Application Note

Interpretation and analysis of Liquid Handling results

Description of most important vocabulary within Liquid Handling applications including the mathematics behind.

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1 About this document

There are several ways and terms to analyze the results of a dispensing or pipetting application. The most common terms are accuracy, trueness, precision, repeatability, reproducibility, coefficient of Variances (CV), mean and standard deviation as well as maximum permissible error (+ and -). Nevertheless, there are many more.

Obviously, misunderstandings between two parties can occur by misusing these terms. Therefore, it is very important to have a common understanding about all these terms. Otherwise, there is a high risk of a huge deviation of the customer expectations and the promised performance of the offered solution.

This application note describes how Festo uses these terms as well as providing an overview of the standards, norms, and mathematics behind them. In addition to that, this application note also describes, which external and internal influences exist, and how these can either worsen or improve results in experiments. Furthermore, some countermeasures are described.

2 Term definitions and simplified explanation

The definition of the most important terms of Liquid Handling can be found within ISO 23783-1:2022, ISO 5725-1:1994, ISO/IEC Guide 99:2007 and ISO 8655-6:2022. In the following chapter 2.1 the relevant terms for this paper are defined formally. In chapter 2.2 simplified visualizations describe those terms in a more comprehensible way.

2.1 Term definitions

2.1.1 Accuracy

Closeness of agreement between a delivered volume and the target volume.

Note 1: The concept "accuracy" is not given a numerical value. A liquid delivery is said to be more accurate when it yields a smaller error.

Note 2: The term "accuracy" shall not be used for "trueness" and the term "precision" should not be used for “accuracy”, which, however, is related to both concepts.

[ISO 23783-1:2022, modified; ISO/IEC Guide 99:2007, ISO 5725-1:1994]

2.1.2 Bias

The difference between the expectation of the delivered volume and the target value.

Note 1: Bias is the total systematic error as contrasted to random error. There may be one or more systematic error components contributing to the bias. A larger systematic difference from the target value is reflected by a larger bias value.

Note 2: Bias can be expressed in an absolute and relative numerical value. Those values describe the trueness of a system.

[ISO 5725-1:1994, modified]

2.1.3 Coefficient of variances

The coefficient of variation (CV) is defined as the ratio of the standard deviation (σ) to the mean (μ).

Note 1: Coefficient of variance (CV) is the relative numerical value of the random error. It helps to describe the precision of a system.

Note 2: To analyze measurement results, there are different approaches to express different influences and conditions. To express repeatability the intra-run CV (channel CV, inter-assay CV) as well as the overall CV (plate CV) can be used. In contrast, the following approaches can be considered for the description of reproducibility: inter-run CV (inter-assay CV), run-order CV, tip-to-tip CV (channel-to-channel CV) as well as the overall CV (plate CV). The named approaches are described in chapter 3.

[Everitt 1998, modified; Bammesberger 2014; ISO 23783-1:2022]

2.1.4 Delivered volume

Quantity delivered by a liquid handling system.

Note 1: Delivered volume is a conceptual term and cannot be known with complete certainty due to measurement error.

Also: Delivered quantity (ISO 8655-6:2022)

[ISO 23783-1:2022]

2.1.5 Individually controlled channel

Liquid handling channel that can be operated independently of other channels.

Note 1: At Festo it means that one valve controls one output (e.g. Festo VTOE dispense head).

[ISO 23783-1:2022, modified]

2.1.6 Maximum permissible error

Upper or lower permitted extreme value for the deviation of the measured volume (dispensed volume) from the target volume.
Also: Control limits (colloquial)

[ISO 23783-1:2022]

2.1.7 Maximum occurred error

The most extreme value (upper or lower) for the deviation of the measured volume (dispensed volume) from the target volume.

Note 1: It may or may not be an outlier.

2.1.8 Measured volume

Quantity reported by a volume measuring system.

Note 1: In practice, all measurements contain some measurement errors. The measured volume is a
quantity value and serves as an estimate of the delivered volume, which is not known with complete certainty.
Note 2: To better evaluate measurement results containing larger datasets the individual successive measured volumes (delivery order n=1...N) can additionally be assigned to different channels used (l= 1...L) and the different runs (r=1...R).
Also: Dispensed volume (colloquial)

[ISO 23783-1:2022, modified]

2.1.9 Measurement uncertainty

Non-negative parameter characterizing the statistical dispersion of the delivered volumes.
Also: Measurement error (ISO/IEC Guide 99:2007) and Correction factor (ISO 8655-6:2022)

Note 1: The measurement uncertainty of the mean delivered volume and the measurement uncertainty of a single delivered volume are two distinct applications of this concept.
Note 2: The measurement uncertainty of the mean delivered volume and the measurement uncertainty of a single delivered volume include contributions from the random errors and uncorrected systematic errors of the automated liquid handling systems.
Note 3: The measurement uncertainty includes contributions from the measuring system uncertainty,
as well as the automated liquid handling systems under test.
Note 4: These measurement uncertainties can be estimated according to ISO/IEC Guide 98-3.

[ISO 23783-1:2022]

2.1.10 Microtiter plate

Flat plate with an array of wells

Note 1: Some dimensions of microplates are defined in ANSI/SLAS standards.

[ISO 23783-1:2022]

2.1.11 Multichannel head

Group of liquid handling channels operated in common.

Note 1: Common arrangements of multichannel heads include more than one channel. Typical arrangements are 2, 4, 8, 96, 384 or 1536 channel configurations.

Note 2: Multichannel heads can be controlled singularly (one valve for all channel-outputs, e.g. Festo VTOI dispense head) or by each channel individually (one valve for each channel-output, e.g. Festo VTOE dispense head).

[ISO 23783-1:2022, modified]

2.1.12 Outlier

Member of a set of values which is inconsistent with the other members of that set.

[ISO 23783-1:2022]

2.1.13 Precision

The closeness of agreement between replicate delivered volumes under specified conditions.

Note 1: Precision is conceptual and not a quantity value.

Note 2: Measurement precision is usually expressed numerically by measures of random error, such as standard deviation (absolute value), variance, or coefficient of variation (CV, relative value) under the specified conditions of measurement.

Note 3: The "specified conditions" can be, for example, repeatability conditions, intermediate precision, or reproducibility conditions (see ISO 5725-1:1994).

[ISO 23783-1:2022, modified]

2.1.14 Random error

Component of liquid handling error that in replicate deliveries varies in an unpredictable manner.

Note 1: The random error can be calculated numerically by the standard deviation (absolute) and coefficient of variance (CV, releative).

[ISO 23783-1:2022, modified; ISO/IEC Guide 99:2007, modified; ISO 8655-6:2022, modified]

2.1.15 Repeatability

Precision of liquid deliveries under a set of repeatability conditions.

Note 1: Repeatability refers to the variability among liquid deliveries made on the same automated liquid handling system under nearly identical circumstances. It is recognized that, because of unknown or uncontrollable factors which influence the liquid handling process, repeated measurements will usually not agree. The extent of this variability can be expressed by a standard deviation, called the repeatability standard deviation.

[ISO 5725-1:1994; ISO 23783-1:2022, modified; ISO/IEC Guide 99:2007]

2.1.16 Repeatability conditions

Condition of liquid delivery, out of a set of conditions that includes the same liquid delivery procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same automated liquid handling system over a short period of time

[ISO 5725-1:1994; ISO 23783-1:2022; ISO/IEC Guide 99:2007]

2.1.17 Repeatability standard deviation

The standard deviation of test results obtained under repeatability conditions.

Note 1: It is a measure of dispersion of test results under repeatability conditions.

Note 2: Similarly, “repeatability variance” and “repeatability coefficient of variation” could be defined and used as measures of the dispersion of test results under repeatability conditions.

[ISO 5725-1:1994]

2.1.18 Reproducibility

Precision of liquid deliveries under reproducibility conditions.

Note 1: Reproducibility refers to the variability of replicate volume deliveries by identical automated liquid handling system under differing conditions. It includes effects caused by differences among the automated liquid handling system and measurement instruments, reagents, operators, laboratories, and environmental conditions. The variability of results under these conditions may be described by a standard deviation, called the reproducibility standard deviation.

[ISO 5725-1:1994; ISO 23783-1:2022]

2.1.19 Reproducibility conditions

Condition of liquid delivery that includes different locations, environmental conditions,
operators, or automated liquid handling systems.

Note 1: Many different factors (apart from variations between supposedly identical specimens) may contribute to the variability of results from a measurement method, including: the operator; the equipment used; the calibration of the equipment; the environment (temperature, humidity, air pollution, etc.); the time elapsed between measurements.

Note 2: The variability between measurements performed by different operators and/or with different equipment will usually be greater than the variability between measurements carried out within a short interval of time by a single operator using the same equipment.

[ISO 5725-1:1994, modified; ISO 23783-1:2022]

2.1.20 Reproducibility standard deviation

The standard deviation of test results obtained under reproducibility conditions.

Note 1: It is a measure of the dispersion of the distribution of test results under reproducibility conditions.

Note 2: Similarly, “reproducibility variance” and “reproducibility coefficient of variation” could be defined and used as measures of the dispersion of test results under reproducibility conditions.

[ISO 5725-1:1994]

2.1.21 Systematic error

Component of liquid handling error that in replicate liquid deliveries remains constant or
varies in a predictable manner

Note 1: Systematic error is estimated by calculating the average volume of a series of deliveries and comparing it to the target volume of the automated liquid handling system (bias). Frequently, this result is expressed as a percentage of the target volume.

Note 2: Systematic liquid handling error, and its causes, can be known or unknown. A correction can be applied to compensate for a known systematic error.

Note 3: The systematic error describes the trueness of a system.

[ISO 23783-1:2022, modified; ISO/IEC Guide 99:2007, modified]

2.1.22 Target volume

Also indicated volume, selected volume, or test/nominal volume (ISO 8655-2)

Volume which is intended to be delivered.

[ISO 23783-1:2022]

2.1.23 Test result

Value of a characteristic obtained by carrying out a specified test method.

Note 1: Test result is a broader concept than measured volume. The test result can be a single measured volume, a set of measured volumes, or descriptive statistics such as the mean or standard deviation of multiple measurements. The test method should specify what form the test results take.

Note 2: Depending on the situation described in Note 1, “measurement result” is another colloquial term, which can be used.

[ISO 23783-1:2022, modified; ISO 5725-1:1994]

2.1.24 Trueness

Closeness of agreement between the average volume delivered in a large series of deliveries and the target volume.

Note 1: Precision is conceptual and not a quantity value.

Note 2: Trueness is inversely related to systematic error but is not related to random error.

Note 3: The term “accuracy” shall not be used for “trueness”.

[ISO 23783-1:2022, modified; ISO/IEC Guide 99:2007]

2.2 Simplified explanation of the terms

2.2.1 Understanding of basic terms

The following visualizations (**Figure 1 & 2**) simplify the understanding of the formal definitions of the terms described above. Within the first step, two exemplary test results are shown.

In **Figure 1** a typical scenario for a dispensing application is shown. An operator has defined a target volume (in that case 100µl) with a range of the maximum permissible error (like for example +/- 5%). That means, the operator will only accept the complete test result, if all measured volumes are within this control limits.

The connection between delivered and measured volumes are illustrated as well. The width of the circular mark of the measured volume represents the measurement uncertainty. The true value of the delivered volume is believed to be somewhere within each circular mark. For the sake of simplicity, it will be assumed in the following that the measured volume is a certain value (at best the delivered value).

In total the test result includes seven measured volumes. The measured volume on the left is far away from the other measured volumes. Therefore it is called an outlier. The distance between the outlier and the target volume is the maximum occurred error.

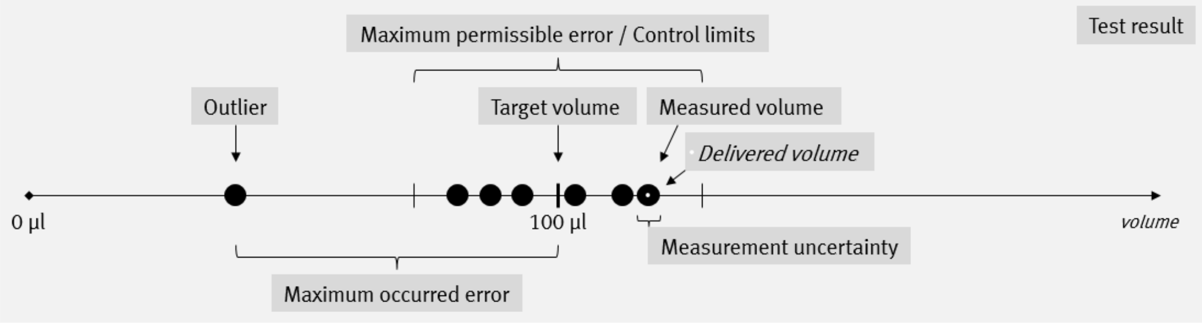

Figure 1: Exemplary test result 1

In the next example (**Figure 2**) eight measured volumes are part of a test result. Two measured volumes are outside of the control limits. However, they aren’t such far away from the rest as the outlier in the first example. In that case those two volumes are not called outlier. Nevertheless, it is possible to calculate the maximum occurred error, which is the volume that has the biggest distance from the target.

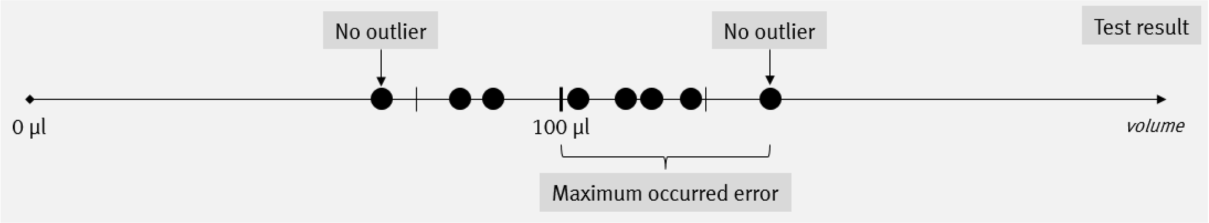

Figure 2: Exemplary test result 2

It is very common, that an operator defines the target of a Liquid Handling test like mentioned above: A certain volume with a maximum permissible error. Attention: The maximum permissible error is often erroneously called CV. In the next chapter, this common misconception will be clarified.

2.2.2 Differentiation between accuracy, trueness, and precision

Many of the terms mentioned in chapter 2.1 depend on each other in some way or are an expression of accuracy and precision. In the market especially these two terms are very common and are used within several data sheets from several Liquid Handling machines and component suppliers.

Several things must be considered to describe a test result:

- How many test results/measurement volumes exist?
- What should be analyzed: repeatability (no condition has changed) or reproducibility (at least one condition has changed)?
    - Which conditions were changed?
    - How many channels and/or runs must be considered?

Figure 3 illustrates additional terms related to the common terms, accuracy and precision. Obviously, there is a third term which must be considered more in detail: trueness.

Concept	Measurement error	Mathematical expression	Category	Conditions	Variants	
Trueness	Systematic error (e_S)	Bias [absolute] Bias [relative]		Large series of test results / measured volumes	Channel; Intra-run; Intra-assay	Overall; Plate
					Inter-run; Inter-assay	
					Run-order	
					Channel-to-channel; Tip-to-tip	
Accuracy	Uncertainty	Complex formulas (see: ISO/IEC Guide 98-3; ISO/TR 20461:2000)		Single test result / delivered volume		
Precision	Random error (S_r)	Standard deviation (σ) [absolute] Coefficient of variance (CV) [relative]	Repeatability	Repeatability conditions	Channel; Intra-run; Intra-assay	Overall; Plate
			Reproducibility	Reproducibility conditions	Inter-run; Inter-assay	
					Run-order	
					Channel-to-channel; Tip-to-tip	
			Intermediate precision	Int. pr.conditions		

Figure 3: Terms and norms of accuracy and precision[1]

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[1] Sources: ISO 23783-1:2022; ISO 23783-3:2022; ISO 5725-1:1994; ISO 5725-3:1994; ISO/IEC Guide 99:2007; ISO 8655-6:2022; ISO/IEC Guide 98-3:2008; ISO/TR 20461:2000; Bammesberger 2014

To explain the difference between precision, trueness, and accuracy more easily, four dart boards are shown in **Figure 4**. In this case it is assumed, that no conditions have been changed between the four dart boards. That means repeatability is analyzed.

Reading from left to right, we see that the first dart board is showing all the darts close to the target. In this case there is a good precision, trueness, and accuracy. To explain the differences between these terms, other situations must be checked.

On the second dart board all the darts are close to each other. Therefore, it has a good repeatability, giving it a good precision. However, the darts are far away from the target – the bullseye. Because of this, it has no good trueness and no good accuracy.

By calculating the mean of all of the darts on the third board, the average result is the bullseye. But even the trueness is perfectly given then the set of darts are far away from each other and partly far away from the target. That means: It has a bad precision and a bad accuracy.

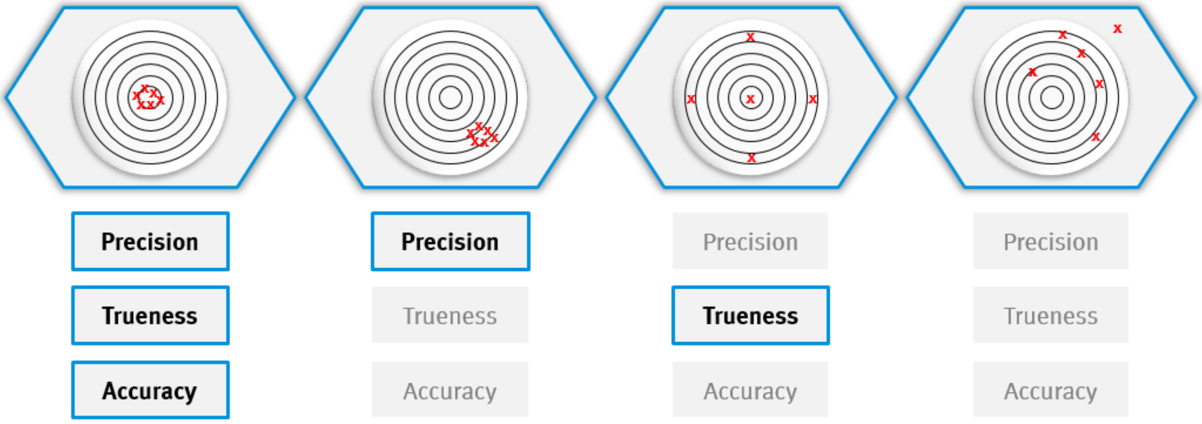


Figure 4: Differentiation between precision, trueness, and accuracy

To summarize: If a test result has a good precision and a good trueness, only then is it possible to have a good accuracy as well. In other words: there is a relation between these three terms, which is illustrated in **Figure 5**.

Attention: Typically, the term accuracy is used to express trueness in the field!

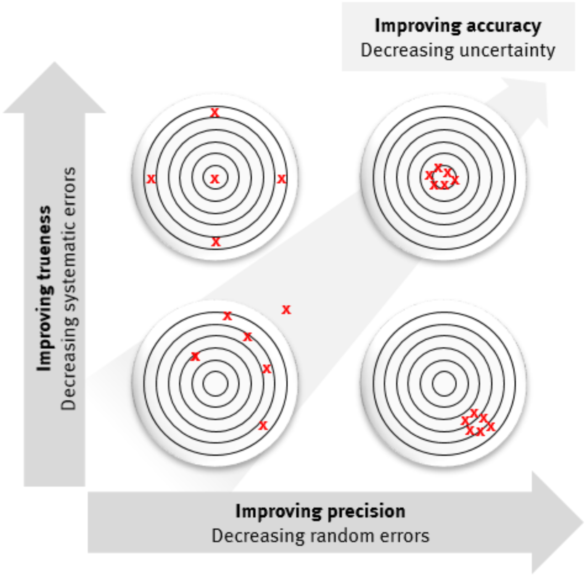


Figure 5: Relationship between precision, trueness, and accuracy

If this representation is extended to include the maximum permissible error (control limits), it becomes clear that the targets defined by the user also play a major role. In the example showed in **Figure 6** the first dart board is modified by adding another (blue) dart and two different definitions of the maximum permissible error (blue dotted circle line). Also, a target for the overall CV is defined by the user (CV should be ‹5%).

On the left dart board, the maximum occurred result is outside of the control limits. Nevertheless, the CV target has been reached. Therefore, a good precision, but no good trueness neither a good accuracy is given.

Be aware: Simply by increasing the limit for the maximum permissible error, an unacceptable result becomes an acceptable result, which can be seen on the second dart board.

The key finding out of this is: At the end, each user defines the target values that must be reached. Precision (standard deviation and CV), trueness (bias), as well as accuracy, are values determined by taking data. The limits are always defined by the operator (by considering the affected application).

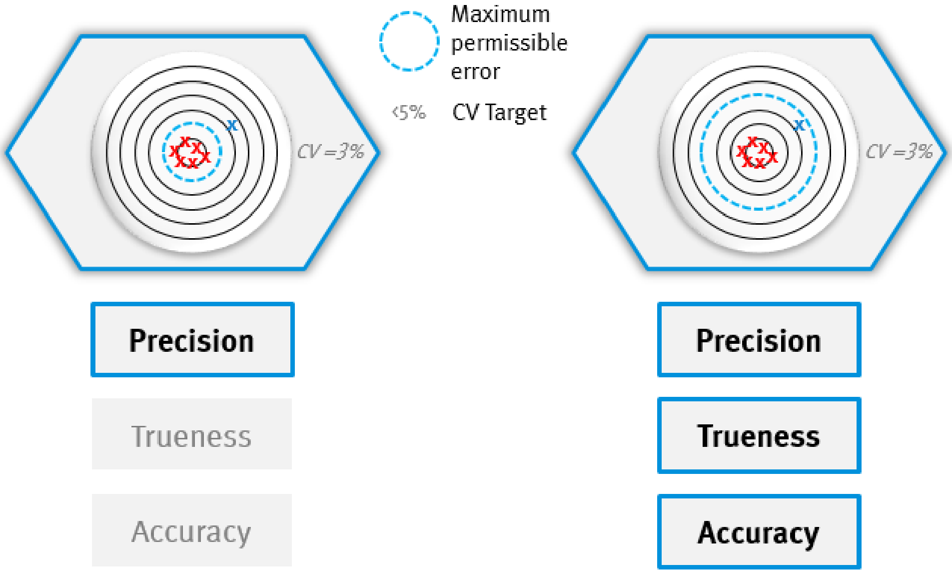


Figure 6: Explanation maximum permissible error

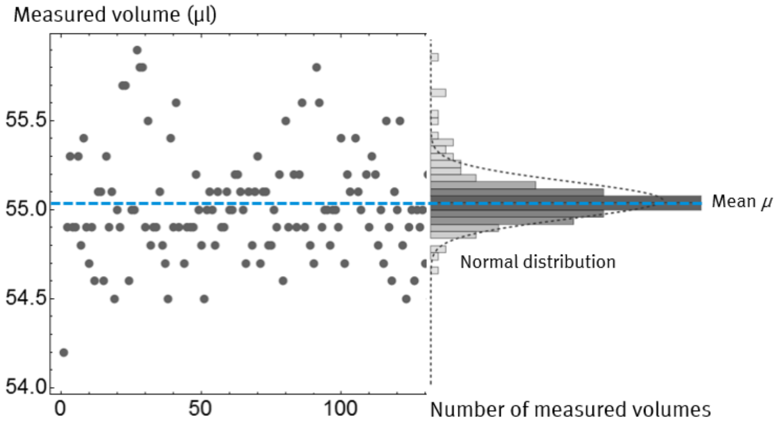
With the above dart board example, repeatability was analyzed. The concept of reproducibility is very similar to this. But at least one condition must be changed: for example, each dart was thrown by another player – or each dart was thrown on another day.

Finally, a link to the mathematics behind completes this section – see also **Figure 7**. Like mentioned above: precision, accuracy and trueness are described by mathematical formulas (coefficient of variances, standard deviation, bias, etc.) out of a set of data. In general, trueness is described by the bias, which is the deviation of the averaged results to the target (similar to that: accuracy is the deviation of a single delivery to the target). To get a relative value, it is divided by the target. This value (absolute or relative) is also called systematic error.

By assuming that the result has a normal distribution, the absolute value for describing precision is the standard deviation σ – also called random error. By dividing the standard deviation by the mean μ, the so-called coefficient of variance (CV) is calculated. The CV is a relative impression of the repeatability or reproducibility.

The closer the CV percentage is to zero, the better the relative results are. However, within each application different limits can be defined by the user itself.

To summarize it: By decreasing the systematic errors, trueness can be improved. By decreasing the random errors, precision can be improved. These two measures lead to an improvement in accuracy and a decrease in uncertainty.



Calculation (relative)

$$CV = \frac{Standard\ Deviation}{Mean}$$

$$Bias = \frac{Mean - Target}{Target}$$

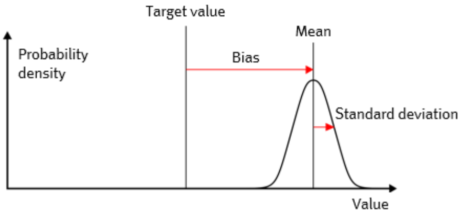


Figure 7: Mathematics behind the coefficient of variances

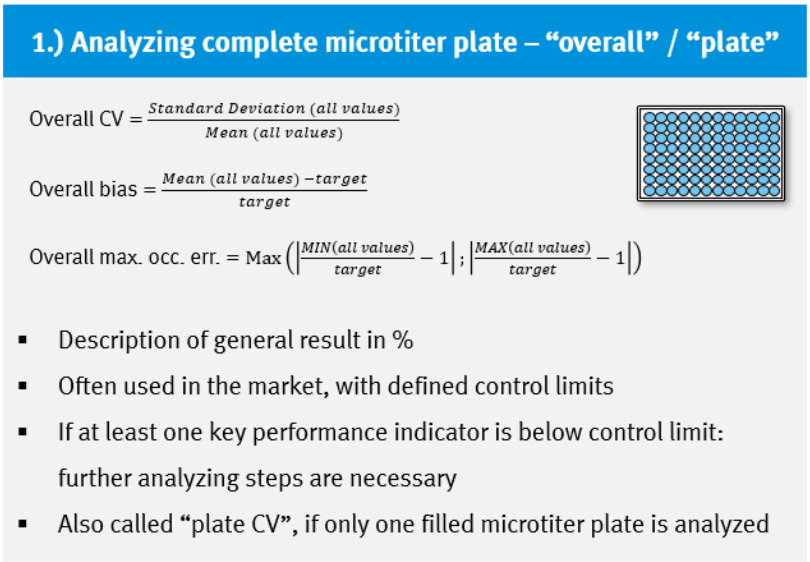
3 Different views on analyzing a measurement result

Others challenges with analyzing results are the various conditions under which they can be conducted. For example, same or different dispense/pipette channels/heads could be used or time differences between some runs must be considered. This leads us to the following situation: We need to differentiate between different values for CV, bias, as well as for the maximum occurred error.

Festo uses a 5-step method for analyzing results of dispensing and pipetting applications. These steps are relating to ISO 23783-3:2022 and Bammesberger 2014. For reasons of simplicity a microtiter plate with 96 wells is considered in the following section.

3.1 Analyzing complete microtiter plate – “overall” / “plate”

First, the complete plate is analyzed by calculating the overall CV, the overall bias as well as the overall maximum occurred error. In **Figure 8** the simplified mathematical formulas are shown to calculate the relative values. These values are describing the general performance of the system. If at least one of these key performance indicators (KPI) is below the control limit defined by the customer (maximum permissible error), further analyzing steps are necessary.



1.) Analyzing complete microtiter plate – “overall” / “plate”

$$\text{Overall CV} = \frac{\mathit{Standard\ Deviation\ (all\ values)}}{\mathit{Mean\ (all\ values)}}$$

$$\text{Overall bias} = \frac{\mathit{Mean\ (all\ values)} - \mathit{target}}{\mathit{target}}$$

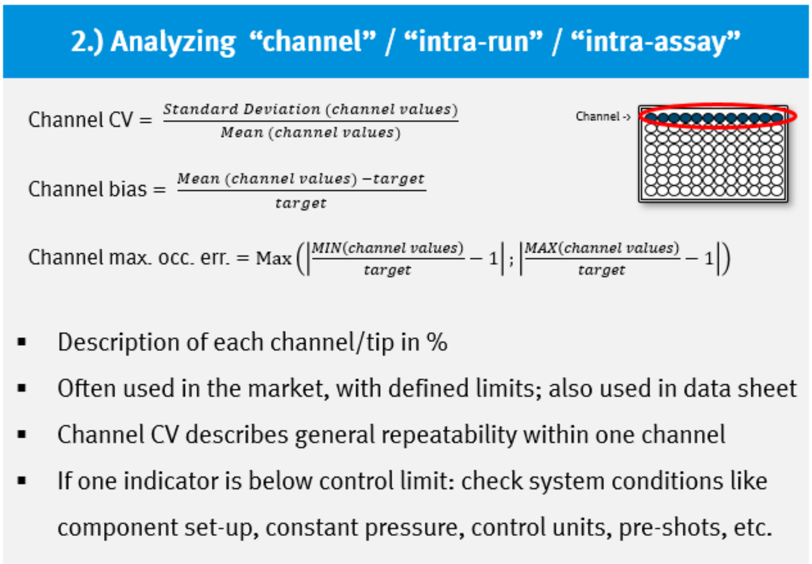
$$\text{Overall max. occ. err.} = \mathrm{Max}\left(\left|\frac{\mathit{MIN(all\ values)}}{\mathit{target}} - 1\right|;\left|\frac{\mathit{MAX(all\ values)}}{\mathit{target}} - 1\right|\right)$$

- Description of general result in %
- Often used in the market, with defined control limits
- If at least one key performance indicator is below control limit: further analyzing steps are necessary
- Also called “plate CV”, if only one filled microtiter plate is analyzed

Figure 8: Plate (overall) perspective on analyzing a microtiter plate

3.2 Analyzing “channel” / “intra-run” / “intra-assay”

Within the second step (**Figure 9**) the channel performance is analyzed, which is also called intra-run or intra-assay analysis. So only one channel (e.g., from an 8-channel-dispense head) is considered. This method is good for identifying principle sources of error and for describing the repeatability of one channel – that means, no conditions are changed within this run. If the channel key performance indicators are not within the defined control limits, several actions can be started: Check system conditions like component set-up, constant pressure and control units; do pre-shots; etc.



2.) Analyzing "channel" / "intra-run" / "intra-assay"

$$\text{Channel CV} = \frac{Standard\ Deviation\ (channel\ values)}{Mean\ (channel\ values)}$$

$$\text{Channel bias} = \frac{Mean\ (channel\ values) - target}{target}$$

$$\text{Channel max. occ. err.} = \text{Max}\left(\left|\frac{MIN(channel\ values)}{target} - 1\right| ; \left|\frac{MAX(channel\ values)}{target} - 1\right|\right)$$

- Description of each channel/tip in %
- Often used in the market, with defined limits; also used in data sheet
- Channel CV describes general repeatability within one channel
- If one indicator is below control limit: check system conditions like component set-up, constant pressure, control units, pre-shots, etc.

Figure 9: Intra-run (channel) perspective on analyzing a microtiter plate

3.3 Analyzing "inter-run" / "inter-assay"

The inter-run or inter-assay analysis (**Figure 10**) describes the stability of the system. This means that the runs are reproducible, even if pauses are made between runs. If inter-run key performance indicators are above the defined limits: Check system conditions like component set-up, constant pressure, control units, pre-shots, etc. – also check environmental conditions like temperature, clogging effects, etc.

Be careful: With inter-run or inter-assay analysis the means of single runs are used to calculate CV, bias and maximum occurred error. This is different from the previous channel analysis where each measured volume is used to calculate CV, bias, and the maximum occurred error.[2]

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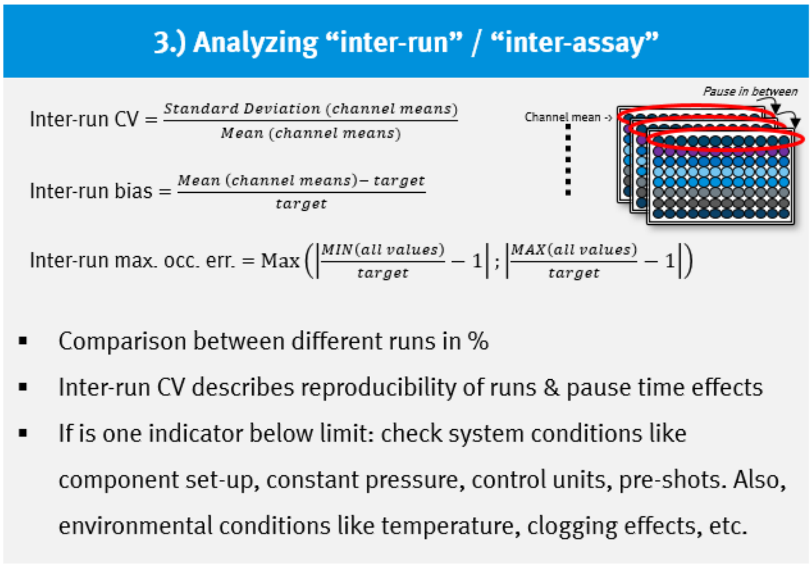
[2] In addition to that: ISO23783-3:2022 is using a different mathematical formula to calculate the CV for inter-run (remark: ISO23783 don't call it "inter-run"). Whereby L are the different channels and M the different runs:

$$CV\ (l) = \sqrt{\frac{\sum_{m=1}^{M} CV(l,m)^2}{M}}$$



3.) Analyzing “inter-run” / “inter-assay”

$$\text{Inter-run CV} = \frac{Standard\ Deviation\ (channel\ means)}{Mean\ (channel\ means)}$$

$$\text{Inter-run bias} = \frac{Mean\ (channel\ means) - target}{target}$$

$$\text{Inter-run max. occ. err.} = \text{Max}\left(\left|\frac{MIN(all\ values)}{target} - 1\right| ; \left|\frac{MAX(all\ values)}{target} - 1\right|\right)$$

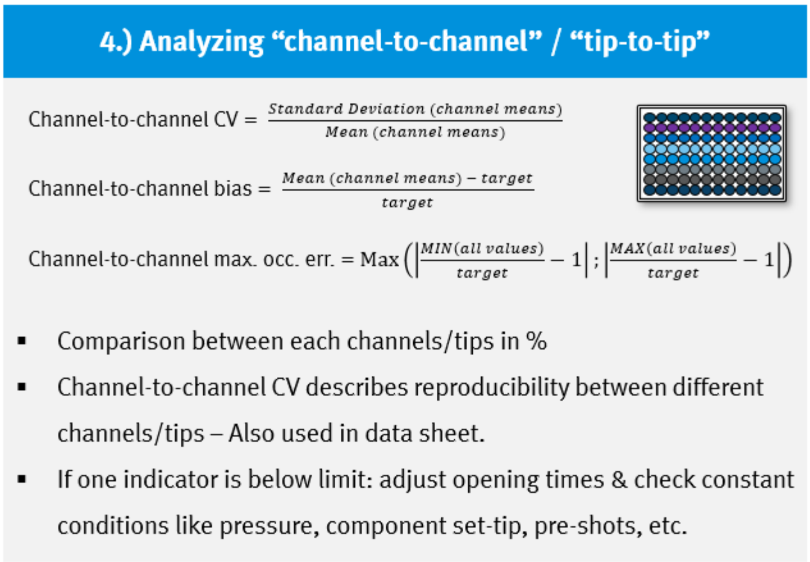
- Comparison between different runs in %
- Inter-run CV describes reproducibility of runs & pause time effects
- If is one indicator below limit: check system conditions like component set-up, constant pressure, control units, pre-shots. Also, environmental conditions like temperature, clogging effects, etc.

Figure 10: Inter-run perspective on analyzing a microtiter plate

3.4 Analyzing “channel-to-channel” / “tip-to-tip”

The channel-to-channel CV (also called tip-to-tip CV) describes the reproducibility between different channels/tips (**Figure 11**). If the channel-to-channel CV is above the defined limit: Adjust opening times & check constant conditions like pressure, component set-tip, pre-shots, etc.

Be careful: Within this method, the channel means of each tip are compared to each other!



4.) Analyzing “channel-to-channel” / “tip-to-tip”

$$\text{Channel-to-channel CV} = \frac{Standard\ Deviation\ (channel\ means)}{Mean\ (channel\ means)}$$

$$\text{Channel-to-channel bias} = \frac{Mean\ (channel\ means) - target}{target}$$

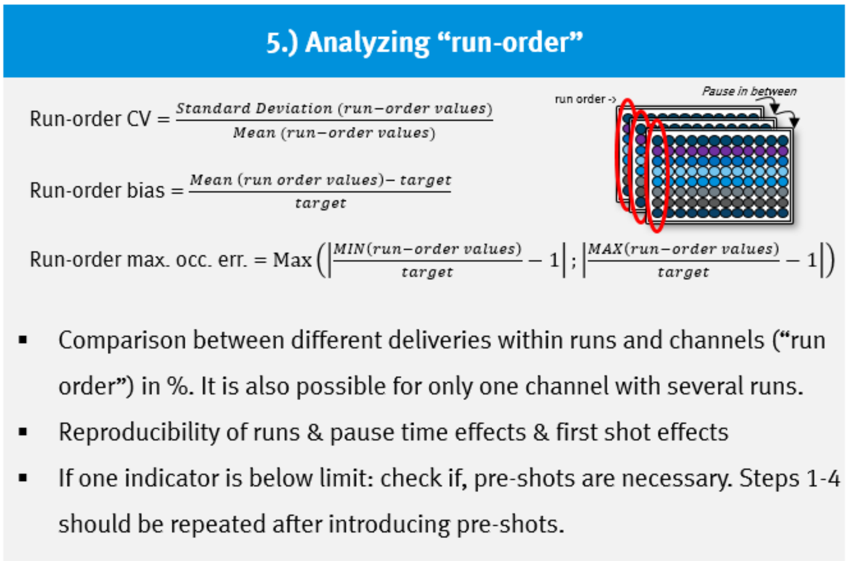
$$\text{Channel-to-channel max. occ. err.} = \text{Max}\left(\left|\frac{MIN(all\ values)}{target} - 1\right| ; \left|\frac{MAX(all\ values)}{target} - 1\right|\right)$$

- Comparison between each channels/tips in %
- Channel-to-channel CV describes reproducibility between different channels/tips – Also used in data sheet.
- If one indicator is below limit: adjust opening times & check constant conditions like pressure, component set-tip, pre-shots, etc.

Figure 11: Tip-to-tip (channel-to-channel) perspective on analyzing a microtiter plate

3.5 Analyzing “run-order”

Finally, the delivery order of each run and each channel can be analyzed (**Figure 12**). Within the so-called run-order the *n*-th dispense of all channels and all runs are analyzed. This method supports to identify and prevent systematic trending effects during the delivery sequence. A typical outcome is the definition of pre-shots, to avoid first shot effect.



5.) Analyzing “run-order”

$$\text{Run-order CV} = \frac{Standard\ Deviation\ (run{-}order\ values)}{Mean\ (run{-}order\ values)}$$

$$\text{Run-order bias} = \frac{Mean\ (run\ order\ values) - target}{target}$$

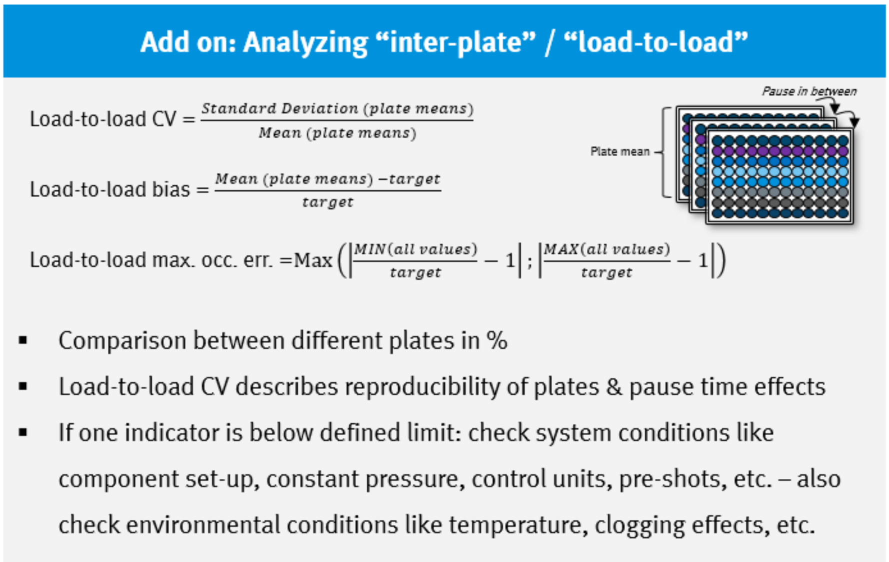
$$\text{Run-order max. occ. err.} = \text{Max}\left(\left|\frac{MIN(run{-}order\ values)}{target} - 1\right| ; \left|\frac{MAX(run{-}order\ values)}{target} - 1\right|\right)$$

- Comparison between different deliveries within runs and channels (“run order”) in %. It is also possible for only one channel with several runs.
- Reproducibility of runs & pause time effects & first shot effects
- If one indicator is below limit: check if, pre-shots are necessary. Steps 1-4 should be repeated after introducing pre-shots.

Figure 12: Run-order perspective on analyzing a microtiter plate

3.6 Add on: Analyzing “inter-plate” / “load-to-load”

Those are the five most important methods for analyzing a measurement result. However, afterwards inter-plate or load-to-load KPIs (**Figure 13**) can be generated to analyze the reproducibility of the whole plate under consideration of pause time effects and other influences (different operators, different locations, etc.).



Add on: Analyzing “inter-plate” / “load-to-load”

$$\text{Load-to-load CV} = \frac{Standard\ Deviation\ (plate\ means)}{Mean\ (plate\ means)}$$

$$\text{Load-to-load bias} = \frac{Mean\ (plate\ means) - target}{target}$$

$$\text{Load-to-load max. occ. err.} = \text{Max}\left(\left|\frac{MIN(all\ values)}{target} - 1\right| ; \left|\frac{MAX(all\ values)}{target} - 1\right|\right)$$

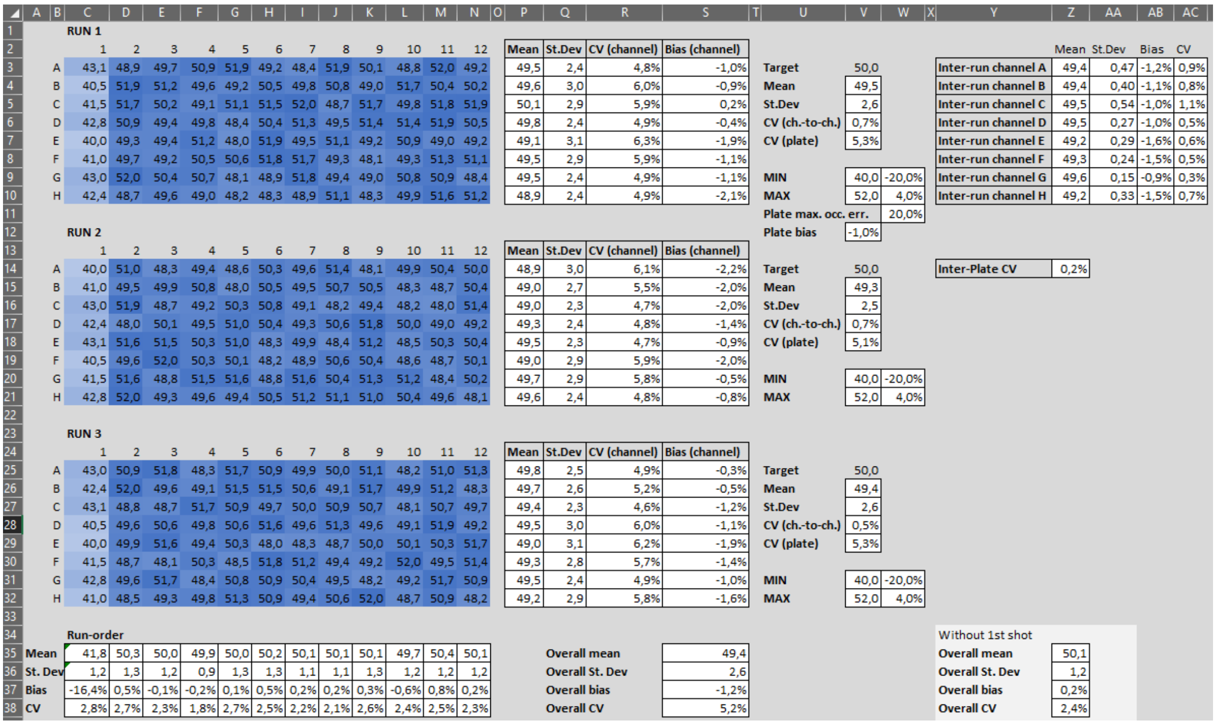
- Comparison between different plates in %
- Load-to-load CV describes reproducibility of plates & pause time effects
- If one indicator is below defined limit: check system conditions like component set-up, constant pressure, control units, pre-shots, etc. – also check environmental conditions like temperature, clogging effects, etc.

Figure 13: Load-to-load (inter-plate) perspective on analyzing a microtiter plate

Note: In ISO 23783-1:2022 and ISO 8655-6:2022 it is defined that 10 values are necessary as a minimum for analyzing a dispensing or pipetting result. This can also be transferred to the mentioned methods above. However, within a microtiter plate you also have a given number of results, depending on the size of the microtiter plate.

4 Example with Excel formulas

The example in **Figure 14** shows three microtiter plates with random numbers. On the right side and under the third microtiter plate selected key performance indicators are shown. These indicators support in the analysis of the data. Immediately differences between the different CV values can be identified. The used Excel formulas are written down in **Table 1**.



RUN 1

	1	2	3	4	5	6	7	8	9	10	11	12	Mean	St.Dev	CV (channel)	Bias (channel)
A	43,1	48,9	49,7	50,9	51,9	49,2	48,4	51,9	50,1	48,8	52,0	49,2	49,5	2,4	4,8%	-1,0%
B	40,5	51,9	51,2	49,6	49,2	50,5	49,8	50,8	49,0	51,7	50,4	50,2	49,6	3,0	6,0%	-0,9%
C	41,5	51,7	50,2	49,1	51,1	51,5	52,0	48,7	51,7	49,8	51,8	51,9	50,1	2,9	5,9%	0,2%
D	42,8	50,9	49,4	49,8	48,4	50,4	51,3	49,5	51,4	51,4	51,9	50,5	49,8	2,4	4,9%	-0,4%
E	40,0	49,3	49,4	51,2	48,0	51,9	49,5	51,1	49,2	50,9	49,0	49,2	49,1	3,1	6,3%	-1,9%
F	41,0	49,7	49,2	50,5	50,6	51,8	51,7	49,3	48,1	49,3	51,3	51,1	49,5	2,9	5,9%	-1,1%
G	43,0	52,0	50,4	50,7	48,1	48,9	51,8	49,4	49,0	50,8	50,9	48,4	49,5	2,4	4,9%	-1,1%
H	42,4	48,7	49,6	49,0	48,2	48,3	48,9	51,1	48,3	49,9	51,6	51,2	48,9	2,4	4,9%	-2,1%

Target	50,0	
Mean	49,5	
St.Dev	2,6	
CV (ch.-to-ch.)	0,7%	
CV (plate)	5,3%	
MIN	40,0	-20,0%
MAX	52,0	4,0%
Plate max. occ. err.		20,0%
Plate bias	-1,0%	

	Mean	St.Dev	Bias	CV
Inter-run channel A	49,4	0,47	-1,2%	0,9%
Inter-run channel B	49,4	0,40	-1,1%	0,8%
Inter-run channel C	49,5	0,54	-1,0%	1,1%
Inter-run channel D	49,5	0,27	-1,0%	0,5%
Inter-run channel E	49,2	0,29	-1,6%	0,6%
Inter-run channel F	49,3	0,24	-1,5%	0,5%
Inter-run channel G	49,6	0,15	-0,9%	0,3%
Inter-run channel H	49,2	0,33	-1,5%	0,7%

Inter-Plate CV	0,2%

RUN 2

	1	2	3	4	5	6	7	8	9	10	11	12	Mean	St.Dev	CV (channel)	Bias (channel)
A	40,0	51,0	48,3	49,4	48,6	50,3	49,6	51,4	48,1	49,9	50,4	50,0	48,9	3,0	6,1%	-2,2%
B	41,0	49,5	49,9	50,8	48,0	50,5	49,5	50,7	50,5	48,3	48,7	50,4	49,0	2,7	5,5%	-2,0%
C	43,0	51,9	48,7	49,2	50,3	50,8	49,1	48,2	49,4	48,2	48,0	51,4	49,0	2,3	4,7%	-2,0%
D	42,4	48,0	50,1	49,5	51,0	50,4	49,3	50,6	51,8	50,0	49,0	49,2	49,3	2,4	4,8%	-1,4%
E	43,1	51,6	51,5	50,3	51,0	48,3	49,9	48,4	51,2	48,5	50,3	50,4	49,5	2,3	4,7%	-0,9%
F	40,5	49,6	52,0	50,3	50,1	48,2	48,9	50,6	50,4	48,6	48,7	50,1	49,0	2,9	5,9%	-2,0%
G	41,5	51,6	48,8	51,5	51,6	48,8	51,6	50,4	51,3	51,2	48,4	50,2	49,7	2,9	5,8%	-0,5%
H	42,8	52,0	49,3	49,6	49,4	50,5	51,2	51,1	51,0	50,4	49,6	48,1	49,6	2,4	4,8%	-0,8%

Target	50,0	
Mean	49,3	
St.Dev	2,5	
CV (ch.-to-ch.)	0,7%	
CV (plate)	5,1%	
MIN	40,0	-20,0%
MAX	52,0	4,0%

RUN 3

	1	2	3	4	5	6	7	8	9	10	11	12	Mean	St.Dev	CV (channel)	Bias (channel)
A	43,0	50,9	51,8	48,3	51,7	50,9	49,9	50,0	51,1	48,2	51,0	51,3	49,8	2,5	4,9%	-0,3%
B	42,4	52,0	49,6	49,1	51,5	51,5	50,6	49,1	51,7	49,9	51,2	48,3	49,7	2,6	5,2%	-0,5%
C	43,1	48,8	48,7	51,7	50,9	49,7	50,0	50,9	50,7	48,1	50,7	49,7	49,4	2,3	4,6%	-1,2%
D	40,5	49,6	50,6	49,8	50,6	51,6	49,6	51,3	49,6	49,1	51,9	49,2	49,5	3,0	6,0%	-1,1%
E	40,0	49,9	51,6	49,4	50,3	48,0	48,3	48,7	50,0	50,1	50,3	51,7	49,0	3,1	6,2%	-1,9%
F	41,5	48,7	48,1	50,3	48,5	51,8	51,2	49,4	49,2	52,0	49,5	51,4	49,3	2,8	5,7%	-1,4%
G	42,8	49,6	51,7	48,4	50,8	50,9	50,4	49,5	48,2	49,2	51,7	50,9	49,5	2,4	4,9%	-1,0%
H	41,0	48,5	49,3	49,8	51,3	50,9	49,4	50,6	52,0	48,7	50,9	48,2	49,2	2,9	5,8%	-1,6%

Target	50,0	
Mean	49,4	
St.Dev	2,6	
CV (ch.-to-ch.)	0,5%	
CV (plate)	5,3%	
MIN	40,0	-20,0%
MAX	52,0	4,0%

Run-order

Mean	41,8	50,3	50,0	49,9	50,0	50,2	50,1	50,1	50,1	49,7	50,4	50,1
St. Dev	1,2	1,3	1,2	0,9	1,3	1,3	1,1	1,1	1,3	1,2	1,2	1,2
Bias	-16,4%	0,5%	-0,1%	-0,2%	0,1%	0,5%	0,2%	0,2%	0,3%	-0,6%	0,8%	0,2%
CV	2,8%	2,7%	2,3%	1,8%	2,7%	2,5%	2,2%	2,1%	2,6%	2,4%	2,5%	2,3%

Overall mean	49,4
Overall St. Dev	2,6
Overall bias	-1,2%
Overall CV	5,2%

Without 1st shot

Overall mean	50,1
Overall St. Dev	1,2
Overall bias	0,2%
Overall CV	2,4%

Figure 14: Excel example of three microtiter plates

In this example a first shot effect exists. By deleting the measured volumes of the first shots from the overall CV and bias calculation, the overall test results get a significant improvement.

View	Value	Cell	Excel formula
Channel	Mean channel	P3	=AVERAGE(C3:N3)
	St. Dev channel	Q3	=STDEV.S(C3:N3)
	CV (channel)	R3	=Q3/P3
	Bias (channel)	S3	=(P3-V3)/V3
Single plate	Mean plate	V4	=AVERAGE(C3:N10)
	St. Dev plate	V5	=STDEV.S(C3:N10)
	CV (channel-to-channel)	V6	=STDEV.S(P3:P10)/AVERAGE(P3:P10)
	CV (plate)	V7	=STDEV.S(C3:N10)/AVERAGE(C3:N10)
	MIN	V9	=MIN(C3:N10)
	MIN (%)	W9	=V9/V3-1
	MAX	V10	=MAX(C3:N10)
	MAX (%)	W10	=V10/V3-1
	Plate max. occ. err.	W11	=MAX(ABS((MIN(C3:N10)/V3)-1);ABS((MAX(C3:N10)/V3)-1))
	Plate bias	V12	=(V4-V3)/V3
	CV (inter-run) Channel A	AC3	=STDEV.S(P3;P14;P25)/AVERAGE(P3;P14;P25)

Several plates	CV (inter-plate)	Z14	=STDEV.S(V4;V15;V26)/AVERAGE(V4;V15;V26)
	CV (run-order)	C38	=STDEV.S(C3:C10;C14:C21;C25:C32)/AVERAGE(C3:C10;C14:C21;C25:C32)

Table 1: Excel formulas for calculating the selected key performance indicators

5 Influences on results

5.1 External influences on results

All the calculations and analyzing steps above depend on good/reliable measuring results. If there is a big gap/error between delivered volumes and measured volumes (e.g., because of poor measuring), misleading key performance indicators will occur.

Therefore, it’s very important to use suitable measuring equipment. In ISO 8655 and ISO 23783 several measuring methods and conditions are described (e.g., a maximum permissible systematic and random errors for measuring different volume ranges). However, a basic requirement is to keep the environmental conditions as constant as possible and eliminate external influences.

Even if the measuring equipment is fine, you can have unsatisfactory results, which are not caused by the dispense or pipette head. Some typical errors and counter measures are:

- Air in the system
  - Possible reasons: non-degassed liquids, too many components, split of channels with enlargement of diameters, etc.
  - ➢ Counter measures: flush system, avoid increasing diameters of channels from start to end, reduce amount of components
- Particles in the system and leaking valves
  - Possible reasons: dirty products, insufficient filtration, wrong fittings, damaged tubing, etc.
  - ➢ Counter measures: clean products before installing, flush system with high pressure, use special liquid fittings, use suitable air and liquid filters
- Bad repeatability
  - Possible reasons: slow valve control units, pressure drop between channels, pressure drop due to reduce volume inside reservoir, tolerances of components, etc.
  - ➢ Counter measures: use fast control unit (e.g., Festo VAEM-V), calibration of each channel/valve, use a liquid pressure sensor after reservoir
- Other environmental conditions
  - Possible reasons: inconsistent temperature, vibrations, long tubing connections, etc.
  - ➢ Counter measures: keep environmental conditions as constant as possible, minimize external influences, reduce the length of tubing to a minimum

5.2 Internal influences on results

As mentioned above, external effects could affect the results dramatically. Nevertheless, also some small changes on a given dispense and pipette head can improve the results – especially with small target volumes. Festo uses a pressure driven dispensing and pipetting approach. In that case it’s mandatory to use a valve with good repeatability and reproducibility without pause time effects.

When it comes to dispensing applications, also the selection of the right needle is very important.

Figure 15: Linearity of VTOE dispensing head illustrates this behavior on an example with Festo dispense head VTOE. Dispensing results with different needle diameters show two major findings:

- High linearity: Depending on the opening time and needle, you will get a linear behavior of the dispensed volume.
- To reach the same amount of dispensed volume with different needles, different impulse times are necessary.

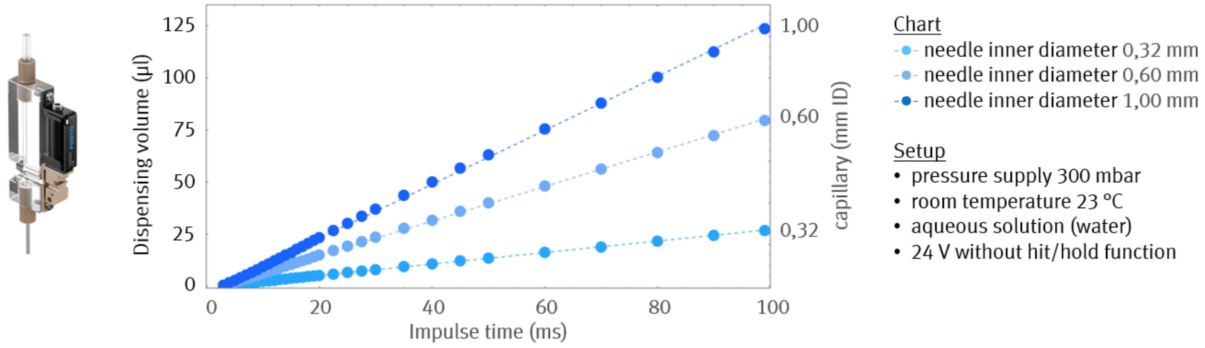


Figure 15: Linearity of VTOE dispensing head

By checking the corresponding channel CV values (also called intra-run or intra assay CV) depending on the dosing results of one needle (**Figure 16**), two other major findings are directly visible:

- The CVs are very low – That means the system has a very good performance.
- If the impulse time gets smaller, the CVs are getting higher.

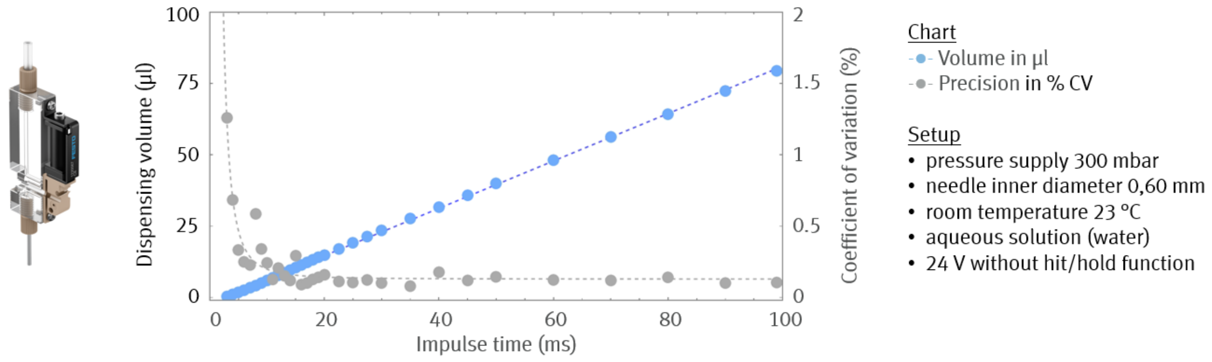


Figure 16: Relationship between CV and dispensed volume

The reason for higher CVs with smaller impulse times is simple: With the opening and closing the valves there are a lot of parameters and properties, which may vary slightly (e.g., time resolution of the control unit, switching behavior of the valve, liquid flow, etc.). If the impulse pulse time is low, the time portion of switching on and off is higher and therefore has a greater influence. This leads to a greater spread of dispensing results and poorer CVs.

To avoid this, the knowledge from the first chart is relevant. By using a needle with a smaller flow rate (e.g., smaller inner diameter) the impulse time can be enlarged to reach a certain amount of volume. By doing so, the CV values are getting better again for the same target volume.

5.3 Summary

To conclude, it is obvious that a good Liquid Handling result depends on several parameters. First, suitable measuring equipment is necessary. Furthermore, it is very important to avoid external influences by designing suitable fluid paths, having a clean working atmosphere, and keeping environmental conditions as constant as possible. Finally, by using the right components for each application internal influences may be eliminated.

6 Sources

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