

Traceability of values for catalytic activity concentration of enzymes: a Certified Reference Material for aspartate transaminase

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Abstract

Background: A new reference material for the liver enzyme aspartate transaminase (AST) (L-aspartate: 2-oxoglutarate-aminotransferase, EC 2.6.1.1), also called aspartate aminotransferase (ASAT), has been developed under the code ERM-AD457/IFCC. This certified reference material (CRM) for AST has been produced from a human type recombinant AST expressed in *Escherichia coli* and a buffer containing bovine serum albumin, and has been lyophilised.

Methods: The homogeneity and the stability of the material have been tested and the catalytic activity concentration has been characterised by 12 laboratories using the reference procedure for AST at 37°C from the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).

Results: The certified catalytic activity concentration and certified uncertainty of AST in the reconstituted material are $(1.74 \pm 0.05) \mu\text{kat/L}$ or $(104.6 \pm 2.7) \text{U/L}$ (with a coverage factor $k=2$; 95% confidence interval).

Conclusions: Both the certified value and uncertainty are traceable to the International System of Units (SI). The material is aiming to control the IFCC reference procedure for AST at 37°C, which will then be used to assign values to calibrants and control materials. The present paper highlights the scientific challenges and innovations which were encountered during the development of this new CRM.

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Keywords: aspartate aminotransferase; aspartate transaminase; Certified Reference Material; IFCC reference measurement procedure; traceability.

Introduction

The standardisation of catalytic activity concentration measurements in clinical chemistry is very demanding, as the catalytic activity of an enzyme is a property measured by the catalysed rate of a chemical reaction under specific experimental conditions. Since the measurement results are procedure-dependent, the comparability of measurement results obtained by different routine procedures can best be ensured by the calibration of these routine procedures using validated calibrants, which carry values traceable to the International System of Units (SI) using a reference measurement procedure (1–4). Consequently, the reference measurement procedure is used to define the measurand and to ensure the highest level of traceability to the SI (1, 4, 5). The link to the SI units is ensured by appropriate calibration of the

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instruments with respect to the influence quantities such as pH, temperature, absorbance and other factors of the reference measurement procedure. At a lower level, the traceability of values obtained by different routine procedures can then be ensured by using the adequate commutable certified reference materials (CRMs) for calibration (6–8).

In 2002, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) published a series of reference measurement procedures for the measurement of catalytic activity concentrations of enzymes at 37°C (9). These procedures are based on the measurement of the change of absorbance over time caused by the product(s) of the enzymatic reaction. Part 5 of the publication series specifically describes the recommended reference procedure for aspartate transaminase (AST) (10). Together with the IFCC reference procedures, CRMs are key components of the enzyme reference systems (1). Therefore, the IFCC and the Institute for Reference Materials and Measurements (IRMM) cooperated to certify the reference materials for γ -glutamyltransferase, lactate dehydrogenase, alanine aminotransferase and creatine kinase, according to the newly developed reference measurement procedures (11). However, the corresponding material for AST was lacking. Therefore, the release of this material represents an important step towards the standardisation of AST measurements.

AST (L-aspartate: 2-oxoglutarate-aminotransferase, EC 2.6.1.1) or aspartate aminotransferase (ASAT) is a vitamin B₆-dependent enzyme involved in amino acid metabolism (12). From the clinical point of view, it represents an important biomarker of liver damage, although it is also increased as a result of heart, muscle and kidney damage, or lyses of erythrocytes. Using methods traceable to the IFCC reference system, the AST upper reference limit for adult serum is 35 U/L (13). In cases of acute liver damage, the catalytic activity concentration of AST may reach values as high as a 100-fold the upper reference limit, although 10-fold to 40-fold increases are most frequently encountered (12).

IRMM has now prepared a lyophilised reference material with a certified catalytic activity concentration for AST in the reconstituted sample. The material has been selected based on two preliminary commutability studies performed in 1999 and 2002 (14).

A test batch of the lyophilised material was first prepared and tested for homogeneity, stability, water content, hygroscopicity and catalytic activity concentration. Then IRMM processed a final batch of the reference material using the same procedure.

Acceptable homogeneity and stability of the reference material have been demonstrated, and the material is intended to be used as a quality control material for the IFCC reference procedure at 37°C. The catalytic activity concentration of AST in the material has been characterised in accordance with ISO Guide 34 and ISO 17025 (15, 16). The certified value is accompanied by an uncertainty value estimated according to the Guide to the expression of uncertainty in measurement (GUM) (17).

This paper highlights the scientific challenges and innovations which were encountered during the development of the new CRM.

Materials and methods

Raw material

A preparation from Asahi Kasei Pharma Corporation (Japan) containing a recombinant form of AST enzyme expressed in *Escherichia coli* was selected as the raw material. The enzyme is a cytosolic isoenzyme and normally originates from human liver. It was dissolved in buffer at pH 7.5 containing bovine serum albumin. Until processing, the bulk material was kept frozen.

Processing

The frozen bulk material from Asahi Kasei Pharma Corporation was allowed to thaw slowly at 4°C. Then the material was filled (1 mL per vial) at IRMM in a clean room (quality of air corresponding to ISO level 5 in the classification according to ISO 14644-1, no particle $\geq 5 \mu\text{m}$) using an automated device and sterile consumables.

The lyophilisation program developed for AST lasts 57 h and includes pre-freezing of the material at -50°C . After lyophilisation, the vials were filled with argon and closed under slight negative pressure (80 kPa) to avoid any loss of material during reconstitution. The vials were then stored at -20°C .

Reconstitution

The material was reconstituted with 2 g of highly purified water. The reconstitution was performed at once by injection through the rubber stopper without opening the vial to prevent contamination and sample loss. Venting the vial during reconstitution could easily be done by introducing a second needle in the stopper, as is common practice for the reconstitution of pharmaceutical drugs in hospitals.

IFCC reference procedure at 37°C

The IFCC reference procedure at 37°C, used for the CRM value assignment, is based on the measurement of the change of absorbance over time caused by the consumption of NADH (β -nicotinamide adenine dinucleotide, reduced form) in the reaction mixture. The calculation of the catalytic activity concentration is performed by use of the absorbance rate and physical constants, including the molar absorption coefficient for NADH. Therefore, the reference measurement procedure does not require calibration with another preparation of the enzyme. However, the appropriate calibration of the spectrophotometer, pH-meter and thermometer is required.

Finally, routine procedures will be calibrated against the reference procedure. But this calibration is only suitable if the reference and routine procedures have very similar analytical specificities for the measured enzyme (18). This implies similar selectivity to isoenzymes and incorporation of coenzymes in the procedure (19).

The procedure was performed in 12 experienced laboratories, referred to by codes from L1 to L12 in the text. The participating laboratories either performed the measurements under ISO/IEC 17025 (16) and ISO 15195 (20) accreditation, or within the scope of a quality system.

Results and discussion

Preliminary evaluation of commutability

The IFCC Committee for Reference Systems for Enzymes (C-RSE) performed preliminary commutability studies on the

raw material. Two experiments were performed: the first in 1999 regarding two preparations from Asahi Kasei Pharma Corporation and another manufacturer, both containing AST of recombinant origin in a mixture with other enzymes. The second experiment was organized in 2002 with a pilot batch of the present preparation.

In the first experiment, 19 small serum pools with AST catalytic activity concentrations from 58 U/L to 240 U/L were prepared in one center (Diagnostica e ricerca San Raffaele), aliquoted, frozen at -80°C and distributed on dry ice to three other centers (Centro Interdipartimentale per la Riferibilità Metrologica in Medicina di Laboratorio, Hôpitaux Universitaires de Strasbourg and Medizinische Hochschule Hannover). In each center, the serum pools together with the two multienzyme preparations, were measured with a provisional Standard Operating Procedure (later published as the IFCC reference procedure at 37°C) and with the local routine methods (duplicate measurements). In total, 11 different routine methods were used. The results from each routine method were compared to the results obtained with the Standard Operating Procedure. The multienzyme preparations were considered commutable if the measurement results were within the 95% confidence interval (CI) defined by the frozen pools [according to the approach proposed by Eckfeldt and Copeland (21)].

The material from Asahi Kasei Pharma Corporation was commutable in five of 11 method comparisons, while the other material was commutable only in two. For this reason, it was decided to choose the recombinant material from Asahi Kasei Pharma Corporation for further batches.

The second experiment was performed only in one center (Diagnostica e ricerca San Raffaele). A new experimental lot of purified AST was prepared by Asahi Kasei Pharma Corporation. AST was measured in 48 fresh sera over five different analytical runs with the IFCC reference procedure (single measurement) and with Roche Modular SWA (AST/GOT reagent, with pyridoxal phosphate, Roche Diagnostics s.p.a, Milan, Italy) and Ortho Vitros (Vitros 950 AT, Ortho Clinical Diagnostics, Milan, Italy) (duplicate measurements). In each of the runs, the material from Asahi Kasei Pharma Corporation was measured once in duplicate with the reference method and once in triplicate with each of the two routine procedures.

The same criterion was applied to evaluate commutability. The material reconstituted with 1 mL of water was commutable in the IFCC-Roche comparison, as shown in Figure 1. It was not commutable in the IFCC-Vitros comparison. It appears that the Vitros and the IFCC methods had different specificities, as expressed by the limited correlation between the two methods (standard error of the regression $Sy_x=9.27$ U/L vs. $Sy_x=1.73$ U/L of the IFCC-Roche comparison).

These preliminary results were convincing enough to process the material and to certify its AST catalytic activity concentration. However, if ERM-AD457/IFCC would be used to directly calibrate commercial procedures, a commutability study should be performed with samples which were value assigned with the IFCC reference procedure.

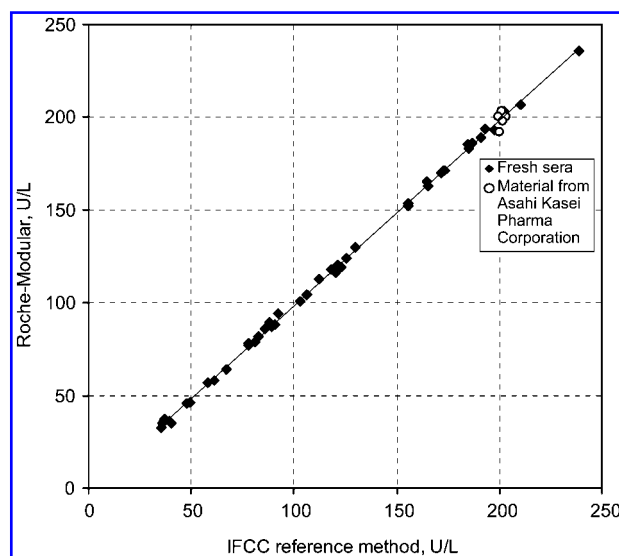


Figure 1 Preliminary commutability study on the material from Asahi Kasei Pharma Corporation reconstituted with 1 mL of water (2002).

Correlation between the Roche Modular and the IFCC reference method: intercept <2.5 U/L and $Sy_x <2.5\%$ of the mean of x .

Particularities of the material processing

As the material is certified for a catalytic activity concentration after reconstitution, the accuracy of the filling is very important to minimise the uncertainty contribution from vial to vial inhomogeneity. Therefore, an automated device was used for the filling and it was calibrated with a solution of sucrose 10% mass fraction, which is close to the viscosity of the AST material. The precision of the robotic arm, the dispensing rate and mode were optimised. Finally, the repeatability (relative standard deviation (RSD)=0.6%, $n=6$), the trueness of the filling (98.6% of the target volume) and the absence of any filling trend were verified gravimetrically during processing of the AST material.

A second critical step for the production of the CRM was the development of a suitable lyophilisation program. The glass transition temperature (T_g) of AST in solution was taken into account in order to guarantee the long-term stability of the enzyme during and after lyophilisation. T_g is the temperature at which an amorphous solid, such as glass or polymer, becomes brittle on cooling, or soft on heating (22). Glass formation, also called vitrification, can occur with a liquid through very rapid cooling, such as a lyophilisation process. Below T_g , the thermodynamic properties of the protein become time dependent and the rates of chemical and physical deterioration decrease with decreasing temperature. Above T_g , these rates increase in a non-Arrhenius manner by several orders of magnitude (23). Therefore, it is only possible to guarantee long-term stability of lyophilised protein product if T_g is reached during the lyophilisation process so that ice sublimation is performed below that temperature (24, 25). T_g is a function of the product composition, including any residual water. Therefore, the glass transition tempera-

ture of the AST solution was determined on the test batch by immersing a probe in the material to measure the temperature, and another probe to measure the electrical resistance. A T_g of approximately -23°C was determined. Consequently, the temperature for freezing the material before water sublimation was set at -50°C .

A third important step was to verify the catalytic activity of the material after lyophilisation. The catalytic activity of the lyophilised material was compared to the catalytic activity of the frozen material using the IFCC reference measurement procedure. The result indicated a mean loss of 11% catalytic activity concentration in the lyophilised material. The resulting catalytic activity concentration of the material, approximately 100 U/L after reconstitution with 2 g of water, remained sufficiently high to fulfil the requirements for effective quality control of the IFCC reference measurement procedure for AST.

The fourth aspect was related to protection of the material against microbial activity. In addition, to the use of sterile devices and a clean laboratory, a mass fraction of residual water in the lyophilised material lower than 1% was targeted. The residual water was determined using Karl-Fisher titration, giving a satisfactory result of 0.6% ($n=3$). In addition, the hygroscopicity of the processed material was investigated using an Oven Sample Processor Coulometric Karl-Fisher titration at 110°C . Mean water uptake of 3.1% and 4.3% (mass fraction) was obtained for 30 min at 43.2% and 75.3% relative humidity, respectively. This means that the vial should not be opened prior to reconstitution. During reconstitution through the pierced septum, no significant water uptake should be expected.

Test batch

The homogeneity of the test batch was first evaluated using the IFCC reference procedure. Ten units taken randomly from the entire batch were reconstituted with 1 g of water and analysed in duplicate. The results showed no outliers. No trend of filling or analytical sequence was detected. This showed that the filling and lyophilisation steps of the material were fully controlled.

The short-term stability of the test batch was also evaluated using the IFCC reference procedure. The study was performed using an isochronous set-up that consists of the simultaneous analysis of reference and test samples (26). For each study a defined set of samples was exposed for different periods of time to increased temperatures and then brought back to the reference temperature (-70°C). At the end of the study, all samples were analysed for the catalytic activity concentration of AST in one analytical run under repeatability conditions. The data were analysed by determining the regression line for the enzyme catalytic activity concentration as a function of time (27). In this case, the material was stored for 2, 4 and 8 weeks at -20°C , 4°C , 18°C and 60°C . No significant slope indicating possible degradation was observed, except at 60°C . On the basis of these promising results, it was decided to produce a large, final batch applying the same processing conditions.

Final batch, ERM-AD457/IFCC

Homogeneity The homogeneity of ERM-AD457/IFCC was tested by measuring the catalytic activity concentration of AST in duplicate in 20 vials randomly selected from the batch. The measurements were performed using a sufficiently repeatable routine AST measurement procedure (Modular P analyser, following the procedure protocol from Roche Diagnostics) which is traceable to the IFCC reference measurement procedure according to the manufacturer. In these experiments, the material was reconstituted with 1 g of water. Results are shown in Figure 2.

Two outlier samples were found at the 95% confidence level using the Double Grubbs test. No technical explanation could be found for these outliers. In addition, the one-way ANOVA showed a significant difference between the samples ($F > F_{\text{crit}}$) when the outliers were included. However, the total RSD of the results, including the outliers, was 0.8%. The repeatability of the method, obtained in the same laboratory using a control material (target concentration: 237 U/L, $n=22$) was 1.1%. Therefore, these outliers were not considered as a sign of heterogeneity. No significant trend (at the 99% confidence level) related to the analysis sequence or to the filling sequence was observed.

The one-way ANOVA was also applied to calculate the between bottle SD (s_{bb}), and the maximum standard uncertainty related to non-homogeneity that can be hidden by the method repeatability (u_{bb}^*), using the following formulas:

$$s_{\text{bb}} = \sqrt{\frac{MS_{\text{bb}} - MS_{\text{wb}}}{n}} \quad u_{\text{bb}}^* = \sqrt{\frac{MS_{\text{wb}}}{n}} \cdot \sqrt{\frac{2}{df_{\text{wb}}}}$$

(MS_{bb} = mean sum of squares between bottles; MS_{wb} = mean sum of squares within bottles; n = number of replicates; df_{wb} = degrees of freedom within bottles).

Both values were converted into relative uncertainties. The relative between bottle heterogeneity ($s_{\text{bb,rel}}$) was 0.72%. The relative maximum hidden heterogeneity ($u_{\text{bb,rel}}^*$) was 0.24%. The largest of these values, i.e., $s_{\text{bb,rel}}$, was included in the calculation of the combined uncertainty of the CRM certified values.

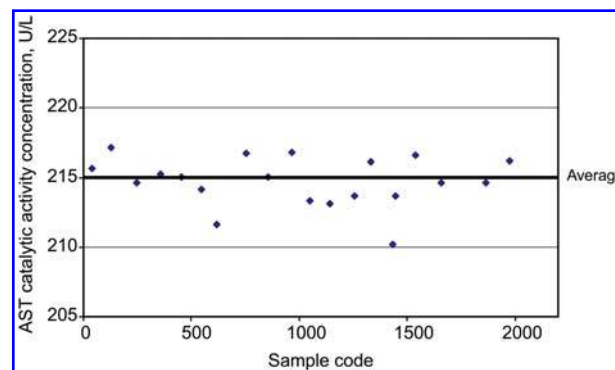


Figure 2 Homogeneity data of ERM-AD457/IFCC plotted by sample mean ($n=2$) (reconstitution with 1 g of water).

Minimum sample intake The data from the homogeneity study performed on ERM-AD457/IFCC could be used to demonstrate that the reconstituted material is at least homogeneous at the level of the sample intake used to perform the homogeneity study. The homogeneity study was performed using a volume of 7 μL from the material reconstituted with 1 g of water. Therefore, for the recommended reconstitution volume of 2 g of water, the sample intake for which there is no indication of heterogeneity is 14 μL .

Stability Short- and long-term stability studies were also performed on the non-reconstituted material using the Modular P analyser. In addition, a short-term stability study was also performed on the reconstituted material mimicking the conditions of laboratory use.

Short-term stability on the non-reconstituted material:

The goal of the short-term stability study was to assess the possible effect of transport at different temperatures on the stability of the material. The reference temperature was -70°C . Test samples were kept for 0, 2, 4 and 8 weeks at -20°C , 4°C , 18°C and 60°C before being brought back to the reference temperature. For each combination of time and temperature, two samples were analysed in triplicate. The results are shown in Table 1.

For each temperature 24 measurements were performed, i.e., 22 degrees of freedom for the linear regression. The slope of the catalytic activity concentration as a function of time is significantly different from 0 if the absolute value of slope b divided by its uncertainty u_b ($|b/u_b|$) is larger than $t_{0.05, 22} = 2.07$. When samples were kept at -20°C , 4°C and 18°C , the slopes were not significantly different from 0. However, at 60°C , a significant (at a 95% confidence level) negative slope was found. It was concluded that the uncertainty due to degradation during shipping is negligible, if the material is shipped with cooling elements.

Long-term stability on the non-reconstituted material:

A 2 year-stability study was then performed in order to confirm the stability of the material upon prolonged storage at -20°C and 4°C . The test samples were kept for 0, 12, 18 and 24 months at -20°C and 4°C (Figure 3). The ANOVA of the data and the test for significance of the slope indicated no significant degradation using a 95% confidence level. Therefore, the material can be considered to be stable for at least 2 years at -20°C and 4°C . The material is stored at -20°C and a stability monitoring will be performed at IRMM at regular intervals to verify the continued stability of the

material. However, an uncertainty contribution of 0.62% for the long-term stability over a period of 24 months at -20°C was taken into account in the combined uncertainty of the certified value of the CRM.

Stability of the reconstituted CRM: The stability of the reconstituted CRM was also investigated in order to determine the time available for users to perform their analyses. Three vials of ERM-AD457/IFCC were reconstituted with 2 g of water and then analysed immediately and after 1, 2, 4 and 6 h at ambient temperature. The measurements were performed in duplicate. Trend analysis, using the t value of Student's test, showed no significant slope ($|b/u_b| < t_{0.05, 4}$). These results indicate that the CRM may be stored at ambient temperature for up to 6 h after reconstitution with 2 g of water. To check the influence of the reconstitution volume on the stability of the reconstituted material, an additional stability test was performed on the material reconstituted with 1 g of water. Results showed that the material was equally stable after reconstitution with 1 g or 2 g of water.

Value assignment

Feasibility study The IFCC reference procedure is, like any procedure for enzymatic activity measurement, very sensitive to input quantities, such as pH, temperature, concentration of reagents, as well as to procedural parameters, such as mixing. Any small deviation from the procedure might lead to bias in the results. Therefore, a feasibility study was first performed for the determination of AST catalytic activity concentration using the IFCC reference procedure at 37°C in the 12 selected laboratories.

The laboratories were provided with two lyophilised samples from the IFCC External Quality Assessment Scheme (EQAS) for Reference Laboratories in Laboratory Medicine (RELA samples A and B, trial 2007), as well as with a pool of human sera (frozen material) obtained from the reference measurement laboratory at the Medizinische Hochschule Hannover (Germany). Each sample was analysed in duplicate on three different days.

As it can be seen in Table 2, the results obtained were satisfactory for both the RELA test material and the serum pool. The RSD and relative uncertainty obtained for the pool of human sera are slightly lower than the results obtained with the EQAS materials. Some laboratories showed systematic effects on trueness (results systematically below or above the average) or precision (larger SD). However, no

Table 1 Short-term stability study, test for significance of the slope: slope (b), standard uncertainty (u_b) and relative standard uncertainty ($u_{\text{sts,rel}}$) after 2 weeks storage at the temperatures indicated.

Temperature, $^\circ\text{C}$	b , (U/L)/week	u_b , (U/L)/week	$ b/u_b $	$t_{0.05, df}$	$u_{\text{sts,rel}}$, %
-20	0.02	0.31	0.065	2.07	0.3
4	0.07	0.18	0.389	2.07	0.2
18	0.03	0.34	0.088	2.07	0.3
60	-8.11	0.45	18.022	2.07	5.1

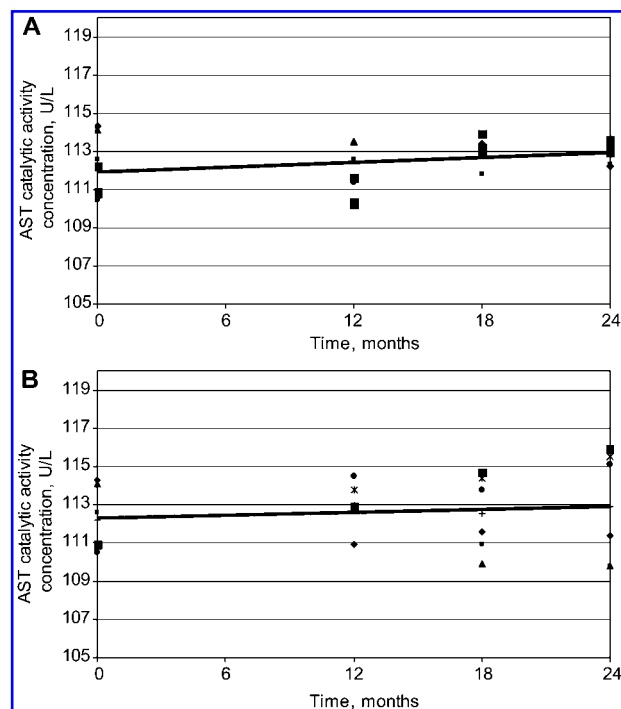


Figure 3 Long-term stability study of ERM-AD457/IFCC for storage: (A) at -20°C ; (B) at 4°C .

Different symbols indicate different vials. The CRM was reconstituted with 2 g of water.

deviation in the experimental conditions could be related to a particular bias or higher SD.

Characterisation study The characterisation of the CRM was performed using the IFCC reference procedure at 37°C . In addition, the same human sera pool samples used in the feasibility study were included in the sequence of analysis as control material. The time interval between the feasibility and the characterisation study was 5 months. Therefore, results obtained on the control material gave as well some information about the intermediate precision of the participating laboratories.

Control samples: The human serum pool sample was analysed over 2 days (one measurement per day). The results

obtained showed one outlier (L9), but no technical explanation could be provided. However, given the large SD of the results for L9 already noted for the same control material during the feasibility study, a lack of control of the experimental conditions of the measurement procedure was indicated. Therefore, the results of this laboratory were removed from the final data set. The resulting data showed a normal distribution. Table 3 shows the mean results from the remaining 11 laboratories for the control sample during the characterisation of ERM-AD457/IFCC. The mean value (110.22 U/L, Table 3) was close to the mean value which was obtained for the same samples in the feasibility study (110.08 U/L, Table 2). The RSD of the mean was 1.74%, much lower than in the feasibility study (2.95%). This might indicate improvements in laboratory performance between the first and the second exercise.

ERM-AD457/IFCC: Six samples of ERM-AD457/IFCC were analysed over 2 days (three samples per day, one measurement). Samples were reconstituted by the addition of $2.00\text{ g} \pm 0.02\text{ g}$ of distilled water through the septum of the vial, in accordance with the reconstitution protocol. Samples were analysed by each laboratory within 6 h following reconstitution, and the measured catalytic activity concentration of AST (U/L) was corrected for the mass of water added in g (m) using the correction factor (f):

$$f = m/m_{\text{target}}$$

where m_{target} is 2 g, the targeted mass of water for reconstitution.

The results of the ERM-AD457/IFCC had a normal distribution. As can be seen on Figure 4, L9 gave the result with the lowest mean. This result could not be classified statistically as an outlier.

The overall results presented in Table 3 were very satisfactory with an RSD of 2.90% and a mean catalytic activity concentration of 104.6 U/L. The catalytic activity concentration was close to the expected value of 100 U/L estimated by Asahi Kasei Pharma Corporation. The uncertainty contribution from characterisation (relative u_{char}) was 0.84%, which was close to the results obtained for the pool of human sera during the feasibility study (relative $u_{\text{char}} = 0.85\%$), and even lower than the relative uncertainty obtained for the EQAS materials A and B (relative $u_{\text{char}} = 1.06$ and 0.95%).

Table 2 Feasibility study for the determination of the catalytic activity concentration of AST in a pool of human sera and in two lyophilised proficiency testing samples (RELA A, RELA B).

	Feasibility study		
	Pool of human sera	RELA A	RELA B
Mean, U/L	110.08	233.10	191.29
SD of mean, U/L	3.25	8.53	6.30
RSD of mean, %	2.95	3.66	3.29
Relative u_{char} , %	0.85	1.06	0.95

Mean results from 12 laboratories (SD, standard deviation; RSD, relative standard deviation; relative u_{char} , relative standard uncertainty of the mean from characterisation).

Table 3 Characterisation study for the determination of the catalytic activity concentration of AST in the same pool of human sera (control material) and in ERM-AD457/IFCC.

	Characterisation study	
	Pool of human sera	ERM-AD457/IFCC
Mean, U/L	110.22	104.62
SD of mean, U/L	1.92	3.03
RSD of mean, %	1.74	2.90
Relative u_{char} , %	0.53	0.84

Mean of 11 laboratories for the pool of human sera and 12 laboratories for ERM-AD457/IFCC.

Certified value

The value assigned to ERM-AD457/IFCC for the catalytic activity concentration of AST is accompanied by uncertainty, which takes into account the uncertainty contributions from characterization, possibly hidden between-bottle heterogeneity and possibly hidden degradation during long-term storage (27). The relative standard uncertainties from each contribution are presented in Table 4, as well as the certified values in U/L and in $\mu\text{kat/L}$.

The relative standard uncertainty from characterisation ($u_{\text{char,rel}}$) was calculated by RSD/\sqrt{p} with RSD, the RSD of the mean of means, and p , the number of datasets. The relative combined standard uncertainty was calculated as:

$$u_{\text{c,rel}} = \sqrt{u_{\text{char,rel}}^2 + u_{\text{bb,rel}}^2 + u_{\text{its,rel}}^2}$$

An expanded uncertainty, U_{CRM} of 2.65 U/L, was finally obtained by multiplying $u_{\text{c,rel}}$ (expressed in percentage) by a coverage factor of 2 (95% level of confidence) and by the mean of dataset means (expressed in U/L). After rounding, the expanded uncertainty is 2.7 U/L.

Table 4 Uncertainty budget and certified values for ERM-AD457/IFCC reconstituted with 2 g of water.

ERM-AD457/IFCC	
$u_{\text{char,rel}}$, %	0.84
$u_{\text{bb,rel}}$, %	0.72
$u_{\text{its,rel}}$, %	0.62
$u_{\text{c,rel}}$, %	1.27
Certified values, U/L	104.6 ± 2.7
Certified values, $\mu\text{kat/L}$	1.74 ± 0.05

$u_{\text{char,rel}}$, Relative standard uncertainty from characterisation; $u_{\text{bb,rel}}$, relative standard deviation between-units; $u_{\text{its,rel}}$, relative standard uncertainty from the 24 months stability study at -20°C ; $u_{\text{c,rel}}$, relative combined standard uncertainty; certified values with $k=2$, 95% confidence interval.

Metrological traceability

As described earlier, the reference measurement procedure for the determination of catalytic activity concentration of AST is defined and standardised by a set of measurement parameters. The laboratories participating in the certification campaign for ERM-AD457/IFCC were requested to adhere strictly to the descriptions of the published reference measurement procedure for AST, and additional instructions for the control of the calibrated measurement equipment. The instructions comprised the control of the linearity of spectrometric absorbance, wavelength accuracy, gravimetric control of the weighing procedures, control of the volumetric devices, control of the accuracy of pH, and control of the measurement temperature 37°C . Therefore, the reference measurement procedure is under control, and traceability of the results to the SI can be ensured. As the measurement of the catalytic activity concentration of AST in ERM-AD457/IFCC is defined in terms of response to the IFCC reference procedure for AST at 37°C , the certified values are traceable to the SI, applying the IFCC reference procedure.

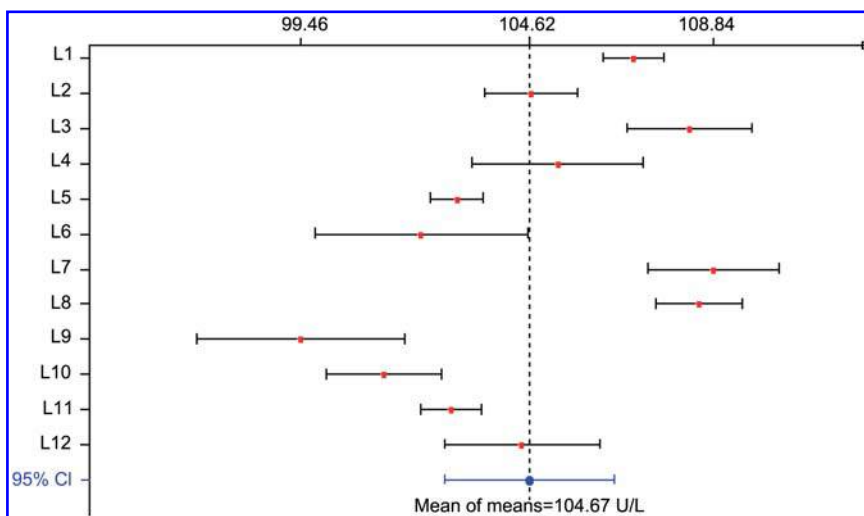


Figure 4 Characterisation study of ERM-AD457/IFCC: laboratory means and corresponding standard deviations (95% CI, 95% confidence interval of the mean of the means).

The CRM was reconstituted with 2 g of water.

Conclusions

A new CRM for AST has been developed. This has required high quality processing technology and extensive stability, homogeneity and characterisation studies involving the international collaboration of expert laboratories. The metrological traceability of the certified value and its uncertainty has been ensured and the values are traceable to the SI. A certificate and a certification report have been produced (28) and are available at <http://irmm.jrc.ec.europa.eu/catalogue>.

The CRM is intended to be used to verify the performances of the IFCC reference procedure at 37°C. If the CRM is used to directly calibrate commercial procedures, a commutability study should be performed with samples that are value assigned with the IFCC reference procedure.

Together with the IFCC reference procedure, the CRM forms the basis of the reference measurement system aiming at the standardisation of AST measurements and at the comparability of patient results in the clinical setting.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Provision of the raw material by Asahi Kasei Pharma Corporation played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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