and measurement conditions to ensure reproducibility.

In summary, the overall procedure of ISO 20391-2 can be described as:

"Dilution series design → Sample preparation → Replicate measurements → Statistical analysis (CV, R^2, PI) → Reporting of results."

Advantages and Limitations of ISO 20391-2

Advantages

◦ Since statistics are calculated directly using the actual cells handled by the researcher, the accuracy of the method can be validated most directly.

◦ Provides numerical values for repeatability, reproducibility, and proportionality, offering objective evidence of whether the counting method in use is reliable.

◦ Reports results using common indicators (CV, R^2, PI), thereby enhancing persuasiveness in inter-laboratory comparisons as well as in multi-center or regulatory contexts.

Limitations

◦ To draw reliable conclusions, sufficient sample size (N) and repeated measurements are required, demanding significant time, materials, and personnel.

◦ Due to cell-type–specific characteristics, results obtained from one cell type are difficult to generalize to others, and the procedure must be repeated whenever the cell type changes.

◦ The procedure is too elaborate and inefficient for use in daily QC of instruments (it is excellent for validating research methods but excessive for routine checks).

◦ There is a burden of statistical design and interpretation. For example, DF (dilution factor) design, PI calculation and interpretation, and CI (confidence interval) estimation require at least basic statistical competence.

◦ There are practical constraints in securing, storing, and ensuring lot-to-lot consistency of reference materials and standard beads. Issues in supply, storage conditions, or batch variations may complicate the interpretation of results.

Conclusion: ISO Rigor + Practicality in Daily QC

ISO 20391-2 provides an international framework for demonstrating the reliability of cell counting data in a quantitative manner. By statistically evaluating repeatability, reproducibility, and inter-laboratory variability, researchers can clearly show how trustworthy their counting method is. This enhances international comparability and offers credible evidence in regulatory and collaborative contexts.

However, in real-world settings, there are burdens associated with statistical design/analysis and constraints in handling reference materials. Applying this framework to daily instrument checks would be excessively demanding.

Therefore, a dual-track strategy is more practical for laboratories:

1. Use ISO 20391-2 to validate “my method” and secure fundamental reliability.
2. Perform routine QC with simple, reproducible standard materials—such as validation slides—consistently and efficiently.

In short, by combining the principles outlined in the international standard with practical QC tools readily applicable in the laboratory, both reliability and efficiency can be achieved.

Frequently Asked Questions (FAQ)

Q1. Is ISO 20391-2 mandatory?

A. It is not strictly mandatory, but it is increasingly required to ensure the reliability of research data and to secure international comparability. In particular, it provides significant advantages in multi-center studies, regulatory submissions, and cell therapy development.

Q2. What is the difference between ISO 20391-2 and ISO 20391-1?

A. ISO 20391-1 focuses on verifying whether an instrument operates properly (IQ/OQ/PQ), while ISO 20391-2 is about validating the cell counting method itself through experimental design and statistical metrics (CV, R^2, PI).

Q3. Should Daily QC also be performed according to ISO 20391-2?

A. No. ISO 20391-2 is excellent for method validation, but it is too complex for daily QC routines. For everyday checks, it is much more practical to use standardized materials such as Validation Slides.

Q4. What do CV, R^2, and PI each mean?

- CV (Coefficient of Variation): Consistency (precision) of results under the same conditions
- R^2 (Coefficient of Determination): Goodness of fit between dilution series and counting results (reference metric)
- PI (Proportionality Index): Degree to which results deviate from the theoretical dilution-proportional relationship

Together, these three metrics provide an objective way to demonstrate the reliability of a cell counting method.