

# EP15A3 Based Precision and Trueness Verification of VITROS HbA1C Immunoassay

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**Abstract** Verification of analytical performance of measurands becomes an essential requirement for the laboratories before proceeding to patients' samples testing. In our study we have verified the performance of HbA1C Immunoturbidimetric assay (VITROS 5600) against manufacturers' claims using CLSI EP15A3 Guidelines. We performed our study using two concentrations of Quality Control from Bio-Rad (Level 1 and Level 2). A precision verification study was carried out using five replicates of QC per day for five days following which imprecision estimates in form of Within Run (Repeatability) %CV and Within Lab %CV were calculated and compared against manufacturer's claims. Second part of our study included derivation of grand mean from the results of 25 replicates of QC used for precision verification. This was compared against the Target Value of the assigned QC obtained from the peer group mean of laboratories participating in inter-laboratory QC program (unity<sup>TM</sup> Interlab-Bio-Rad) for %bias estimation. The findings of our precision study showed an acceptable Within Lab imprecision (%CV<sub>WL</sub>-0.6%), while the %CV -repeatability (%CV<sub>R</sub>-0.54%) was greater than the manufacturer's claim ( $\sigma_R$ -0.5%). Hence upper verification limit for the manufacturer's claim

(0.65%) was calculated against which the %CV Repeatability was compared and was found to be acceptable. The trueness verification showed that our grand mean (5.488%) was within the verification interval of the target value (5.462–5.497%) and hence the actual %bias was not statistically significant. Our study demonstrates that HbA1C immunoassay shows an acceptable performance consistent with the manufacturer's claims.

**Keywords** Verification · EP 15 A3 · Precision · Trueness · HbA1c

## Abbreviations

ISO	International organisation for standardisation
CLSI	Clinical laboratory standards institute
QC	Quality control
HbA1c	Glycosylated haemoglobin
SD	Standard deviation
CV	Co-efficient of variation

## Introduction

Laboratory medicine has tremendously evolved to establish itself as a connecting bridge between the patient and the clinicians. Quality assurance involves monitoring and maintenance of quality in all phases of testing process including Pre examination, examination and post examination [1]. Quality of testing of examination phase is determined by the method used for testing a Measurand. The essential requirement which determines the quality of a method is its validity. The current laboratory practice worldwide involves use of manufacturer validated methods

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[2]. Even then, there is a distinct difference in the quality of testing between laboratories. This difference can be explained through various confounding variables including quality control procedures, machine maintenance procedures, training related issues etc. According to Westgard “quality you buy is the quality you own” [3]. Having selected an appropriate methodology based on the requirements of the individual laboratory, verification becomes an essential responsibility of the laboratory. According to ISO 15189, clause 5.5.1.2 “Validated examination procedures used without modification shall be subject to independent verification by the laboratory before being introduced into routine use” [2]. Having become a requirement, method verification becomes a responsibility for a clinical laboratory. Blue books from CLSI provide the essential traceability for verification of methods in laboratories.

In this study, we have tried to verify the precision and trueness of HbA1c (Immunoturbidimetric assay, VITROS 5600) based on CLSI EP15A3 guidelines- User verification of precision and estimation of bias [4].

## Materials and Methods

In our study, we have tried to verify HbA1c by Immunoturbidimetric (VITROS 5600), validated based on CLSI EP5A2 Guidelines [5], for precision verification and trueness estimation using CLSI EP 15 A3 guidelines. We used two levels of quality control materials from Bio-Rad for verification of precision and estimation of trueness. These testing materials were labelled as level 1 and level 2 accordingly. The unit of measurement of HbA1c was expressed in % [6].

Precision verification included testing of twenty five replicates of level 1 and 2 Bio-Rad QC. Testing was done for five consecutive days. This included testing five replicates per day under similar operating conditions which constituted a run and precision estimates were determined. Trueness estimation of HbA1c was done by comparing the grand mean obtained from the precision study (Level 1 and 2 QC) against the peer group mean (unity<sup>TM</sup> Interlab-Bio-Rad).

## Results

### Verification of Precision

We followed a step wise approach to verify precision. The experiment was carried with two levels (Level 1 and Level 2) of QC material from Bio-Rad and for study purpose; we have illustrated the findings of Level 1 QC.

Step: 1 Compilation of data-

Results of twenty five replicates of were plotted as a table (Table 1). Visual inspection of the data showed that there were no gross outliers. Significant statistical outliers were ruled out by Grubbs’ test [7, 8].

Step: 2 Grubbs’ Test for outliers

According to Grubbs’ test, a result qualifies as an outlier if that value lies more than the G SDs from the sample mean (Grubbs’ limits) where G is the Grubbs’ factor, and SD is the standard deviation of the raw data including the suspected outliers. Grubbs’ factor G was calculated using Grubbs’ table (Table 2). G was calculated as 3.135. This was followed with calculation of Grubbs’ limits.

Grubbs’ limits = mean  $\pm$  G  $\times$  SD, where, Mean = 5.488%, SD = 0.03% and G = 3.135

Grubbs’ limits =  $5.488 \pm (3.135 \times 0.033) = 5.488 \pm 0.103$

Grubbs’ lower limit = 5.385% and Grubbs’ upper limit = 5.591%

Since all results fell within these limits, statistical outliers were ruled out.

Step: 3 Imprecision estimate by one way analysis of variance (ANOVA)

Step: 3.1 In our study, we used one way ANOVA to find out the imprecision estimates of HbA1c in form of within run and between run variability [9]. A one way ANOVA format was prepared using automated ANOVA calculation software (Table 3). From the ANOVA Table, DF (Degrees of Freedom), DF<sub>Total</sub> (Total Degrees of Freedom), MS

**Table 1** Compilation of data

%	Run 1	Run 2	Run 3	Run 4	Run 5
Rep 1	5.5	5.4	5.5	5.5	5.5
Rep 2	5.5	5.4	5.5	5.5	5.5
Rep 3	5.5	5.5	5.4	5.5	5.5
Rep 4	5.5	5.5	5.5	5.5	5.5
Rep 5	5.5	5.5	5.5	5.5	5.5

REP Replicate

**Table 2** Calculation of Grubbs’ factor

5 Runs		
N	G	n0
23	3.087	4.565
24	3.112	4.792
25	3.135	5

Where, n0 is the average number of results per run, N is the total number of results for five runs, five replicates per run

**Table 3** Imprecision estimate by one way analysis of variance (ANOVA)

Source of variation	SS	DF	MS
Between run	0.0064	4	0.0016 (MS1)
Within run	0.02	20	0.001 (MS 2)
Total	0.026	24	

*DF* Degrees of freedom, *DF Total* Total degrees of freedom, *MS* Mean squares, *SS* Sum of squares, *SS Total* Total sum of squares

**Table 4** Comparison of imprecision estimates against manufacturer's claim

Testing material	Mean	N	Repeatability				Within lab imprecision		
			Estimate (R)	Claim (R)	UVL (R)	Status	Estimate (WL)	Claim (WL)	Status
Level 1 QC	5.488%	25	0.54%	0.5%	0.65%	PASS	0.6%	1.1%	PASS

(Mean Squares), SS (Sum of Squares),  $SS_{Total}$  (Total Sum of squares) were calculated.

Step: 3.2 Calculation of variance:

The two components of variance were calculated from the ANOVA table (Table 3)

$$V_w = MS2$$

$$V_B = (MS1 - MS2)/n_0$$

where,  $V_w$  = Repeatability (within run) variance,  $V_B$  = between run variance,  $V_{WL}$  = within lab variance,  $n_0$  = average number of results per run, MS1 = mean square of between run variation and MS2 = mean squares of within run variation.

$$V_w = MS2 = 0.001$$

$$V_B = \frac{(0.0016 - 0.001)}{5} = 0.0001$$

The sum of two variance ( $V_w + V_B$ ) yielded within lab variance ( $V_{WL}$ ),

$$V_{WL} = V_w + V_B = 0.001 + 0.0001 = 0.0011$$

Step: 3.3 Calculation of imprecision in terms of SD

The square root of variance yielded the standard deviation. Three components of standard deviation were estimated including,

$$S_R = \sqrt{V_w} = \sqrt{0.001} = 0.03$$

$$S_B = \sqrt{V_B} = \sqrt{0.0001} = 0.01$$

$$S_{WL} = \sqrt{V_w} + \sqrt{V_B} = \sqrt{0.001} + \sqrt{0.0001} = \sqrt{0.0011} = 0.033$$

where,  $S_R$  = Repeatability (within run) standard deviation,  $S_B$  = between run standard deviation, and  $S_{WL}$  = within lab standard deviation.

Step: 3.4 Conversion of SD to %CV

$$\%CV_R = (S_R \times 100)/x, \text{ where } x = \text{grand mean}$$

$$\%CV_R = (0.03 \times 100)/5.488 = 0.54\%$$

$$\%CV_B = (S_B \times 100)/x = (0.01 \times 100)/5.488 = 0.18\%$$

$$\%CV_{WL} = (S_{WL} \times 100)/x = (0.033 \times 100)/5.488 = 0.6\%$$

Step: 4 Comparison of imprecision estimates with manufacturer's claims

Step: 4.1 The imprecision estimates obtained from our study were compared against the manufacturer's claims. The repeatability %CV (0.54%) was greater than the manufacturer's claim,  $\sigma_R$  (0.5%). Within lab %CV (0.6%) was lesser than the manufacturer's claim,  $\sigma_{WL}$  (1.1%).

Step: 4.2 Since, the imprecision estimate of repeatability was greater than the manufacturer's claim, we attempted to calculate the upper verification limit for repeatability ( $UVL_R$ ), to limit the rate of failure due to chance and we compared our imprecision estimate against the UVL. The three steps of calculation of  $UVL_R$  included:

Step: 4.2.1 Calculation of Degree of Freedom of repeatability ( $df_R$ )

$df_R = N - K$ , where  $N$  = No. Of replicates and  $K$  = No. Of runs

$$df_R = 25 - 5 = 20$$

Step: 4.2.2 Calculation of UVL Factor:

UVL Factor ( $F$ ) for repeatability was calculated from  $df_R$  by using Table: 7 of EP 15 A3 guidelines [4].

$$F \text{ for repeatability} = 1.31$$

Step: 4.2.3 Calculation of  $UVL_R$ :

$$UVL \text{ (repeatability)} = F * \sigma_R = 1.31 * 0.5 = 0.65\%$$

Step: 4.3 Comparison of imprecision estimates against manufacturer's claim:

The comparison of imprecision estimates with manufacturer's claim was tabulated (Table 4).

### Estimation of bias

In our study, the bias of HbA1c was estimated by comparing the grand mean of 25 replicates of level 1 and level 2 assayed QC (Bio-Rad) against the peer group mean of participating laboratories in interlaboratory QC program (unity<sup>TM</sup> Interlab-Bio-Rad).

The following example illustrates the bias estimation of level 1 QC against the peer group mean (target value or TV).

$$TV = 5.480\%$$

Grand mean obtained from precision verification study = 5.488%.

The following were the steps employed for trueness estimation study:

Step: 1 Calculation of standard error of mean ( $se_X$ )  $se_X = \sqrt{1/nRun \times (S_{WL}^2 - (nRep-1)/nRep) \times S_R^2}$ ; where,  $nRun = 5$ ,  $nRep = 5$   
 $se_X = \sqrt{1/5 (0.033^2 - (4/5) \times 0.03^2)} = \sqrt{1/5 \times (0.00012)} = 0.005$ .

Step: 2 Calculation of standard error of the target value (TV) ( $se_{RM}$ ):

$se_{RM} = SD_{RM}/\sqrt{nLab}$ ; where,  $SD_{RM}$  = standard error of the reference material (Bio-Rad QC) and  $nLab$  = number of participant laboratories in interlaboratory QC program

$$se_{RM} = 0.10/\sqrt{234} = 0.006$$

Step: 3 Calculation of combined standard error ( $se_C$ ) of mean and TV:

$$se_C = \sqrt{se_X^2 + se_{RM}^2} = \sqrt{0.005^2 + 0.006^2} = \sqrt{0.000061} = 0.007$$

Step: 4 Calculation of tau:

$$\tau = se_{RM}/se_X = 0.006/0.005 = 1.20$$

Step: 5 Calculation of combined degree of freedom ( $df_c$ ):

The combined degree of freedom was calculated as a function of the ratio of the standard error of the peer group data to the standard error of the mean, for five runs with five replicates per run for 234 laboratories. The calculation was done using Table 15 A of EP15 A3 guidelines [4].

$$df_c = 23$$

Step: 6 setting multiplier ( $m$ ) to the students't quantile for a probability of 95% with 23 as degree of freedom and number of samples being 2.

$$m = t(1 - \alpha/nsam, df_c), \text{ where } \alpha = 0.025, \text{ nsam} = \text{number of samples}$$

$$m = t(1 - (0.025/2), 23)$$

$$m = t(0.987, 23)$$

$$m = 2.5$$

Step: 7 Calculation of verification interval (VI):

$$VI = TV \pm (m \times se_C) = 5.48 \pm (2.5 \times 0.007) = 5.48 \pm (0.0175)$$

$$VI = 5.462\text{--}5.497\%.$$

Our grand mean (5.488%) was within the verification Interval; hence the bias was not statistically significant.

Step: 8 Calculation of %bias

$$\text{Acceptable \% bias} = \frac{TV - \text{Lower verification limit}}{TV} \times 100$$

$$= \frac{5.48 - 5.46}{5.48} \times 100 = 0.36\%$$

$$\text{Actual \% bias} = \frac{TV - \text{grand mean}}{TV} \times 100$$

$$= \frac{5.48 - 5.488}{5.48} \times 100 = -0.14\%$$

## Discussion

To err is human but to accept and act is divine. Every laboratory makes errors. Quality of a patient result is inversely proportional to the magnitude of error made in a laboratory. Quality assessment of the errors made in a laboratory should be done periodically to understand the degree of impact of these errors on patients' safety. For this to occur, a basic understanding of what, where and how an error can happen in a laboratory is essential. Imprecision (%CV) and inaccuracy (%bias) are estimates of errors made in analytical testing process of a quantitative measurement. Before proceeding to patients' sample testing, each clinical laboratory needs to specify its analytical performance in form of acceptable imprecision (%CV) and acceptable inaccuracy (%bias), usually through comparison with the published data such as the manufacturers' claims. Good quality practice demands laboratories to have such verification procedures traceable to national/international guidelines. Several laboratories worldwide have adopted the CLSI guidelines for specification and verification of their performance. EP15 is a published guideline available in CLSI for verification of precision and trueness of a quantitative procedure. EP15 has undergone four iterations and the latest iteration, EP15A3 was released on September 2014 [4].

A significant change which was made in the recent version of EP15 (EP15A3) included a single 5 day experiment to verify precision and estimate trueness (accuracy) whereas the previous versions demanded two different experiments including a minimum 5 day replication experiment for precision verification and a minimum of 20 patient samples comparison between the testing method and a comparative method for trueness verification [10]. The bias obtained from such a study is compared against the manufacturers' claimed bias. Though the precision

experiment model has been retained in the present version, a refinement in the comparison experiment has been brought in the new version of EP15 due to following reasons

- (1) Difficulty in getting access to the measurement procedure used for comparison by the manufacturer while establishing his bias claims.
- (2) Difficulty in availing patients' samples especially if the measurand under testing is not a commonly utilized one in daily practice.
- (3) Difficulty in comparing the testing laboratory's bias against the manufacturer especially when the manufacturer does express his claims as regression statistics and not as bias claims.

Due to want of a singleton experiment which serves for dual purpose, the latest version of EP15 (EP15A3) is more user friendly and is less time consuming than the previous versions.

Hence in our present study, we tried to specify the analytical performance of HbA1c (Immunoturbidimetric method, VITROS 5600), by adopting EP15A3 guidelines. Two different concentrations (Level-1 and Level-2) of assayed QC materials (Bio-Rad) were used as testing materials and for study purpose, we had illustrated the findings of Level-1 QC material as an example.

The findings of precision verification study yielded imprecision estimates in form of  $\%CV_R$  (0.54%) and  $\%CV_{WL}$  (0.6%), both of which were compared against the manufacturer's claims ( $\sigma_R=0.5\%$  and  $\sigma_{WL}=1.1\%$ ). The comparison showed that the actual  $\%CV$  of repeatability obtained from our study was greater than the manufacturer's claims while within lab  $\%CV$  was within acceptable limits. Hence we attempted to calculate the UVL (upper verification limit) for repeatability, which is the 95<sup>th</sup> percentile expected for imprecision estimates obtained in an experiment similar in size and design to our precision verification study when the claim is correct. Use of UVL has been recommended by CLSI especially when the actual imprecision of the study exceeds the manufacturers' claims, in order to protect the testing laboratories from inappropriately failing solely due to chance more than about 5% of the time [4].

Hence, we proceeded with verifying our repeatability estimate with UVL as the acceptance criterion. The findings showed that the actual repeatability  $\%CV$  obtained from our study were within acceptable limits when compared to UVL ( $UVL_R=0.65\%$ ). Hence the laboratory imprecision of HbA1C Immunoturbidimetric assay (VITROS 5600) was deemed to be consistent with the manufacturer's claims.

The second part of our experiment included the assessment of results obtained from the precision study for trueness estimation. A grand mean was derived from the results of 25 replicates of our testing materials (level 1 and level 2 QC from Bio-Rad). The mean obtained from the study was compared against the Target Value (TV) of the assayed QC, which was the peer group mean of the laboratories participating in the inter laboratory QC program (unity<sup>TM</sup> Interlab-Bio-Rad). The percentage of difference was expressed as  $\%bias$ . We assessed the statistical significance of the actual bias as per EP15A3 guidelines, for which we followed a step wise approach towards finding the Verification Interval (VI) of Target Value (TV) which had the 95% probability of true difference.

For the study purpose we have illustrated an example of trueness estimation of Level 1 QC. The findings of our study showed that the grand mean of the 25 replicates of Level 1 QC (5.49%) was within the verification Interval of Target Value ( $5.48 \pm 0.0175$ ). The acceptable bias was  $\pm 0.36\%$ . Hence the actual  $\% bias$  ( $-0.14\%$ ) obtained from our study was not statistically significant.

Our study demonstrates that HbA1C (Immunoturbidimetric, VITROS 5600) shows an acceptable imprecision and inaccuracy consistent with the manufacturer's claims. One major limitation of our study with respect to bias estimation, being use of QC as a testing material since there is a high probability of skewness of peer group data depending on the participant laboratories interlaboratory QC program which could influence the reliability of standard error of Target Value of the QC material.

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**Compliance with Ethical Standards**

**Conflict of interest** None of the authors of this study have any conflict of interest.

**Ethical Approval** This article contains study only with quality control materials commercially obtained. No patient samples were involved.

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