METHOD VALIDATION IN MEDICAL LABORATORIES

DR. IRINI LEIMONI

COUNTRY QUALITY ASSURANCE MANAGER & DATA PROTECTION OFFICER,

AFFIDEA GREECE

METHOD EVALUATION

VALIDATION:

Establishing standards performance through a defined process.

- Long studies

- For Non- FDA approved testing

VERIFICATION:

- Short studies to demonstrate that a test performs in significant compliance to previously established claims.

- For FDA approved testing

METHOD VALIDATION - QUANTITATIVE TESTS

Precision

- Repeatability
- Reproducibility

Accuracy

- CRM or Control Sample
- Recovery

METHOD VALIDATION - QUANTITATIVE TESTS

Limit of Detection

- Is calculated when they are critical for drawing conclusions from the parameter examined

Limit of Quantitation

- Is calculated when they are critical for drawing conclusions from the parameter examined

Estimation of Uncertainty

METHOD VERIFICATION

Is performed, either by using commercial control samples, or by using CRM (Certified Reference Materials), or mixtures obtained by mixing patient samples (pooled samples), at two concentration levels at least, within and above the reference range (normal - abnormal) or within and below the reference range (normal - sub normal), according to the critical medical decision limits of the parameter measured

METHOD VALIDATION

PRECISION: is the quantitative expression of random error usually by the coefficients of variation monitored under specific conditions.

Repeatability conditions exist when the same examination procedure, same operators, same measuring system, same operating conditions and same location are used for replicate measurements on the same or similar objects over a short period of time, usually less than a working day of 8 h.

Reproducibility conditions includes the same or different measurement procedure, different location, and replicate measurements on the same or similar objects over an extended period, but may include other conditions involving changes

METHOD VALIDATION

PRECISION:

Intermediate precision: includes conditions in between the extremes of repeatability and reproducibility. It is usually estimated by daily examinations over extended periods of time for at least 1 year.

All sources of variation included in intermediate precision including, e.g. lot number changes are included in appropriate number of occurrences. The intermediate precision can refer to one measuring system or to all measuring systems in the conglomerate of laboratories.

METHOD VALIDATION

Intermediate precision/intra-laboratory reproducibility:

At least six (6) measurements of the same control sample under different conditions, e.g. different days and using at least two different batches of the analyte reagents.

The results of the internal quality control charts (QC) may also be used. The CVR% of the measurements is calculated

METHOD VALIDATION

REPEATABILITY:

At least six (6) measurements of the same control sample, divided into aliquots or a pooled sample, divided into aliquots. The measurements should cover the entire duration of the daily operation of the laboratory. A 15 min interval at least, is required between the six measurements. The CVr% of the measurements is calculated.

METHOD VALIDATION

TRUNESS:

Bias is an estimate of a systematic measurement error.

The qualitative concept trueness—in this case lack of trueness— is quantitatively expressed as bias.

It is optimally estimated using commutable certified RM or by comparing the average concentration measured in a natural patient sample with the method to be tested with the average concentration measured in the same sample using a reference method.

METHOD VALIDATION - QUANTITATIVE TESTS

TRUNESS:

is calculated by recovery experiments (mixing with control sample or calibrator with different concentrations) and measuring samples of known concentration six (6) times at least, within the same day.

The %recovery (R%) and the %error (bias) are calculated, respectively. It is supplemented and monitored by participation in proficiency testing schemes.

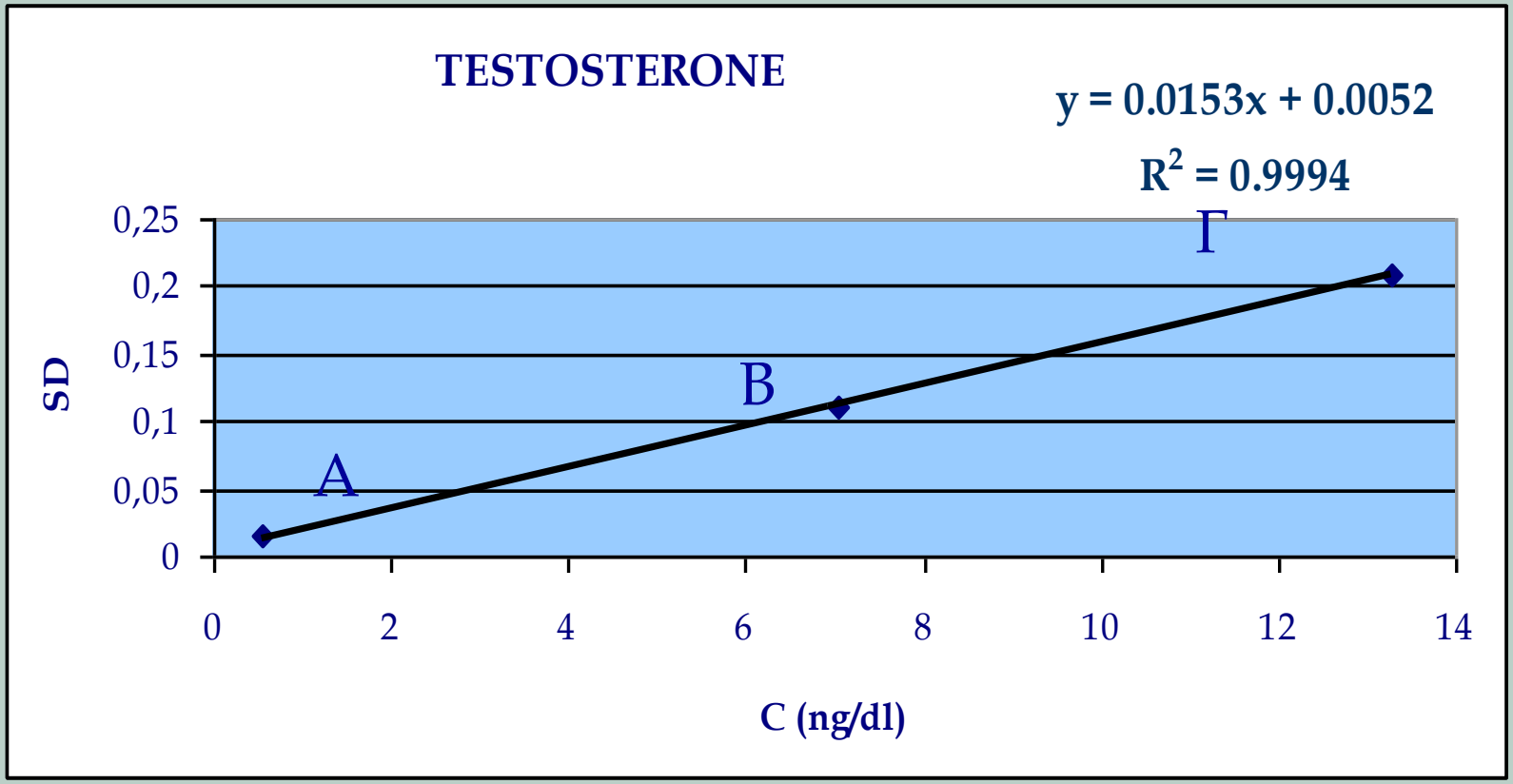
METHOD VALIDATION - QUANTITATIVE TESTS

LIMIT OF DETECTION (LOD) & LIMIT OF QUANTIFICATION (LOQ)

are calculated when they are critical for drawing conclusions from the parameter examined. The characteristics are calculated by sextet measurement of a diluted sample near the LOQ stated by the manufacturer, within the same day. Particularly, in the case of immunochemical techniques, imprecision plots (SD versus concentration diagram) could also be used.

Limit of Detection & Quantification

Immunochemical Test: Testosterone



Limit of Detection & Limit of Quantification

Immunochemical test: Testosterone

- 10 measurements of calibrator in low concentration:

$\bar{x}$: 0,548 ng/ml και SD: 0,015 ng/ml (spot A)

- 10 measurements of calibrator in medium concentration:

$\bar{x}$: 7,02 ng/ml και SD: 0,11 ng/ml (spot B)

- 10 measurements of calibrator in high concentration:

$\bar{x}$: 13,28 ng/ml και SD: 0,21 ng/ml (spot Γ)

Limit of Detection & Limit of Quantification

Immunochemical tests: Testosterone

- Formula: y=0,0153x+0,0052
- (LOD) = (3 x β), means (3 x 0,0052) = 0,02 ng/ml
- (LOQ) = (10 x β), means (10 x 0,0052) = 0,05 ng/ml

METHOD VALIDATION

ESTIMATION OF UNCERTAINTY

Type A: By the standard deviation of at least six(6) measurements under conditions of laboratory reproducibility, at all concentration levels tested during verification and the standard deviation of six (6) measurements of each parameter of the recovery experiments.

METHOD VALIDATION

ESTIMATION OF UNCERTAINTY

Type B: Appraisal of sources of uncertainty for the calibrators used by the automated analyzer. In the case of a calibrator being reconstituted, the contribution of the uncertainty of the volumetric equipment used can also be estimated.

Generally, all sources of type B uncertainty are recorded, and advice is given in order to restrict their contribution to the final result (equipment, sampling, sample transportation, pre-analytical sample preparation e.t.c.

METHOD VALIDATION

ESTIMATION OF UNCERTAINTY

The combined uncertainty is calculated by the law of uncertainty propagation. To assess the suitability of the application of the method by the laboratory, the expanded uncertainty is compared with the values provided by the manufacturer of the device and / or the values mentioned in the literature.

METHOD VALIDATION

The mixing process and the process of calculation of recovery can not be applied in the cases of free forms of parameters, such as fT3, fT4.

In order to calculate the accuracy of these parameters during verification, only analysis of control samples and the results of participation in interlaboratory comparison schemes (external quality control) are used.

METHOD VALIDATION

QUALITY ASSURANCE

Internal Quality Control: is always performed for all tests and is based on the manufacturer's instructions, methodology, biological and analytical variation.

External Quality Control: Annual participation in appropriate proficiency testing schemes (PTs) is required for all tests included in the laboratory accreditation scope. For clinical tests applied to matrices apart from peripheral blood (e.g. urine or CSF), at least one participation in a suitable interlaboratory proficiency scheme is required.

In case of calculated parameters (e.g. LDL) participation in a PT scheme is not required provided that there is participation for the parameters used for the calculation, having set an acceptable z–score, ranging from -2 to 2 and within the range of concentrations that the relative equation applies.

METHOD VALIDATION - QUALITATIVE METHODS

The verification is achieved by determining the following relevant performance characteristics:

Trueness: At least six (6) negative and six (6) positive samples with a known value should be analysed. Trueness is calculated from the ratio [true positive + true negative] / [total number of samples]. In case the kit contains both a negative and a positive control sample, these could be analyzed in duplicate for six (6) days.

In case of semi-quantitative methods with a qualitative final expression, samples with a known value should be analysed (at different concentration levels) and the % error bias) or the % recovery should be determined.

METHOD VALIDATION - QUALITATIVE METHODS

Repeatability: A control sample (positive and negative) or a pooled sample (positive and negative), divided into aliquots, should be measured for six (6) times. The measurements should cover the entire duration of the daily operation of the laboratory. Between measurements, a time interval of at least 15 min is required.

Intermediate precision/intra-laboratory reproducibility: For purely qualitative tests, analysis of positive and negative samples by different analysts during six (6) different days is required. In the case of semi-quantitative methods with an ultimate qualitative result, analysis of samples (known or unknown) between different dates is required as well as calculation of the coefficient of variation (CV%) of the measurements. Measurements have to be made at concentration levels with clinical significance. Trueness experiments could also be used.

METHOD VALIDATION - QUALITATIVE METHODS

Limit of detection (LOD): For purely qualitative tests, analysis of serial dilutions of a positive control material is required and the LOD is defined as the concentration level above which the sample is reliably classified as positive (through repeated measurements).

In case of semi-quantitative methods with an ultimate qualitative result, standards or low concentration positive samples is required, as well as calculation of the standard deviation (SD) of multiple measurements (e.g. 6).

The detection limit is 3.3 times the SD. It may, also, be necessary to determine the Cut Off Value (COV) and the area around it for which the outcome is uncertain (Unreliability Region, from the level with False Positive Rate to level with False Negative Rate)

METHOD VALIDATION - QUALITATIVE METHODS

Estimation of Uncertainty:

Qualitative tests belong to the category of tests where strict metrological and statistical calculations of uncertainty cannot be implemented.

For semi-quantitative methods which result in a qualitative output the estimation of uncertainty is required

METHOD VALIDATION - QUALITATIVE METHODS

QUALITY ASSURANCE:

Internal Quality Control for the qualitative tests is performed by analyzing positive and negative control samples (controls), before analyzing patient samples. Moreover, in accordance with the quality standards used (e.g. CLSI), these procedures should, where appropriate, be incorporated into the daily laboratory workflow.

METHOD VALIDATION - MOLECULAR TESTS

Verification of CE IVD qualitative tests

Trueness: Assessment of trueness is achieved by using control samples provided commercially, reference materials, results from interlaboratory schemes and recovery.

In multiparametric methodologies where more than one microorganisms / genes / mutations / polymorphisms are detected, it is not always easy to control all of them (e.g. 32 mutations in CFTR), especially if they are many. However, the laboratory must obtain a satisfactory panel of control samples with e.g. at least the most frequent mutations / polymorphisms detected in the Greek population in order to verify the trueness of the kit used.

METHOD VALIDATION - MOLECULAR TESTS

Verification of CE IVD qualitative tests

Repeatability/Reproducibility: In qualitative molecular methods, repeatability has to be assessed with three (3) measurements of control samples (as the ones mentioned above) and reproducibility with six (6) measurements under different conditions.

Limit of Detection (LOD): In the case of determination of parameters with clinical significance, confirming the Limit of Detection is required and is achieved by analyzing five (5) samples (control or reference material provided by the manufacturer) diluted close to LOD (up to +20% LOD)

METHOD VALIDATION - MOLECULAR TESTS

Verification of CE IVD quantitative tests

In addition to the requirements for the quantitative tests, the calibration curve should be checked three (3) times, by using at least 4 calibration points (in duplicate), if calibrators are available.

METHOD VALIDATION - MOLECULAR TESTS

Validation of laboratory-developed internal (in-house) tests

In-house methodologies are validated the same way CE-IVD methodologies are validated by their manufacturers. In the case of accreditation according to ISO 15189, in addition to analytical validation, clinical validation is also required (diagnostic sensitivity and specificity, NPV, PPV, clinical utility). For Research-use-only tests the laboratory may be accredited according to ISO 17025, without the need for clinical validation.

Requirements regarding the validation of in-house laboratory developed methods are the following:

METHOD VALIDATION - MOLECULAR TESTS

Validation of laboratory-developed internal (in-house) tests

Trueness: Assessment of trueness is achieved either by using control samples commercially provided or reference materials, results from proficiency testing schemes and recovery experiments.

Furthermore, trueness is checked by:

- **Method comparison**: For in-house methods, it is required to compare the method with a CE-IVDKit (or when absent, with another commercial kit or other established methodology) with 40 samples (e.g. 20 negative and 20 positive in qualitative parameters, 10 negative and 30 positive with a broad range of values in quantitative parameters)and appropriate statistical analysis

METHOD VALIDATION - MOLECULAR TESTS

Validation of laboratory-developed internal (in-house) tests

Trueness: In multiparametric methodologies where more than one microorganism / gene / mutation / polymorphism is detected, the laboratory must have a complete panel of control samples in order to validate the accuracy of the method used.

Repeatability/ Intermediate precision/intra laboratory reproducibility: At least ten (10) measurements of control samples in at least two levels (negative / weakly positive, 2-3 x LOD) on different days and using different lots of reagents. The CV% of the measurements is calculated (it can be drawn from control charts within a period of 3 or 6 months).

METHOD VALIDATION - MOLECULAR TESTS

Validation of laboratory-developed internal (in-house) tests

Limit of Detection (LOD): Validation of LOD is calculated by applying Probit Analysis at five concentration levels of the reference material, around the LOD (each level with 8-10 samples). The verification of LOD should be done with samples at -20% of LOD and +20% of LOD (at least 5 times). For multiparametric methodologies, the process should be repeated for each detected parameter.

Limit of quantification (LOQ) in quantitative methods: the point where a satisfactory CV% (e.g. 20%) is achieved.

METHOD VALIDATION - MOLECULAR TESTS

Validation of laboratory-developed internal (in-house) tests

Linearity and Measuring Range for quantitative methods: For an in-house Q-PCR methodology able to measure in the range of 10 log units, using at least 7 points (in triplicate) by diluting appropriate reference material is necessary for obtaining the standard curve (n = 5)and performing linearity check (dilutions shall cover at least 5 log units).

Analytical Specificity: check with electrophoresis and DNA Sequencing the obtained PCR product (and Tm in Q-PCR), check for interfering substances that could inhibit or block the measurement (e.g. haemoglobin, heparin, etc.), in Molecular Microbiology check for the presence of genetically-similar organisms or organisms that are often found in the samples analyzed in the laboratory, in Molecular Biology/Genetics additional check for pseudo genes or homologous regions

METHOD VALIDATION - MOLECULAR TESTS

Validation of laboratory-developed internal (in-house) tests

Estimation of Uncertainty As the Quantitative tests

Quality Assurance

Internal Quality Control

In conventional / Q-PCR: always use a blank (noDNA), a negative and a positive control (in molecular microbiology and in somatic mutation detection, a weak positive shall be used in place of the positive, e.g. 2-3 x LOD), in Molecular Genetics at least two control samples shall be used: blank and mutant.

METHOD VALIDATION - MOLECULAR TESTS

Validation of laboratory-developed internal (in-house) tests

Quality Assurance

Internal Quality Control

For high complexity tests (e.g. microarrays) where additional method or DNA isolation controls exist, the laboratory should use one positive or negative control sample either per kit of 20-40 samples or at least once a month, for small number of samples.

METHOD VALIDATION - MOLECULAR TESTS

Validation of laboratory-developed internal (in-house) tests

External Quality Control

For Molecular Diagnostics laboratories, participation in external quality assessments for each category of tests is done according to the following:

For initial assessment and at any extension of the scope of accreditation, the laboratory must have successful results of interlaboratory comparisons for all laboratory tests for which accreditation is requested.

The laboratory could then make groups according to parameter and technique/equipment and rotate on a yearly basis.

Within the four-year accreditation cycle, the full scope of accreditation should be covered (matrices/tests/techniques).

METHOD VALIDATION - MOLECULAR TESTS

Validation of laboratory-developed internal (in-house) tests

External Quality Control

In the molecular detection of pathogens or somatic mutations, external quality control shall be more demanding in order to check the sensitivity of the method (to include rare strains or very dilute samples).

After the first accreditation cycle (4 years) and on condition that the laboratory has demonstrated excellent results in all the previous External Quality Assessments, the frequency of interlaboratory comparisons may be reduced (once every 2 years) per group of parameters and/or techniques/equipment,

METHOD VALIDATION

BIOLOGICAL VARIATION

is commonly measured at two basic levels: variation within individual communities, and the distribution of variation over communities or within a metacommunity.

BV data are reference data that have many applications in laboratory medicine. The data describe the variability of clinically important measurands around homeostatic set points within subjects (CVI) and between subjects (CVG).

The availability of well characterised data enables the interpretation of laboratory results in clinical settings and can be used to define analytical performance specifications (APS) and other applications. The literature describing studies of BV stretches back over 45 years. Reviews of BV data identify widely varying estimates for many measurands, calling for a new approach to deliver robust BV estimates for safe clinical application.

TERMINOLOGY

$$CV_T^2 = CV_A^2 + CV_I^2$$

CV_I : Individual Biological Variation

CV_A : Analytical Variation

CV_T : Total Variation

CV_W : within subject variation

Total Individual Variation

CV: Radom Analytical Error

$$CV < 0{,}5\ CV_W$$

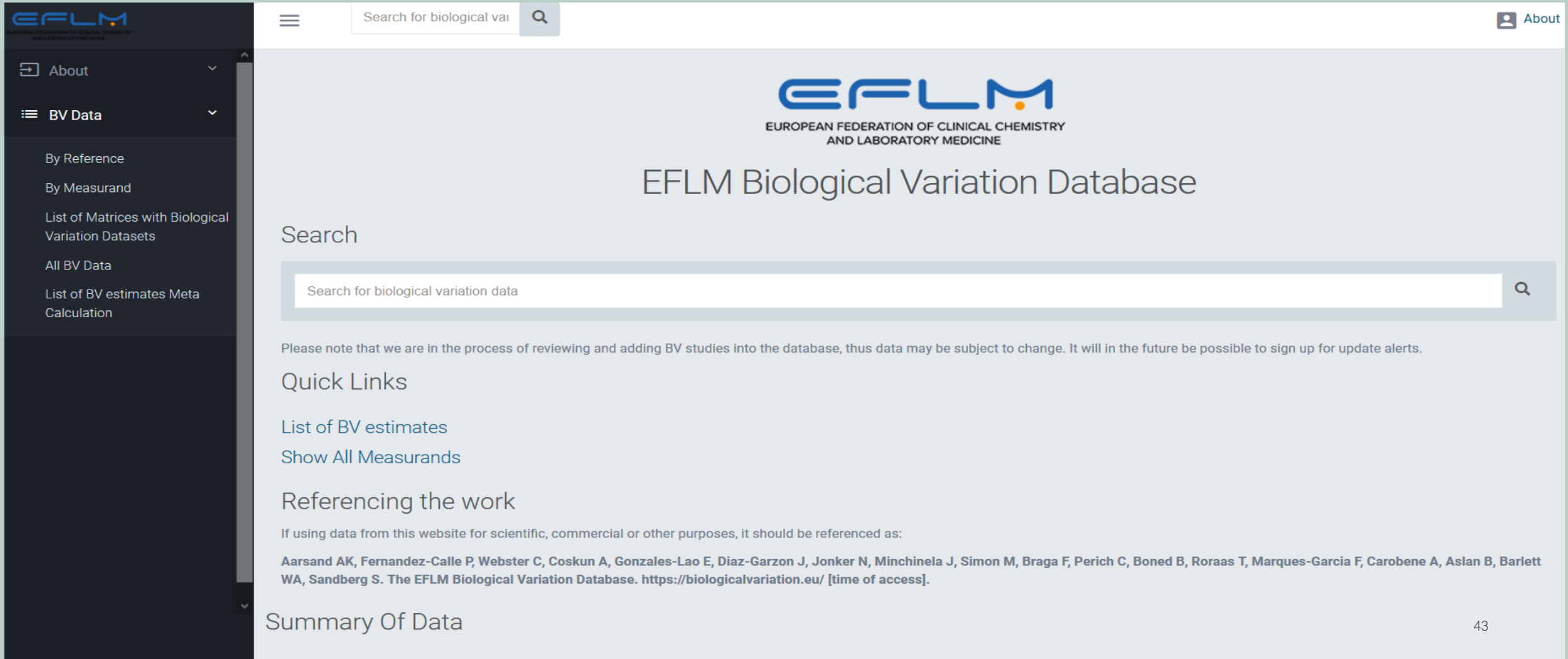
CV_W = Intra-Individual Variation

The Systematic Analytical Error:

$$B < 0{,}25\sqrt{CVw^2 + CV_g{}^2}$$

Total error < 1,65 (Random error)+(Bias%)

BIOLOGICAL VARIATION DATA



BIOLOGICAL VARIATION DATA

Analyte	Intra-individual variation (%)	Inter-individual variation (%)	Typical analytical imprecision (%)	Desirable analytical precision (%)	Desirable analytical bias (%)
Sodium	0.7	1.0	1.1	0.4	0.3
Potassium	4.8	5.6	1.5	2.4	1.8
Calcium (total)	1.9	2.8	2.6	1.0	0.8
Magnesium	3.6	6.4	4.5	1.8	1.8
Phosphate	8.5	9.4	3.0	4.3	3.2
Bicarbonate	4.8	5.3	7.4	2.4	1.8
Urea	12.3	18.3	4.6	6.2	5.5
Creatinine	6.0	14.7	7.9	3.0	4.0
Urate	9.0	17.6	3.3	4.5	4.9
Glucose	4.5	5.8	2.5	2.3	1.8
Bilirubin (total)	23.8	39.0	6.0	11.9	11.4
Cholesterol (total)	5.4	15.2	3.0	2.7	4.0
Triglycerides	20.9	37.2	4.1	10.5	10.7
Total protein	2.7	4.0	3.7	1.4	1.2
Albumin	3.1	4.2	3.4	1.6	1.3
Alkaline phosphatase	6.4	24.8	4.7	3.2	6.4
Alanine aminotransferase	18.0	42.0	4.7	9.0	11.4
γ-Glutamyl transferase	13.8	41.0	4.2	6.9	10.8
Creatine kinase	22.8	40.0	4.2	11.4	11.5

Data from various sources, including UKNEQAS (UK National External Quality Assurance

METHOD VERIFICATION_ CASE

CA 19-9) (U/mL)		
ROCHE		
REREATABILITY		
	ΕΠΙΠΕΔΟ 1	**ΕΠΙΠΕΔΟ 2**
ΗΜΕΡ. ΜΕΤΡΗΣΕΩΝ	8/22/2019	8/22/2019
Responsible	xxxxxx	xxxxxx
MEASUREMENTS	31.19	114.00
	29.55	112.60
	30.48	115.90
	31.26	112.40
	31.19	115.70
	31.29	116.90
	30.76	114.70
	31.73	112.6
	31.15	115.6
	30.95	114.6
MEAN	**30.955**	**114.50**
SD	**0.596**	**1.57**
%CV	**1.92**	**1.4**
% CV MANUFACTORER	***9.02***	***7.00***

METHOD VERIFICATION_CASE

REPRODUCIBILTY		
	CONTROL ΕΠΙΠΕΔΟ 1	CONTROL ΕΠΙΠΕΔΟ 2
12/8/2019	26.80	100.60
	28.62	104.60
	27.56	100.20
	29.91	105.70
	28.49	98.39
	26.84	99.65
	26.66	97.41
	29.02	101.4
	27.54	100.67
	29.55	100.54
MEAN	**28.10**	**100.94**
SD	**1.18**	**2.87**
%CV	**4.21**	**2.8**
% CV MANUFACTORER	***4.4***	***2.9***

METHOD VERIFICATION_CASE

UNCERTAINTY		
ΣΧ. ΤΥΠ. ΑΒΕΒΑΙΟΤΗΤΑ ΑΝΑΠΑΡΑΓΩΓΙΜΟΤΗΤΑΣ (SD/MEAN)	0.04	0.03
ΣΧΕΤΙΚΗ ΣΥΝΔΙΑΣΜΕΝΗ ΑΒΕΒΑΙΟΤΗΤΑ (ΑΝΑΠΑΡΑΓΩΓΙΜΟΤΗΤΑΣ)	0.04	0.03
ΣΧΕΤΙΚΗ ΔΙΕΥΡΥΜΕΝΗ ΑΒΕΒΑΙΟΤΗΤΑ (ΑΝΑΠΑΡΑΓΩΓΙΜΟΤΗΤΑΣ)	**0.08**	**0.06**
ΣΧ. ΤΥΠ. ΑΒΕΒΑΙΟΤΗΤΑ ΟΡΘΟΤΗΤΑΣ (SD/MEAN)	0.02	0.01
ΔΙΕΥΡΥΜΕΝΗ ΑΒΕΒΑΙΟΤΗΤΑ CALIBRATOR (ROCHE) CA 19-9 CalSet 11776215	3.25	6.55
ΑΒΕΒΑΙΟΤΗΤΑ CALIBRATOR (%)	1.63	3.28
ΣΧΕΤΙΚΗ ΔΙΕΥΡΥΜΕΝΗ ΑΒΕΒΑΙΟΤΗΤΑ (k=2)	3.25	6.55

METHOD VERIFICATION_CASE

TRUNESS		
DATE OF MEASUREMENTS	1st LEVEL	2nd LEVEL
MEAN	30.96	114.50
TARGET	31.19	114.00
SD	0.60	1.57
%ΑΝΑΚΤΗΣΗ (RECOVERY)	99.25	100.44
% ΣΦΑΛΜΑΤΟΣ (%BIAS)	-0.75	0.44
ERROR (BIAS)	-0.01	0.00
TE (TOTAL ERROR)	1.18	3.15

METHOD EVALUATION	
I%	1.00
B%	4.03
Teb	5.68

METHOD
VERIFICATION_CASE

THANK YOU VERY MUCH !!!