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CERTIFICATION REPORT

The certification of the catalytic activity concentration of alanine aminotransferase in ERM®-AD454k/IFCC

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Abstract
This report describes the production of ERM®-AD454k/IFCC, which is a material certified for the catalytic activity concentration of alanine aminotransferase (ALT). This material was produced in collaboration with the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) following ISO Guide 34:2009 and it is certified in accordance with ISO Guide 35:2006.
The starting material was a recombinant form of human cytosolic ALT expressed in E. coli. It was produced, purified, filled and lyophilised by Asahi Kasei Pharma Corporation (Tokyo, Japan).
Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006.
The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025:2005.
Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [ ] and include uncertainties related to possible inhomogeneity, instability and characterisation.
The material is intended for the assessment of method performance of the IFCC primary reference measurement procedure for the measurement of the catalytic activity concentration of ALT at 37 °C. As with any reference material, it can be used for establishing control charts or validation studies.
The CRM is available in glass vials containing lyophilised material from 1 mL of ALT solution. The minimum amount of sample to be used is 23 µL after reconstitution of the whole content in one vial.
The CRM was accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

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The certification of the catalytic activity concentration of alanine aminotransferase in ERM®-AD454k/IFCC

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Summary

This report describes the production of ERM®-AD454k/IFCC, which is a material certified for the catalytic activity concentration of alanine aminotransferase (ALT). This material was produced in collaboration with the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) following ISO Guide 34:2009 [1] and it is certified in accordance with ISO Guide 35:2006 [2].

The starting material was a recombinant form of human cytosolic ALT expressed in *E. coli*. It was produced, purified, filled and lyophilised by Asahi Kasei Pharma Corporation (Tokyo, Japan).

Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [2].

The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025:2005 [3].

Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible inhomogeneity, instability and characterisation.

The material is intended for the assessment of method performance of the IFCC primary reference measurement procedure for the measurement of the catalytic activity concentration of ALT at 37 °C. As with any reference material, it can be used for establishing control charts or validation studies. The CRM is available in glass vials containing lyophilised material from 1 mL of ALT solution. The minimum amount of sample to be used is 23 µL after reconstitution of the whole content in one vial.

The CRM was accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

The following value was assigned:

	Certified value[2)]	Uncertainty[3)]
Catalytic activity concentration [1)]	103.8 U/L 1.73 µkat/L	2.6 U/L 0.05 µkat/L

[1)] Catalytic activity concentration of alanine aminotransferase (ALT) in the reconstituted material, as obtained by the IFCC primary reference measurement procedure for the measurement of catalytic activity concentration of alanine aminotransferase at 37 °C.

[2)] Certified values are values that fulfil the highest standards of accuracy and represent the unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory. The certified value and its uncertainty are traceable to the International System of Units (SI). Values were converted from U/L into µkat/L by multiplication with the factor $f = 0.01667$.

[3)] The uncertainty is the expanded uncertainty of the certified value with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

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Glossary

ANOVA	Analysis of variance
ALT	Alanine transaminase
b	Slope in the equation of linear regression $y = a + bx$
CRM	Certified reference material
C-RSE	IFCC Committee for Reference Systems of Enzymes
EC	European Commission
E. coli	*Escherichia coli*
ERM®	Trademark of European Reference Materials
EU	European Union
GUM	Guide to the Expression of Uncertainty in Measurements [ISO/IEC Guide 98-3:2008]
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IRMM	Institute for Reference Materials and Measurements of the JRC
ISO	International Organization for Standardization
k	Coverage factor
kat/L	Katal per litre
m	Mass
$MS_{between}$	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
n	Number of replicates per unit
p	Number of datasets
rel	Index denoting relative figures (uncertainties etc.)
s	Standard deviation
s_{bb}	Between-unit standard deviation; an additional index "rel" is added when appropriate
$s_{between}$	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate
SI	International System of Units
s_{rel}	Relative standard deviation of all results of the stability study
s_{within}	Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate
s_{wb}	Within-unit standard deviation
$\bar{t}$	Mean of all t_i
t_i	Time point for each replicate
t_{sl}	Proposed shelf life

t_{tt}	Proposed transport time
u	Standard uncertainty
U	Expanded uncertainty
U/L	Units per litre
u^*_{bb}	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability; an additional index "rel" is added as appropriate
u_{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{Δ}	Combined standard uncertainty of measurement result and certified value
U_{Δ}	Expanded standard uncertainty of measurement result and certified value
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{meas}	Standard measurement uncertainty
u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
v	Volume
$\overline{x}$	Arithmetic mean
Δ_{meas}	Absolute difference between mean measured value and the certified value
$\nu_{MSwithin}$	Degrees of freedom of MS_{within}

1 Introduction

1.1 Background

Alanine aminotransferase (ALT) is a cytosolic enzyme with a dimeric structure composed of two identical polypeptide subunits of about 400 amino acid residues that catalyses the conversion of L-alanine and 2-oxoglutarate to pyruvate and L-glutamate. This enzyme is mainly expressed in the liver, but low amounts can also be found in the kidney, skeletal muscle and the heart. However, its predominance in the liver and its localisation in the cytoplasm account for its relative specificity in the diagnosis of hepatic disorders. Elevated levels of the catalytic activity of ALT can be found even before clinical symptoms appear [5]. Determination of the catalytic activity concentration of ALT is also used for samples stored in blood banks to detect samples potentially infected with hepatitis [6, 7]. Measurement of this enzyme in serum is requested to the clinical laboratory to detect diseases that affect the integrity of liver cells (e.g., liver cell inflammation or necrosis). Oftentimes aspartate aminotransferase (AST) is measured along with ALT and the ratio of these two enzymes can be useful for the diagnosis of fibrosis in patients with chronic liver disease. However, ALT is more liver-specific and has a longer half-life [8].

The catalytic activity of an enzyme is a property that is measured by the rate of a specified chemical reaction under certain experimental conditions. The measurement of this property is very important in clinical chemistry, but the standardisation of catalytic activity measurements is challenging as a number of parameters influence the enzyme activity (e.g. temperature, pH, substrate nature and concentration, activators, inhibitors). Therefore, the measurement results are heavily dependent on the measurement procedure used to attain them. This led to the development of universally recognised measurement procedures for enzymes commonly measured in clinical chemistry, such as the IFCC primary reference measurement procedure for the measurement of catalytic activity concentrations of enzymes at 37 °C [9].

The EU directive on *in vitro* diagnostic medical devices (Directive 98/79/EC) requires traceability of the assigned values of calibrants and control materials to reference measurement procedures and/or reference materials of higher order.

In collaboration with the IFCC Committee for Reference Systems of Enzymes (C-RSE), the Institute for Reference Materials and Measurements (IRMM) developed a CRM certified for the catalytic activity concentration of ALT. This material, ERM-AD454k/IFCC, is intended to be used as a quality control material for the IFCC primary reference measurement procedure for ALT at 37 °C [10]. The homogeneity and the stability of ERM-AD454k/IFCC were demonstrated and the certified value was assigned using the IFCC reference measurement procedure at 37 °C in an interlaboratory comparison of expert laboratories.

1.2 Choice of the material

A recombinant ALT material from Asahi Kasei Pharma Corporation was selected as starting material based on the outcome of a preliminary commutability study carried out by IRMM. The purity was guaranteed by the provider as containing no other enzyme with a relative catalytic activity concentration of more than 1.0 % of the total catalytic activity concentration. The material was solubilised in a buffer, frozen and lyophilised to improve long-term stability. The aim of the production process was to obtain a material that once reconstituted with 1.0 mL of distilled/deionised water would have a catalytic activity concentration of about 100 U/L. The selection of the material and the catalytic activity concentration was based on a recommendation received from the IFCC C-RSE in 2011.

1.3 Design of the CRM project

A commutability study including nine routine measurement procedures was completed to select the most appropriate starting material for the production of ERM-AD454k/IFCC.

The material was certified by interlaboratory comparison using data from expert laboratories using the IFCC primary reference measurement procedure for the measurement of the catalytic activity concentration of ALT at 37 °C [10]. The performances of the laboratories were assessed using two serum based control materials.

The homogeneity and stability of the material were assessed using a UniCel® DxC 800 Synchron Clinical System with ALT reagent cartridges (Beckman Coulter, Inc., Clare, IE). This test kit showed a low average relative standard deviation during the commutability study. Statistical analysis of the data was done using the software SoftCRM (version 2.0.21) [11].

2 Participants

2.1 Project management and evaluation

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

2.2 Processing

Asahi Kasei Pharma Corporation, Tokyo, JP

2.3 Homogeneity and stability study

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

Beckman Coulter, Inc., Clare, IE

2.4 Characterisation

The laboratories are listed below in alphabetic order. This order does not necessarily correspond to the ranking of the laboratories L01 to L10 described in the Tables of this report.

*Affiliated Hospital of Nantong University, Reference Laboratory, Nantong, CN
(measurements under the scope of ISO/IEC 17025:2005 and ISO 15195:2003 accreditation, CNAS No. L6260)

*Beijing Aerospace General Hospital, Reference Laboratory, Beijing, CN
(measurements under the scope of ISO/IEC 17025:2005 and ISO 15195 accreditation, CNAS No. L5536)

Biosystems, S.A., Barcelona, ES

*INSTAND e.V., Düsseldorf, DE
(measurements under the scope of ISO/IEC 17025:2005 accreditation, DAkkS No. D-K-15027-01-00)

LabWest HagaZiekenhuis, Klinisch Chemisch en Hematologisch Laboratorium, Den Haag, NL

Servizio Di Medicina Di Laboratorio Diagnostica e Ricerca, Ospedale San Raffaele, Milano, IT

*Sichuan Maker Biotechnology Co., Ltd., Reference System Department, Chengdu, CN
(measurements under the scope of ISO/IEC 17025:2005 and ISO 15195:2003 accreditation, CNAS No. L6172)

*Stiftung für Pathobiochemie und Molekulare Diagnostik, Referenzinstitut für Bioanalytik, Kalibrierlaboratorium II, Medizinische Hochschule Hannover, Institut für Klinische Chemie, Hannover, DE
(measurements under the scope of ISO/IEC 17025:2005 and ISO15195:2004 accreditation, DAkkS No. D-K-15117-02-00)

*Universitat Autònoma de Barcelona, Departament de Bioquímica i Biologia Molecular, Unitat de Bioquímica de Medicina, Barcelona, ES
(measurements under the scope of ISO/IEC 17025:2005 and ISO 15195:2004 accreditation, ENAC No. 195/LC10.141)

*Università degli Studi di Milano, Laboratorio Analisi Chimico-Cliniche, Centro Interdipartimentale di Ricerca sulla Riferibilità Metrologica in Medicina di Laboratorio, Milano, IT
(measurements under the scope of ISO/IEC 17025 and ISO 15195:2004 accreditation, ACCREDIA No. 217/01)

*Listed in the JCTLM database for reference measurement services

3 Material processing and process control

3.1 Origin of the starting material and processing

The starting material was produced and processed by Asahi Kasei Pharma Corporation. It was a recombinant form of human cytosolic ALT expressed in *E. coli*. The enzyme was purified by hydrophobic and anion-exchange chromatography and then dissolved in a buffer (pH 7.5) containing among others bovine serum albumin and polysaccharides. The liquid preparation was filled in glass vials, frozen and lyophilised. The vials were labelled in the same order as the filling sequence using the labels provided by IRMM.

3.2 Process control

The final starting material was prepared and tested for activity, stability and homogeneity by Asahi Kasei Pharma Corporation. This batch was shown to be homogenous and stable for at least one year when stored at -20 °C.

4 Homogeneity

A key requirement for any reference material aliquotted into units is equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value, but it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 [1] requires reference material producers to quantify the between-unit variation. This aspect is covered in between-unit homogeneity studies.

The within-unit inhomogeneity does not influence the uncertainty of the certified value when the minimum sample intake is respected, but determines the minimum size of an aliquot that is representative for the whole unit.

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified value of the CRM is valid for all vials of the material, within the stated uncertainty.

The number of vials selected corresponds to approximately the cube root of the total number of vials produced. The 20 vials were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this, the batch was divided into 20 groups (with a similar number of vials) and one vial was selected randomly from each group. After reconstitution, three samples were taken from each selected vial, and analysed with a UniCel® DxC 800 Synchron Clinical System using ALT reagent cartridges. The measurements were performed under repeatability conditions, and in a randomised manner to be able to separate a potential trend in the analytical sequence from a trend in the filling sequence.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. No trend in the filling sequence was observed at a 95 % confidence level. A significant (95 % confidence level) trend in the analytical sequence was visible, pointing at a changing parameter, e.g. a signal drift in the analytical system. The correction of biases, even if they are statistically not significant, was found to combine the smallest uncertainty with the highest probability to cover the true value [12]. Correction of trends is therefore expected to improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. As the analytical sequence and the unit numbers were not correlated, trends significant on at least a 95 % confidence level were corrected as shown below:

$$x_{i\_corr} = x_i - b \cdot i \qquad \textbf{Equation 1}$$

b **=** slope of the linear regression

i **=** position of the result in the analytical sequence

The trend-corrected dataset was assessed for consistency using Grubbs outlier tests at a confidence level of 99 % on the individual results and on the unit means. No outlying individual results and outlying unit means were detected and all the data were retained for statistical analysis.

Quantification of between-unit inhomogeneity was undertaken by analysis of variance (ANOVA), which separates the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the method repeatability if the individual samples were representative for the whole vial.

Evaluation by ANOVA requires mean values per vial, which follow at least a unimodal distribution and results for each vial that follow unimodal distributions with approximately the same standard deviations. The distribution of the mean values per vial was visually tested using histograms and normal probability plots.

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the standard deviations and are therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u^*_{bb}, the maximum inhomogeneity that could be hidden by method repeatability, was calculated as described by Linsinger *et al.* [13]. u^*_{bb} is comparable to the limit of detection of an analytical method. It describes the maximum inhomogeneity that might be hidden in the frame of the given study setup.

Method repeatability ($s_{wb,rel}$), between-unit standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{x}}$$ **Equation 2**

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{x}}$$ **Equation 3**

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}}\sqrt[4]{\frac{2}{v_{MSwithin}}}}{\bar{x}}$$ **Equation 4**

MS_{within} mean of squares within-unit from an ANOVA

$MS_{between}$ mean of squares between-unit from an ANOVA

$\bar{x}$ mean of all results of the homogeneity study

n mean number of replicates per unit

$v_{MSwithin}$ degrees of freedom of MS_{within}

The results of the evaluation of the between-unit variation are summarised in Table 1. The resulting values from the above equations were converted into relative uncertainties.

Table 1: Results of the homogeneity study

ERM-AD454k/IFCC	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]
Catalytic activity concentration of ALT in reconstituted material	1.0	0.6	0.3

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore the between-unit standard deviation can be used as estimate of u_{bb}. As u^*_{bb} sets the limits of the study to detect inhomogeneity, the larger value of s_{bb} and u^*_{bb} is adopted as uncertainty contribution to account for potential inhomogeneity.

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus should be used in an analysis. Using sample sizes equal or above the minimum sample intake guarantees the certified value within its stated uncertainty.

Homogeneity and stability experiments were performed using a 23 µL sample intake. This sample intake gives acceptable repeatability, demonstrating that the within-unit inhomogeneity no longer contributes to analytical variation at this sample intake.

5 Stability

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for dispatch of the materials to the customers (short-term stability).

Time, temperature, light (including ultraviolet radiation) and water content were regarded as the most relevant influences on the stability of the materials. The influence of ultraviolet or visible light was minimised by storing the material in the dark and dispatched in boxes, thus removing any possibility of degradation by light. The water content was reduced by freeze-

drying to obtain a stable material. Therefore, only the influences of time and temperature needed to be investigated.

During transport, especially in summer time, temperatures up to 60 °C can be reached and stability under these conditions must be demonstrated, if the samples are to be transported without any additional cooling.

The stability studies were carried out using an isochronous design [14]. In this approach, samples were stored for a particular length of time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples were analysed simultaneously under repeatability conditions which greatly improves the sensitivity of the stability tests.

5.1 Short-term stability study

For the short-term stability study, samples were stored at -20 °C, 4 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to -70 °C. Two vials per storage time were selected using a random stratified sampling scheme. After reconstitution, three samples were measured from each vial with a UniCel DxC 800 Synchron Clinical System using ALT reagent cartridges. The measurements were performed under repeatability conditions, and a randomised sequence was used to differentiate any potential analytical drift from a trend over storage time.

The data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test on a confidence level of 99 %. No outlying result was found (Table 2).

In addition, the data were evaluated against storage time, and regression lines of catalytic activity concentration versus time were calculated, to test for potential increases/decrease of the measurand due to shipping conditions. The slopes of the regression lines were tested for statistical significance.

The results of the measurements are shown in Annex B. The results of the statistical evaluation of the short-term stability are summarised in Table 2.

Table 2: Results of the short-term stability test

ERM-AD454k/IFCC	Number of individual outlying results*			Significance of the trend**		
	-20 ºC	4 °C	60 ºC	-20 ºC	4 °C	60 ºC
Catalytic activity concentration of ALT	none	none	none	no	yes	yes

* 99 % confidence level

** 95 % confidence level

One statistical outlier was detected and it was retained for the estimation of u_{STS}. Two of the trends were statistically significant on a 95 % confidence level for storage at 4 °C and 60 °C.

The material shall be shipped on dry ice.

5.2 Long-term stability study

For the long-term stability study, samples were stored at -20 °C and 4 °C for 0, 4, 8 and 12 months (at each temperature). The reference temperature was set to -70 °C. Two samples per storage time were selected using a random stratified sampling scheme. After reconstitution, three samples were measured from each vial with a UniCel DxC 800 Synchron Clinical System using ALT reagent cartridges. The measurements were performed

under repeatability conditions, in a random sequence to be able to separate any potential analytical drift from a trend over storage time.

The long-term stability data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. No outlying individual results were found and all data were retained for statistical analysis.

In addition, the data were plotted against storage time and linear regression lines of catalytic activity concentration versus time were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage). A significant trend was detected at a 95 % confidence level for storage at 4 °C.

The results of the long-term stability measurements are shown in Annex C.

The material shall be stored at -20 °C.

5.3 Estimation of uncertainties

Since there is always variations on measurement results, no study can entirely rule out degradation of materials, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation. This degradation could be hidden in the frame of the study set up, i.e. to estimate the uncertainty of stability. This means that, even under ideal conditions, the outcome of a stability study can only be that there is no detectable degradation within an uncertainty to be estimated.

The uncertainties of stability during dispatch and storage were estimated, as described in [15]. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions u_{sts} and u_{lts} were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{s_{rel}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{tt} \qquad \textbf{Equation 5}$$

$$u_{lts,rel} = \frac{s_{rel}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{sl} \qquad \textbf{Equation 6}$$

s_{rel} relative standard deviation of all results of the stability study

t_i time elapsed at time point *i*

$\bar{t}$ mean of all t_i

t_{tt} chosen transport time (1 week at -20 ºC)

t_{sl} chosen shelf life (12 months at -20 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the short-term stability studies at -20 °C. The uncertainty describes the possible change during a dispatch at -20 °C lasting for one week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the long-term stability studies at -20 °C. The uncertainty contribution describes the possible degradation during 12 months storage at -20 °C.

The results of these evaluations are summarised in Table 3.

Table 3: Uncertainties of stability during dispatch and storage. $u_{sts,rel}$ was calculated for a temperature of -20 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of -20 °C and 12 months

ERM-AD454k/IFCC	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
Catalytic activity concentration of ALT in reconstituted material	0.2	0.8

The material showed significant degradation at 60 °C but no significant degradation was observed for transport below 4 °C. Transport on dry ice is therefore necessary.

After the certification study, the material will be included in the IRMM's regular stability monitoring programme, to control its further stability.

6 Characterisation

The material characterisation is the process of determining the property value of a reference material.

This was based on an interlaboratory comparison of data from expert laboratories, i.e. the catalytic activity concentration of ALT in the material was determined in different laboratories. All participants used the IFCC primary reference measurement procedures for the measurement of catalytic activity concentration of ALT at 37 °C [10] for the measurements. Using an interlaboratory comparison aims at randomisation of laboratory bias, which reduces the combined uncertainty.

6.1 Selection of participants

Ten laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of catalytic activity concentration measurements of enzymes using the IFCC primary reference measurement procedure for the measurement of catalytic activity concentrations at 37 °C. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2).

6.2 Study setup

Each laboratory received six vials of ERM-AD454k/IFCC and was requested to provide six independent results, one per vial. The vials for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. The sample preparations and measurements had to be spread over at least two days to ensure intermediate precision conditions. Each participant was also required to analyse two blinded quality control samples.

Laboratories were also requested to give estimations of the expanded uncertainties of the mean value of the six results. All GUM approaches were regarded as equally valid procedures.

6.3 Method used

All laboratories used the IFCC primary reference measurement procedure for the measurement of the catalytic activity concentration of ALT at 37 °C [10,16].

6.4 Evaluation of results

The characterisation study resulted in eight accepted datasets. All accepted individual results of the participants are displayed in tabular and graphical form in Annex D.

6.4.1 Technical evaluation

The following criteria were considered during the evaluation:

- compliance with the analysis protocol: sample preparations and measurements performed on two days
- performance in measuring the quality control samples

Based on the above criteria, the dataset from one laboratory (L05) was not included in the evaluation based on their performance in measuring the quality control samples.

6.4.2 Statistical evaluation

The datasets accepted based on technical reasons were tested for normality of dataset means using kurtosis/skewness tests and normal probability plots and were tested for outlying means using the Grubbs test and using the Cochran test for outlying standard deviations, (both at a 99 % confidence level). Standard deviations within (s_{within}) and between ($s_{between}$) laboratories were calculated using one-way ANOVA. The results of these evaluations are shown in Table 4.

Table 4: Statistical evaluation of the technically accepted datasets for ERM-AD454k/IFCC. *p*: number of technically valid datasets

ERM-AD454k/IFCC	*p*	Outliers		Normally distributed	Statistical parameters			
		Means	Variances		Mean [U/L]	*s* [U/L]	$s_{between}$ [U/L]	s_{within} [U/L]
Catalytic activity concentration of ALT	9	none	none	yes	103.81	1.83	1.78	1.04

The laboratory means follow normal distributions. None of the data contains outlying means and variances. The datasets are therefore consistent and the mean of laboratory means is a good estimate of the true value. Standard deviations between laboratories are considerably larger than the standard deviation within laboratories, showing that confidence intervals of replicate measurements are unsuitable as estimate of measurement uncertainty.

The uncertainty related to the characterisation is estimated as the standard error of the mean of laboratory means (5).

Table 5: Uncertainty of characterisation for ERM-AD454k/IFCC

ERM-AD454k/IFCC	*p*	Mean [U/L]	*s* [U/L]	$u_{char,rel}$ [%]
Catalytic activity concentration of ALT	9	103.8	1.83	0.6

7 Value Assignment

Based on the results of the characterisation study a certified value for the catalytic activity concentration of ALT was assigned to ERM-AD454k/IFCC.

Certified values are values that fulfil the highest standards of accuracy. Procedures at IRMM require generally pooling of not less than six datasets to assign certified values. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [4] were established.

7.1 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets as shown in Table was assigned as certified value.

The assigned uncertainty consists of uncertainties relating to characterisation, u_{char} (Section 6), potential between-unit inhomogeneity, u_{bb} (Section 4.1), and potential degradation during transport, u_{sts}, and long-term storage, u_{lts} (Section 5). These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{CRM,\ rel}$) with a coverage factor k given as:

$$U_{CRM,rel} = k \cdot \sqrt{u_{bb,rel}^2 + u_{sts,rel}^2 + u_{lts,rel}^2 + u_{char,rel}^2} \qquad \textbf{Equation 7}$$

- u_{char} was estimated as described in Section 6
- u_{bb} was estimated as described in Section 4.1
- u_{sts} and u_{lts} were estimated as described in section 5.3

A coverage factor k of 2 was applied, to obtain the expanded uncertainty. The certified value and its uncertainties are summarised in Table 6.

Table 6: Certified value and its uncertainties for ERM-AD454k/IFCC

ERM-DA454k/IFCC	Certified value [U/L]	$u_{char,rel}$ [%]	$u_{bb,rel}$ [%]	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]	U_{CRM} [1)] [U/L]
Catalytic activity concentration of ALT	103.8	0.6	0.6	0.2	0.8	2.6

[1)] Expanded (k = 2) and rounded uncertainty.

The International Union of Pure and Applied Chemistry and the International Union of Biochemistry recommended that enzyme concentration is expressed in terms of katals per liter (kat/L) [16]. This name and symbol were approved by the General Conference on Weights and Measures and is consistent with the International System of Units (SI) as kat = mol/s. However, different units have been introduced in the past. Therefore, the Commission on Enzymes of the International Union of Biochemistry proposed the term international unit (U) as the quantity of enzyme that catalyses the reaction of 1 µmol of substrate per minute. Catalytic activity concentration is then to be expressed in terms of U/L [17, 18]. Enzyme units (U) are still more commonly used than the katal, especially in biochemistry. Therefore, the certified value for ERM-AD454k/IFCC is expressed both in µkat/L and in U/L in this certification report as well as on the certificate. The catalytic activity concentration in µkat/L can easily be converted to U/L by multiplying with the factor f = 60.
1 µkat/L = 60 U/L
1 U = 10^{-6} mol/60 s = 16.7 x 10^{-9} mol/s

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

The catalytic activity concentration of ALT is a method-defined measurand and can only be obtained by following the procedure specified in the IFCC primary reference measurement procedure at 37 °C [10]. Adherence to this procedure was confirmed by agreement of the laboratories' results with the assigned value for the samples that were used as quality control samples and by comparison among laboratory results. The measurand is therefore operationally defined by *method*.

Quantity value

Traceability of the obtained results is based on the traceability of all relevant input factors. Instruments in individual laboratories were verified and calibrated with measurement standards ensuring traceability to the SI. Consistency in the interlaboratory comparison demonstrates that all relevant input factors were covered. As the assigned value is a combination of agreeing results individually traceable to the SI, the assigned quantity value itself is traceable to the SI.

8.2 Commutability

Many measurement procedures include one or more steps which select specific (or specific groups of) analytes from the sample for the subsequent whole measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is summarised in a concept called 'commutability of a reference material'. There are various definitions that define this concept. For instance, the Clinical and Laboratory Standards Institute Guideline C53-A [19] recommends the use of the following definition for the term *commutability*:

> "The equivalence of the mathematical relationships among the results of different measurement procedures for an RM and for representative samples of the type intended to be measured."

The commutability of a CRM defines its fitness for use and is therefore a crucial characteristic when applying different measurement methods. When the commutability of a CRM is not established, the results from routinely used measurement procedures cannot be legitimately compared with the certified value to determine whether a bias does not exist in calibration, nor can the CRM be used as a calibrant. For instance, CRMs intended to be used to establish or verify metrological traceability of routine clinical measurement procedures must be commutable for the routine clinical measurement procedures for which they are intended to be used.

A commutability study was carried out on a trial batch of the starting material for ERM-AD454k/IFCC in collaboration with the IFCC C-RSE. The results were convincing enough to process the final batch of the material and to certify its ALT catalytic activity concentration. However, if ERM-AD454k/IFCC would be used to calibrate routine measurement procedures, extended commutability studies should be performed with these procedures.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

The material is for *in vitro* use only.

9.2 Storage conditions

Unopened vials of the material should be stored at (-20 ± 5) °C in the dark. After reconstitution, the material must be kept cold (2-8 °C) and must be used within four hours.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially for opened vials.

9.3 Reconstitution

To prepare ERM-AD454k/IFCC for use, the lyophilised material shall be reconstituted according to the following procedure:

1) Remove vial from freezer and let equilibrate to room temperature (20-25 °C).
2) Tap the vertically positioned vial gently to ensure that the lyophilized material is at the bottom of the vial.
3) Carefully open vial, avoiding the loss of lyophilised material.
4) Weigh the vial with its content to the nearest 0.1 mg.
5) Reconstitute with (1.00 ± 0.01) mL distilled water (20-22 °C) slowly added to the sides of the vial.
6) Weigh the vial after adding the water and record the weight.
7) Carefully close the vial.
8) Allow to stand at room temperature for ten minutes.
9) Slowly stir up the vial to dissolve the lyophilised material completely.
10) Calculate the volume of water at 20 °C from the mass of water added taking into account the temperature dependent density.
11) Keep the vial cold (2-8 °C) until use.
12) The catalytic activity concentration of ALT must be measured within four hours following the reconstitution*.

*The activity is not guaranteed after four hours after reconstitution.

9.4 Minimum sample intake

The minimum sample intake representative for the catalytic activity concentration of ALT in ERM-AD454k/IFCC is 23 µL after reconstitution of the whole vial according to the procedure outlined in 9.3 as this was the sample intake for the homogeneity study.

9.5 Use of the certified value

The main purpose of the material is to control the performance of the IFCC primary reference measurement procedure for the measurement of catalytic activity concentration of ALT at 37 °C [10]. As any reference material, it can be used for establishing control charts or validation studies.

Use as a calibrant

It is not recommended to use this material as calibrant. If used nevertheless, the uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty. When the material is used as a calibrant in a routine measurement procedure the commutability should be verified for the assay concerned.

Comparing an analytical result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, www.erm-crm.org [20].

When assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is summarised here:

- Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).
- Combine the measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_{Δ}) from the combined uncertainty ($u_{\Delta,}$) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %.
- If $\Delta_{meas} \leq U_{\Delta}$ then no significant difference exists between the measurement result and the certified value, at a confidence level of approximately 95 %.

Use in quality control charts

The materials can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart.

10 Acknowledgments

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Annexes

Annex A: Results of the homogeneity measurements

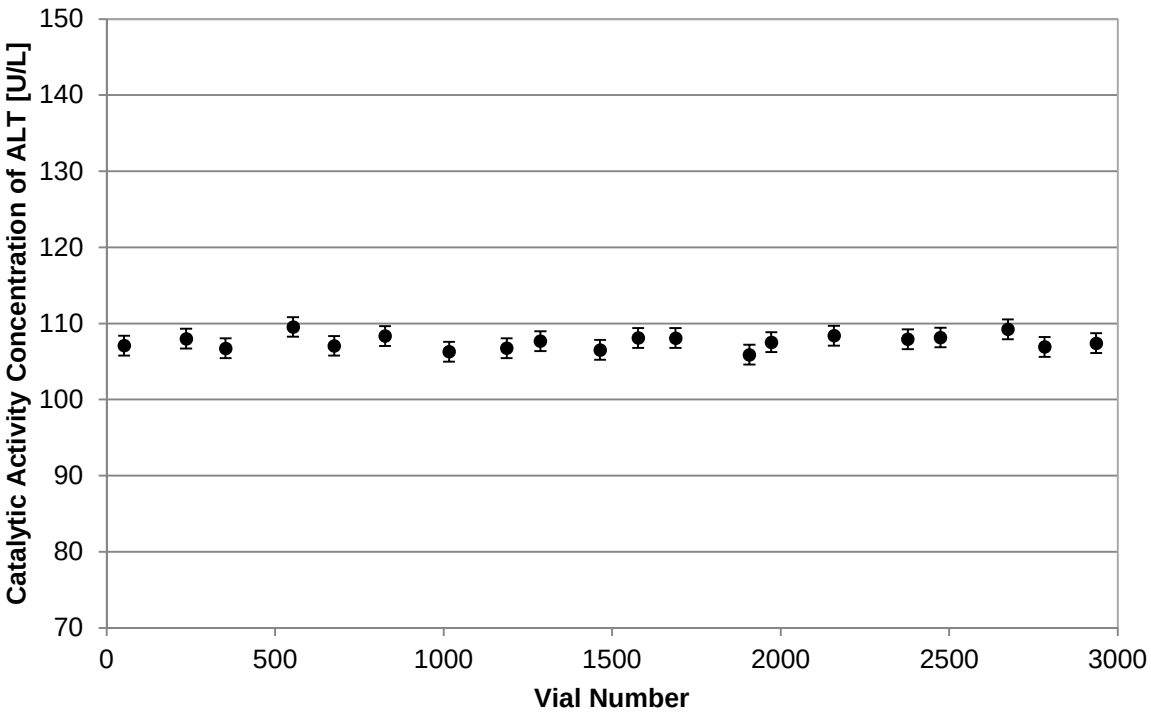

Figure A1: Homogeneity data of ALT in ERM-AD454k/IFCC as measured with a UniCel DxC 800 Synchron Clinical System with ALT reagent cartridges. Shown are the averages per vial number and their 95 % confidence interval based on the standard deviation as derived from a one-way ANOVA of all data after correction of the analysis trend.

Annex B: Results of the short-term stability measurements

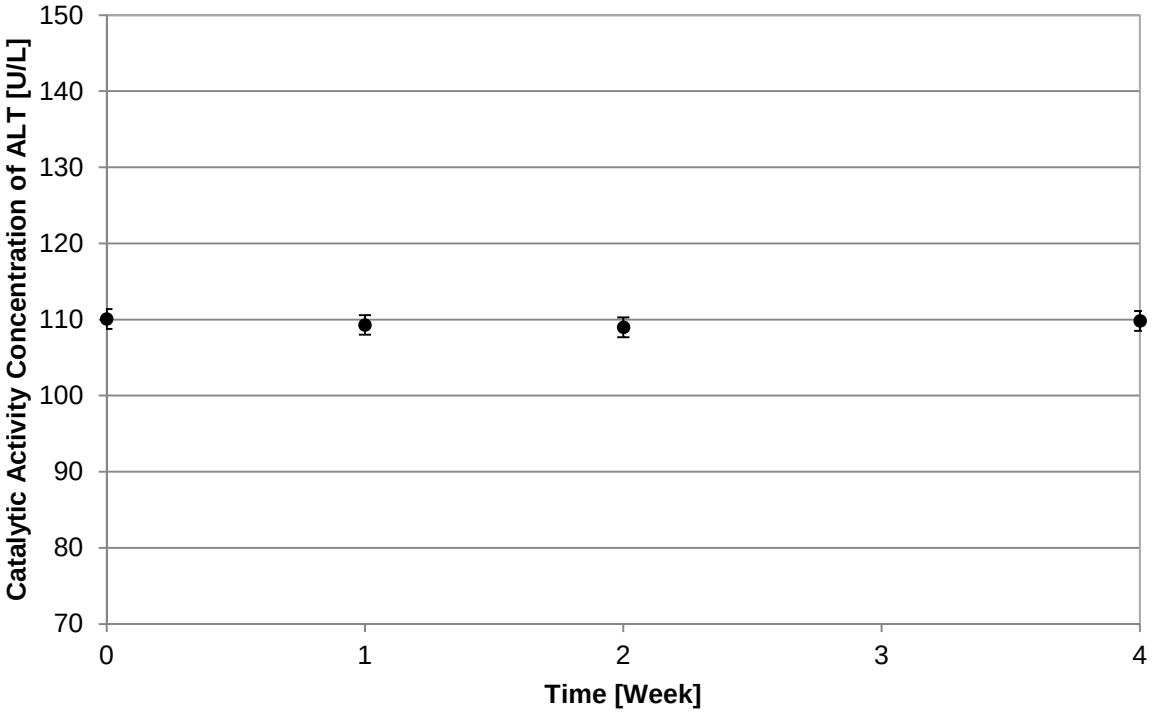

Figure B1: Short-term stability data of ALT in ERM-AD454k/IFCC (stored at -20 °C) as measured with a UniCel DxC 800 Synchron Clinical System with ALT reagent cartridges. Shown are the averages per time point and their 95 % confidence interval based on the standard deviation as derived from a one-way ANOVA of all data.

Annex C: Results of the long-term stability measurements

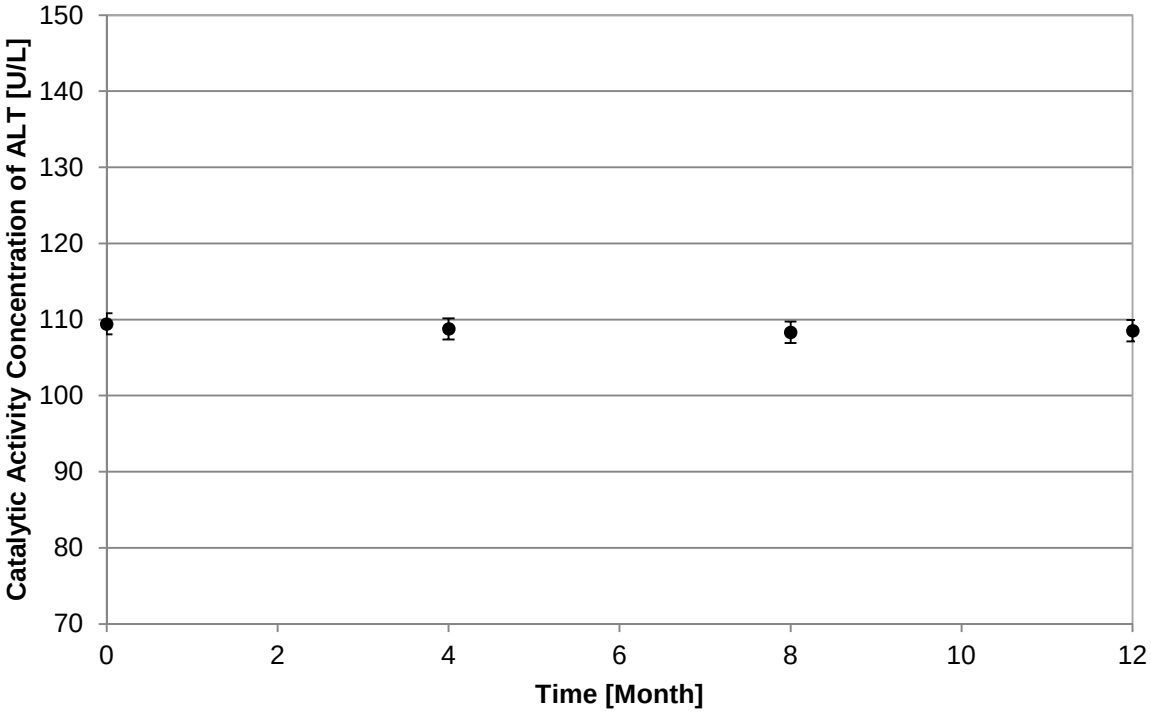

Figure C1: Long-term stability data of ALT in ERM-AD454k/IFCC (stored at -20 °C) as measured with a UniCel DxC 800 Synchron Clinical System with ALT reagent cartridges. Shown are the averages per time point and their 95 % confidence interval based on the standard deviation as derived from a one-way ANOVA of all data.

Annex D: Results of the characterisation measurements

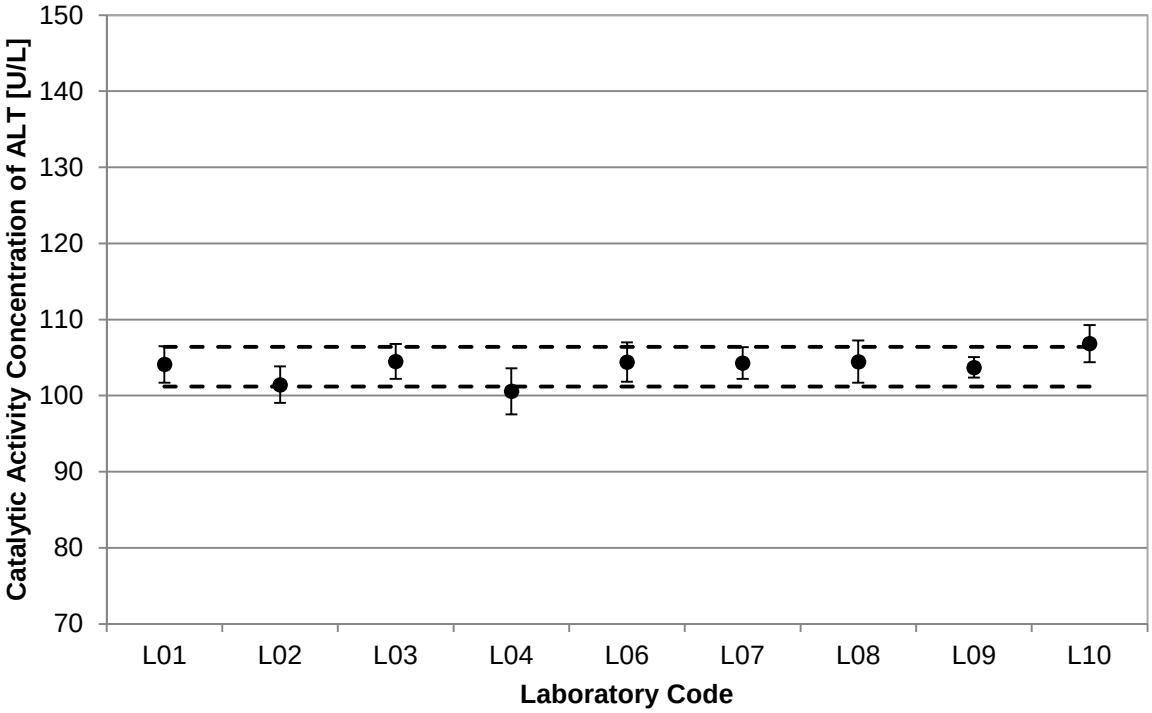

Figure D1: Graph showing average catalytic activity concentrations of ALT in ERM-AD454k/IFCC as measured with the IFCC primary reference measurement procedure at 37 °C with expanded uncertainties as stated by the laboratories and the 95 % certified interval (dotted lines).

Table D1: Summary of all accepted individual results, the mean value and the expanded uncertainty as stated by the laboratories for the catalytic activity concentration measurements of ALT in ERM-AD454k/IFCC.

Laboratory code	Replicate 1 [U/L]	Replicate 2 [U/L]	Replicate 3 [U/L]	Replicate 4 [U/L]	Replicate 5 [U/L]	Replicate 6 [U/L]	Mean [U/L]	Expanded uncertainty [U/L]
L01	104.4	104.3	104.3	105.0	104.4	102.2	104.1	2.4
L02	101.12	101.26	100.95	102.38	101.39	101.51	101.4	2.4
L03	102	105	106	104	104	106	105	2.3
L04	99.1	99.5	100.5	101.0	101.6	101.7	100.6	3.02
L06	104.1	103.0	104.9	104.0	105.4	105.1	104.4	2.6
L07	104.50	103.48	103.49	105.39	104.24	104.61	104.29	2.1
L08	104.80	105.21	104.35	105.50	103.52	103.47	104.48	2.78
L09	103.4	103.3	104.1	102.7	104.4	104.3	103.7	1.345
L10	107	108	109	105	105	107	107	2.5

Disclaimer

Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

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