Catalytic concentration of lactate dehydrogenase isoenzyme 1 (LD1) determined by IFCC method at 37 °C
Mission

The mission of IRMM is to promote a common European measurement system in support of EU policies, especially internal market, environment, health and consumer protection standards.
Catalytic concentration of lactate dehydrogenase isoenzyme 1 (LD1) determined by IFCC method at 37 °C

IRMM/IFCC-453

N. Kristiansen, T. Linsinger, H. Schimmel, J. Pauwels

European Commission, Joint Research Centre
Institute for Reference Materials and Measurements
Geel, Belgium

L. Siekmann

Deutsche Gesellschaft für Klinische Chemie e.V.
Reference Institute of Bioanalysis
Bonn, Germany


International Federation of Clinical Chemistry Laboratory Medicine, on behalf of the Working group for calibrators in clinical enzymology
*President of the IFCC

Directorate General
Joint Research Centre

2000
LEGAL NOTICE

Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use which might be made of the following information.

A great deal of additional information on the European Union is available on the Internet. It can be accessed through the Europa server (http://europa.eu.int).
ABSTRACT
The Institute for Reference Materials and Measurements (IRMM) and the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) have certified a reference material (CRM) under the name and number IRMM/IFCC-453. This report describes the certification of the catalytic concentration of lactate dehydrogenase isoenzyme 1 (LD1) in a purified lyophilised material of human origin when measured by the IFCC recommended method at 37 °C. The catalytic concentration of LD1 in reconstituted material is certified to $502 \pm 7$ U/L or $8.37 \pm 0.12$ μkat/L. It is the intention that the reference material should be used to control and optimise the performance of enzyme measurements, verify the comparability of results from different laboratories and be used as a reference material for manufacturers of reagents and diagnostic kits.
GLOSSARY

ALAT  alanine aminotransferase
ANOV A analysis of variance
ASAT  aspartate aminotransferase
BCR   Community Bureau of Reference
CK-MB  creatine kinase-muscle/brain
CRM   certified reference material
EDTA  Ethylenediaminetetraacetic acid
GGT   gamma-glutamyltransferase
GUM   Guide to the Expression of the Uncertainty in Measurement
HIV   human immunodeficiency virus
IFCC  International Federation of Clinical Chemistry and Laboratory Medicine
IRMM  Institute for Reference Materials and Measurements
ISO   International Organisation for Standardisation
LD 1,2,3,4,5 lactate dehydrogenase isoenzyme 1,2,3,4,5
MSB   mean square between groups (ANOVA)
MSW   mean square within groups (ANOVA)
NAD+  nicotinamide adenine dinucleotide, oxidised form
NADH  nicotinamide adenine dinucleotide, reduced form
PAGE  polyacrylamid gel electrophoresis
RNA ribonucleic acid
RSD relative standard deviation
SI international system of units
SOP  standard operating procedure
WG-CCE working group for calibrators in clinical enzymology
U_{CRM} expanded (k=2) combined uncertainty of the CRM
u_{char} standard uncertainty of the characterisation
s_{betw} standard deviation between certification laboratories (ANOVA)
u_{sb} standard uncertainty of homogeneity
s_{wib} standard deviation within groups from the hom. study (ANOVA)
s_{sb} standard deviation between groups from the hom. study (ANOVA)
u_{tu} standard uncertainty of stability during storage
u_{as} standard uncertainty of stability during transport

DEFINITIONS

Catalytic activity ($x$) of an enzyme is a property quantified by the catalysed rate of conversion of a specified chemical reaction, produced in a specified assay system.

Unit of catalytic activity: katal (kat) = mole per second (mol/s). For any measurement procedure 1 U = 1 μmol/min = 16.67 nmol/s = 16.67 nkat.

Catalytic (activity) concentration ($b$) is the catalytic activity of the component (enzyme) divided by the volume of the original system containing the enzyme (not the assay system).

Unit of catalytic (activity) concentration: 1 kat/L = 10^3 mol . s^{-1} . m^{-3}, 1 U/L = 16.67 nkat/L.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2 PARTICIPATING LABORATORIES</td>
<td>2</td>
</tr>
<tr>
<td>3 PREPARATION OF THE MATERIAL</td>
<td>2</td>
</tr>
<tr>
<td>3.1 CHOICE OF THE SOURCE OF THE ENZYME</td>
<td>3</td>
</tr>
<tr>
<td>3.2 EXTRACTION AND PURIFICATION OF THE ENZYME</td>
<td>3</td>
</tr>
<tr>
<td>3.3 CHOICE AND ADDITION OF THE MATRIX FOLLOWED BY LYOPHILISATION</td>
<td>3</td>
</tr>
<tr>
<td>3.4 PRODUCTION CONTROL</td>
<td>3</td>
</tr>
<tr>
<td>4 CERTIFICATION MEASUREMENTS</td>
<td>4</td>
</tr>
<tr>
<td>4.1 CERTIFICATION PROCEDURE</td>
<td>4</td>
</tr>
<tr>
<td>4.2 QUALITY ASSURANCE</td>
<td>4</td>
</tr>
<tr>
<td>4.3 COMPONENTS OF CRM UNCERTAINTY</td>
<td>4</td>
</tr>
<tr>
<td>5 RESULTS</td>
<td>5</td>
</tr>
<tr>
<td>5.1 CERTIFICATION COLLABORATIVE STUDY</td>
<td>5</td>
</tr>
<tr>
<td>5.2 STABILITY</td>
<td>6</td>
</tr>
<tr>
<td>5.3 HOMOGENEITY</td>
<td>6</td>
</tr>
<tr>
<td>5.4 ESTIMATION OF THE COMBINED UNCERTAINTY</td>
<td>6</td>
</tr>
<tr>
<td>6 CERTIFIED VALUE</td>
<td>7</td>
</tr>
<tr>
<td>7 USE OF THE CRM</td>
<td>8</td>
</tr>
<tr>
<td>7.1 DISPATCH AND INSTRUCTIONS FOR USE</td>
<td>8</td>
</tr>
<tr>
<td>7.2 RECONSTITUTION OF THE MATERIAL</td>
<td>8</td>
</tr>
<tr>
<td>7.3 INTENDED USE</td>
<td>9</td>
</tr>
<tr>
<td>8 REFERENCES</td>
<td>9</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

Lactate dehydrogenase is an ubiquitous oxidoreductase that catalyses the reversible conversion of lactate to pyruvate and thereby plays an important role both in aerobic glycolysis as well as in lactate oxidation. Lactate dehydrogenase is a tetrameric protein of a relative molecular mass of 145000 [1, 2]. The enzyme is normally present in human plasma as five isoenzymes resulting from the association of two polypeptides A (or M: Muscel) and B (or H: Heart). These isoenzymes are called on the basis of their electrophoretic anodic mobility: LD1 (B4), LD2 (B3A1), LD3 (B2A2), LD4 (BA3), LD5 (A4) where LD1 presents the greatest mobility toward the anode. The isoenzymes profiles are different in the various tissues [1, 3] where LD1 and LD2 are present predominantly in heart muscle, kidney and erythrocytes, LD4 and 5 in liver and skeletal muscle and LD2, 3 and 4 are responsible for the LD activity in many tissues such as leukocytes, endocrine glands, spleen, lymph nodes, lung and non pregnant uterus. The subcellular location of lactate dehydrogenase has been accepted to be primarily the cytosol, and much of the work regarding this subcellular location has been done with LD1 from human cardiac tissue [4].

Measurement of LD1 and LD2 in serum are frequently used in the clinical laboratory for monitoring myocardial infarction [5, 6] as a complement to total creatine kinase, CK-MB, aspartate amino transferase (ASAT), myoglobin and troponin [7, 8] measurements. Also higher levels of the total LD concentration have been described in sera of patients with haematological and liver diseases [9], but also different forms of leukaemia [10, 11].

The Community Bureau of Reference (BCR) of the European Commission has in the past co-ordinated the preparation and certification of enzyme reference materials that are of significance when validating measurements in clinical chemistry. Amongst these lyophilised materials are the gamma-glutamyltransferase (GGT) (CRM 319), alkaline phosphatase (CRM 371), creatine kinase BB (CRM 299), alanine aminotransferase (ALAT) (CRM 426), prostatic acid phosphatase (CRM 410) and lactate dehydrogenase isoenzyme 1 (LD1) (CRM 404), creatine kinase MB (CK-MB) (CRM 608) and pancreatic α-amylase (CRM 476) [12-19]. With the exception of α-amylase, these enzymes have been certified at 30 °C. The continuous development of new methods by manufacturers based on different substrates, temperature, pH, cofactors etc. causes difficulties related to interpretation of interlaboratory results. The effort of certifying BCR reference materials using international accepted reference methods has to a certain extent improved comparability of the results in the area of enzyme measurements, however, still many tasks have to be performed before a global standardisation can be reached [20]. Since most laboratories today endorse a reaction temperature at 37 °C, the first task was to develop new reference systems for the mentioned enzymes. On the 1998 IFCC General Conference in Seville, the working group for calibrators in clinical enzymology (WG-CCE) and the manufactures of diagnostic enzyme kits agreed to change the measurement temperature for the IFCC reference procedures from 30 °C to 37 °C. This has resulted in a co-operation project between IRMM, IFCC and the diagnostic companies with the objective to prepare new standard operating procedures (SOPs) and recertify in addition to LD1 also CK-MB, GGT and ALAT at 37 °C [21-23]. Recent regulatory initiatives [24, 25] will have a major impact on the design of test systems, including those for enzyme measurements. European legislation foresees that manufacturers include in the technical documentation of their test systems “adequate performance evaluation data showing the performances claimed by the manufacturer and supported by a reference measurement system with information on the reference methods, the reference
materials, the known reference values, the accuracy and measurement units used’’ [24].
Also, EN 45001 (to be substituted by the ISO/DIS 17025), used in several countries for
accreditation of testing laboratories, requires demonstration of traceability of results: “the
overall programme of calibration of equipment shall be designed and operated so as to
ensure that wherever applicable measurements made in the testing laboratory are traceable
to national and international standards of measurement where available’’ [25].

2 PARTICIPATING LABORATORIES

Purification of the enzyme
Laboratoire de Biochimie Pédiatrique, Hôpital Debrousse, Lyon (FR)

Lyophilisation and ampouling
National Institute for Biological Standards and Control, Potters Bar (UK)

Homogeneity and stability studies
Laboratoire de Biochimie Pédiatrique, Hôpital Debrousse, Lyon (FR)
National Institute for Biological Standards and Control, Potters Bar (UK)

Certification exercise
Ceriotti F, Laboratorio Centrale, Instituto Scientifico San Raffaele (IT)
Ferard G, Centre Traumatologie et Orthopedie, Illkirch Grafenstaden (FR)
Franck F.H, Department of Clinical Chemistry, Ziekenhuis Leyenburg, Den Haag (NL)
Gella J, Biosystems S.A., Barcelona (ES)
Hölzel W, Roche Diagnostics, Tutzing (DE)
Jørgensen P, Department of Clinical Chemistry, Odense University Hospital, Odense (DK)
Kanno T, Clinical Laboratories, Hamasatu University Hospital, Hamasatu City (JP)
Kessner A, Beckman-Coulter, Inc., Brea, California (US)
Panteghini M, Laboratory of Clinical Chemistry, Spedali Civili, Brescia (IT)
Schumann G, Institut für Klinische Chemie, Medizinische Hochschule, Hannover (DE)
Vialle A, Laboratoire d’Enzymologie, Hospital Debrousse, Lyon Cedex, (FR)
Weidemann G, Institut für Klinische Chemie und Labormedizin, Klinikum der Stadt
Nürnberg, Nürnberg (DE)
Yoshida K, Health Care Business Administration, ASAHI Chemical Industry Co., Tokyo
(JP)

Evaluation
Institute for Reference Material and Measurements (IRMM), Geel (BE)

Project management
The IRMM/IFCC-453 was produced and certified in a close co-operation between the above
mentioned institutions on behalf of IFCC in the frame of a project on standardisation of
enzyme measurement initiated by WG-CCE. The work of certification was co-ordinated by
the German Society of Clinical Chemistry (Deutsche Gesellschaft für klinische Chemie,
DGKC), Reference Institute of Bioanalysis (DE) and the IRMM (BE).

3 PREPARATION OF THE MATERIAL
The preparation of the lyophilised material is described in detail in the previous certification
report [17]. However, a short summary of the main steps involved in the purification and
characterisation of the enzyme that were performed in 1989, is given below.
3.1 Choice of the source of the enzyme

The preparation of a suitable enzyme reference material involves the selection of an appropriate source of the enzyme. Important factors that have to be considered when selecting the source of the reference material are material stability, kinetic properties, catalytic concentration, interfering enzymes, commutability and matrix effects. After carefully checking these factors, LD1 was extensively purified from human erythrocytes and characterised.

3.2 Extraction and purification of the enzyme

The erythrocytes were washed three times with NaCl 154 mmol/L containing EDTA Na, 2.7 mmol/L and centrifuged before the red blood cells were lysed by addition of distilled water containing sodium azide. The final concentration of the latter was 3 mmol/L. The lysate was then heated, cooled and centrifuged. Thereafter, different steps of precipitation and gel/affinity chromatography clean-up were performed in order to purify the enzyme. After affinity chromatography, the specific catalytic activity of the protein was determined to 300 U/mg.

3.3 Choice and addition of the matrix followed by lyophilisation

The susceptibility of enzymes to denaturation with loss of catalytic activity is a serious problem in the development of enzyme reference materials. A carefully selected matrix is generally needed to give ease of handling, adequate physical bulk to the lyophilised material and to maintain the stability of the preparation. Purified LD1 was stabilised by dilution in a matrix with the following composition: Triethanolamine hydrochloride buffer 50 mmol/L, pH 6.2, EDTA 0.5 mmol/L, human albumin 30 g/L and saccharose 10 g/L. The matrix was added to give a final volume of 1.020 l with a LD1 catalytic concentration of 1090 U/L (measured at 30 °C). The human albumin was shown to be free from LD1 and from detectable contaminating enzyme activities. The material was thereafter lyophilised in 1 ml portions and checked for inhomogeneity and stability of its catalytic concentration.

3.4 Production control

- Viral markers: the material has been tested for hepatitis B antigen, anti-HIV 1/2 antibodies and hepatitis C virus ribonucocies acidby polymerase chain reaction and found negative.

- Contaminating enzymes: the purified preparation was tested for the presence of possible contaminating enzymes. Catalytic activities of ASAT, ALAT, malate dehydrogenase, adenylate kinase, pyruvate kinase, sorbitol dehydrogenase, glutamate dehydrogenase and aldolase were not detected. The presence of contaminating isoenzymes was checked by electrophoresis, but the result was negative.

- Protein purity: the purity of the LD1 preparation was checked by PAGE under denaturing conditions and the electrophoresis pattern showed only one polypeptide band of relative molecular mass of 35 000 corresponding to the LD1 monomer.

- Water content: the moisture mass fraction of 10 ampoules was measured by an automated iodometric method. The results showed that the moisture content was below 1%.

- Testing of filling procedure: the mass of 54 ampoules of the solution taken at intervals throughout the filling procedure had a mean value of 1.0028 g with a range 1.0018 g to
1.0038 g. There was no evidence of any trend in the variation of mass through the filling procedure. The average dry weight of the of the content was found to be 0.0380 g.

4 CERTIFICATION MEASUREMENTS

4.1 Certification procedure

Experienced laboratories were invited to participate in the certification of the catalytic concentration of LD1 in lyophilised material. The certification was based on the agreement between the results obtained in the different laboratories, all of them using the same SOP to measure the catalytic concentration at 37 °C [26]. In advance to the certification campaign, the laboratories volunteered to perform a training exercise by analysing 5 different commercial enzyme solutions. By this exercise it was possible to ensure the validity of the standard operating procedure for measuring enzyme as well as the performance of the individual laboratories. The new IFCC recommended method for LD1 is optimised with regard to both kinetic reactions and technical aspects of the measurements. The SOP contains information about instructions for calibration of pipettes, specifications and instructions for the calibration and verification of apparatus used in measurement, specifications for reagents and procedure for the preparation of solutions, specifications for the measurement conditions for the procedure and calculation of results. The reaction, where LD catalyses the reversible reaction of lactate to pyruvate is monitored by following the reduction of NAD* at 339 nm. The reaction principle is as follows:

\[ L(+)\text{-Lactate} + NAD^+ \xrightarrow{LD} \text{Pyruvate} + NADH + H^+ \]

Each participant received seven vials of the lyophilised LD1 material together with the samples to be used for internal quality control, the SOP, the reconstitution procedure and data sheets for reporting results and the requested information. Three vials were reconstituted on each of two days, and one measurement of catalytic concentration of LD1 on each vial was performed on the day of reconstitution. The remaining vial was a spare sample. The two standard solutions received were used to verify the photometric performance. Six vials containing a commercial calibrator were used in all laboratories for internal quality assessment. All vials were shipped on dry ice to the participants on March 1999 and were delivered in less than 2 days.

4.2 Quality assurance

Traceability of gravimetry, volumetry and thermometry was demonstrated and documented. Traceability of spectrophotometry was assured through potassium dichromate solutions certified by the German Metrology Institute (Physikalisch-Technische Bundesanstalt).

4.3 Components of CRM uncertainty

Uncertainty of the CRM was estimated according to the Guide to the Expression of Uncertainty in Measurement (GUM) [27]. For this, estimates of the uncertainty of the characterisation \( u_{char} \), homogeneity \( u_{rhom} \) and stability \( u_{stab} \) are required as described by Pauwels et al. [29]. Uncertainty of the characterisation was derived from the certification measurements. An estimation for the inhomogeneity was derived from the homogeneity study of the original material. For assessment of stability, data from the original stability study and results from stability monitoring afterwards were available. The method is described in greater detail elsewhere [28].
5 RESULTS

5.1 Certification collaborative study

The individual results together with laboratory averages of 6 measurements, standard deviations and relative standard deviations (RSDs) from 12 laboratories are given in Table 1 (results rounded to the same decimal position). The results were obtained from single measurements of each of six vials that were performed in March/April 1999. On beforehand, it had been decided that an intralaboratory RSD above 2.5 % could not be accepted for the certification measurements. All 13 laboratories that participated in the certification exercise matched this particular criterion. During the technical discussion, however, one laboratory found by using the internal quality control material, an 8% deviation of the results from the target value. The problem could not be identified, but it was decided to exclude the set of results from this laboratory prior to the statistical evaluation.

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Experimental results [U/L]</th>
<th>Mean [U/L]</th>
<th>Stddev [U/L]</th>
<th>RSD [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>lab 01</td>
<td>514.6 512.0 512.8 517.2 513.4 514.0</td>
<td>514.0</td>
<td>1.8</td>
<td>0.4</td>
</tr>
<tr>
<td>lab 03</td>
<td>489.0 491.0 481.0 505.3 506.9 490.7</td>
<td>494.0</td>
<td>10.1</td>
<td>2.0</td>
</tr>
<tr>
<td>lab 04</td>
<td>489.2 496.5 492.9 491.1 489.2 489.2</td>
<td>491.4</td>
<td>2.9</td>
<td>0.6</td>
</tr>
<tr>
<td>lab 05</td>
<td>496.3 499.5 494.8 499.2 499.5 489.7</td>
<td>496.5</td>
<td>3.9</td>
<td>0.8</td>
</tr>
<tr>
<td>lab 08</td>
<td>497.9 497.9 504.3 506.2 516.1 510.0</td>
<td>505.4</td>
<td>7.1</td>
<td>1.4</td>
</tr>
<tr>
<td>lab 09</td>
<td>494.9 493.5 492.1 490.2 487.9 487.8</td>
<td>491.1</td>
<td>2.9</td>
<td>0.6</td>
</tr>
<tr>
<td>lab 10</td>
<td>500.7 502.1 502.0 496.2 500.0 501.4</td>
<td>500.4</td>
<td>2.2</td>
<td>0.4</td>
</tr>
<tr>
<td>lab 11</td>
<td>507.2 505.6 508.0 505.3 505.0 505.9</td>
<td>506.2</td>
<td>1.2</td>
<td>0.2</td>
</tr>
<tr>
<td>lab 13</td>
<td>507.3 508.5 509.9 504.4 512.2 506.8</td>
<td>508.2</td>
<td>2.7</td>
<td>0.5</td>
</tr>
<tr>
<td>lab 14</td>
<td>494.3 496.9 491.2 491.3 489.6 507.3</td>
<td>495.1</td>
<td>6.5</td>
<td>1.3</td>
</tr>
<tr>
<td>lab 15</td>
<td>513.1 516.5 515.6 514.3 514.5 518.9</td>
<td>515.5</td>
<td>2.0</td>
<td>0.4</td>
</tr>
<tr>
<td>lab 16</td>
<td>503.1 506.4 504.9 508.2 510.0 507.9</td>
<td>506.8</td>
<td>2.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 1. Individual results accepted for the certification.

Estimation of individual uncertainties of the catalytic concentration of LD1 in the lyophilised material was done in compliance with the Guide to the Expression of the Uncertainty in Measurement (GUM) [27]. The uncertainty from the interlaboratory study is referred to as $u_{\text{char}}$ and consists of the uncertainties obtained from the between-lab reproducibility and within-lab repeatability. The standard error of the mean ($\frac{s}{\sqrt{n}}$) was used as the best estimation of the combined effect of these influences. The result of an analysis of variance (ANOVA) shows that the standard deviation between laboratories is the main influence for $u_{\text{char}}$ (Table 2). 12 laboratories participated in the certification exercise.

<table>
<thead>
<tr>
<th></th>
<th>mean of means</th>
<th>standard deviation between means $s$</th>
<th>number of laboratories $n$</th>
<th>standard error of the mean of the means $\frac{s}{\sqrt{n}}$</th>
<th>ANOVA $s$ within labs</th>
<th>$s$ between labs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>502.03 U/L</td>
<td>8.49 U/L</td>
<td>12</td>
<td>0.49 %</td>
<td>4.60 U/L</td>
<td>8.28 U/L</td>
</tr>
</tbody>
</table>

Table 2. Results of the statistical analysis of accepted results.
5.2 Stability

The stability data from the time of the certification and the data originated from the continued stability monitoring arranged by IRMM showed no sign of instability [28]. The material had been monitored over a period of about 5 years. Because reproducibility was not good enough to obtain a reliable estimate of the uncertainty due to the stability, additional stability data involving two units with the method used for certification were obtained. One sample had been stored at +4 °C for 30 months while another sample was stored at the normal storage temperature of −20 °C. After this time period, 18 analyses were conducted for each ampoule stored at the two temperatures.

<table>
<thead>
<tr>
<th></th>
<th>ampoule stored at +4 °C</th>
<th>ampoule stored at −20 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>average</td>
<td>514.6 U/L</td>
<td>514.4 U/L</td>
</tr>
<tr>
<td>standard deviation</td>
<td>2.77 U/L</td>
<td>2.25 U/L</td>
</tr>
<tr>
<td>RSD</td>
<td>0.54 %</td>
<td>0.44 %</td>
</tr>
</tbody>
</table>

*Table 3. Stability testing of ampoules stored at different temperatures.*

As can be seen from Table 3, there was virtually no difference between the results of these two ampoules. The ratio +4 °C/-20°C was 100.0 % with a standard uncertainty of 0.16 %. It was decided to base the estimation of the stability on this single point rather than on the less reliable original stability study. The value of 0.16 % was taken threefold to have a wide security margin for the extrapolation up to 6 years. The uncertainty of the stability ($u_{\text{st}}$) is therefore 0.48%. This would give the certificate validity until 3/2005.

5.3 Homogeneity

A new homogeneity study was performed together with the certification at 37 °C. In total 30 vials of the material were analysed in 8 replicates each. One vial contained a gelatinous droplet instead of a powder and was apparently not completely lyophilised. The data from this ampoule were excluded from the data set. The uncertainty due to inhomogeneity, $s_{bb}$, is the real between-bottle-effect and was used as the best estimate for the uncertainty due to inhomogeneity. This bare between-bottle-effect was derived from the ANOVA shown in Table 4. $s_{bb}$ was calculated as 0.11 % (0.59 U/L). This uncertainty was larger then the minimum between bottle uncertainty of 0.05 %, which was estimated according to Linsinger et al. [30]

<table>
<thead>
<tr>
<th></th>
<th>527.14 U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSB:</td>
<td>7.24 U²/L²</td>
</tr>
<tr>
<td>MSW:</td>
<td>4.41 U²/L²</td>
</tr>
<tr>
<td>$s_{wb}$:</td>
<td>0.40 %</td>
</tr>
<tr>
<td>$s_{bb}$:</td>
<td>0.11 %</td>
</tr>
</tbody>
</table>

*Table 4. Results of the ANOVA over the homogeneity study with MSB and MSW as the mean squares between and within groups of the ANOVA, respectively. $s_{wb}$ and $s_{bb}$ are the within bottle and between bottle variation, respectively.*

5.4 Estimation of the combined uncertainty

The uncertainty of the CRM can be estimated by summation of the contributions of characterisation, homogeneity and stability [29]. The individual uncertainty components for characterisation, inhomogeneity and instability described in the equation below are added,
and multiplied by a coverage factor of 2 to give a combined expanded uncertainty. As uncertainties have the format of standard deviations, addition is done quadratically.

\[ U_{\text{CRM}} = k \cdot \sqrt{u_{\text{char}}^2 + u_{\text{bb}}^2 + u_{\text{lu}}^2 + u_{\text{srt}}^2} \]  

- \( U_{\text{CRM}} \) : expanded uncertainty of the CRM
- \( k \) : coverage factor
- \( u_{\text{char}} \) : uncertainty of the certified property of the batch
- \( u_{\text{bb}} \) : between-bottle inhomogeneity
- \( u_{\text{lu}} \) : uncertainty of long-term stability (storage)
- \( u_{\text{srt}} \) : uncertainty of stability during transport

Transport conditions will be chosen that make the uncertainty of stability during transport negligible. \( u_{\text{char}} \) of the batch amounts to 0.49 %. The \( u_{\text{bb}} \) was estimated to be 0.11 % and the calculated \( u_{\text{lu}} \) was 0.48 %. Thus, expanded uncertainty of the CRM \( (U_{\text{CRM}}) \) amounts to

\[ U_{\text{CRM}} = 2 \cdot \sqrt{0.49^2 + 0.11^2 + 0.48^2} = 1.39 \% \approx 6.98 \text{ U/L} \]

6 CERTIFIED VALUE

Certified value and the corresponding expanded uncertainty are given below

Catalytic concentration of LD1 at 37 °C as determined by the 30 °C IFCC reference method:

\[ 502 \pm 7 \text{ U/L} \]

or

\[ 8.37 \pm 0.12 \text{ µkat/L} \]

The catalytic activity in µkat/L was calculated by multiplication of the results in U/L by 0.01667. The certified value is valid until 3/2005. The results of the laboratory averages and the certified value with its expanded uncertainty calculated according to the GUM are given in Figure 1. The figure was obtained by calculating the expanded uncertainties for the laboratories using the between laboratory standard deviation \( (s_{\text{betw}}) \) of the certification exercise from the ANOVA as the best estimate of laboratory bias. Thus, laboratory uncertainty was calculated using \( (2 \cdot \sqrt{s_{\text{betw}}^2 + s_{\text{lab}}^2}) \), with slab being the standard deviation of the individual laboratories for the 6 measurements performed.
In contrast to previously certified materials for which the 95% confidence interval of the mean of laboratory means was used as uncertainty, an expanded combined uncertainty according to the GUM was calculated this time. This included also influences of homogeneity and stability. The certified uncertainty of the present certification is therefore not readily comparable with the certified uncertainty of the certification at 30 °C.

7 USE OF THE CRM

7.1 Dispatch and instructions for use
Dispatch to the customer will be done under cooled conditions. Upon receipt by the customer, it is advisable to keep the material at -20 °C for long-term storage. Upon arrival, the material can be stored at 2-8 °C for not longer than 3 months until used.

7.2 Reconstitution of the material
1. Take the ampoule out of the freezer and allow reaching room temperature.
2. Tap the vertically positioned ampoule gently to ensure that the lyophilised material is at the bottom of the ampoule.
3. Score the ampoule at the constriction with a sharp file and open, by applying a red hot glass rod to the score for about 1 s, while holding the ampoule almost horizontally to prevent glass from entering the ampoule.
4. Weigh the ampoule with its contents to the nearest 0.1 mg.
5. Reconstitute by slow addition to the sides of the ampoule of (1.00 ± 0.01) ml distilled water (20-22 °C) with calibrated volumetric equipment. Note the temperature.
6. Seal the ampoule with an inert plastic film, invert several times and mix contents by gentle swirling. Allow to stand at +4 °C temperature for 20 min. After this time, swirl ampoule again every 10 minutes during 1 hour at +4 °C and keep cold (0 °C - 4 °C) until use.
7. Calculate the volume of water at 20 °C from the weight of the volume taking into account the temperature-dependent density.

8. The catalytic concentration of lactate dehydrogenase must be measured within 4 hours following the reconstitution.

7.3 Intended use

The material is intended to provide, when reconstituted, a solution with a known catalytic concentration of human LD1 that can be used for intra-laboratory quality control of the measurement procedure and to verify comparability of results from laboratories using this measurement procedure. The certified reference material can also be used for evaluation of in vitro test systems for LD1 measurements by method comparison with the 37 °C IFCC reference measurement procedure. The material can also be used for the calibration of lower order procedures for measuring LD1 activities provided they have the same or similar analytical specificities as the reference measurement procedure used for the certification. Commutability of the results from the undiluted material to dilutions has to be checked thoroughly before using the material at several concentration levels for calibration.

8 REFERENCES


12. Schiele F, Siest G, Moss DW, Colinet E. The certification of the catalytic concentration of gamma-glutamyltransferase in a reconstituted lyophilized material (CRM 319), 1986; CEC Report EUR 10628 EN.


15. Schiele F, Siest G, Colinet E, Profilis C. The certification of the catalytic concentration of alanine aminotransferase from pig heart in a reconstituted lyophilized material (CRM 426), 1992; CEC Report EUR 14475 EN.

16. Moss DW, Francis JM, Colinet E, Profilis C. The certification of the catalytic concentration of human prostatic acid phosphatase in a reconstituted lyophilized material (CRM 410), 1992; CEC Report EUR 14476 EN.


25. The joint European Standards Institution. EN 45001. General criteria for the operation of testing laboratories, 1989; Brussels: CEN/CENELEC.


