Multienzyme control serum (Seraclear-HE) containing human enzymes from established cell lines and other sources. 3: Evaluation as candidate working enzyme reference material for γ-glutamyltransferase

Akira Eto,1 Tomoko Oishi,1 Naomi I. Nakano,2* and Yasushi Chikaura2

Seraclear-HE, containing γ-glutamyltransferase (GGT, EC 2.3.2.2) derived from the cell culture of a human macrophage cell line, was evaluated as a candidate enzyme reference material (ERM) to calibrate routine methods in terms of a Reference Method for GGT. We have compared this preparation with commercially available materials, including Certified Reference Material 319, at 30 °C and 37 °C with respect to kinetic properties and with respect to commutability between the Reference Method and each of 15 analytical procedures involving five structurally different donor substrates. GGTs of human origin are far more commutable with reagents of varied types than are GGTs derived from animals. Calibration of 44 patients' sera with Seraclear-HE decreased average intermethod variation from 20% to ~4%, whereas GGTs of animal origin showed intermethod variations of ~30% with no benefit from calibration. Seraclear-HE is promising as a secondary or working ERM to be used as an intermethod calibrator.

INDEXING TERMS: enzyme activity • commutability • intermethod calibrator • enzyme standardization • human macrophage cell line

In Part 1 of this series of articles, we reported on the preparation of a new multienzyme control serum, Seraclear-HE, and its properties [1]. The control serum was developed to be a candidate enzyme reference material (ERM) that could function as an intermethod calibrator, as well as a control material for enzyme assays in the field of wet reagent chemistry.3 In Part 2 of this series [2], we demonstrated, by applying the concept of the International Clinical Enzyme Scale (ICES) originally proposed by Bowers and McComb [3, 4], that Seraclear-HE is promising as a common calibrator for the activity measurements of aspartate and alanine aminotransferases under varied methodologies and conditions.3

We have extended our effort here to evaluate the γ-glutamyltransferase (GGT, EC 2.3.2.2) present in the preparation for a similar application. The GGT derived from cell culture of a human macrophage cell line constitutes ~70% of total GGT present in the abnormal range control [Seraclear-HE (Abnormal)]. The other part of the set, the normal range control [Seraclear-HE (Normal)], is supplemented to a lesser extent with the same human-origin GGT. We have evaluated the commutability of this human-derived GGT in Seraclear-HE and in a related preparation, as well as GGTs of animal origin present in preparations such as the Certified Reference Material (CRM) 319 of the Community Bureau of Reference (BCR) of the European Communities for GGT [5–8]. We have selected as the Reference Method the method [9] recommended by the IFCC, at a reaction temperature of 37 °C instead of 30 °C. The commutability of each candidate ERM was measured between the Reference Method and each of 15 different procedures involving five structurally different types of the γ-glutamyl group donor substrates.

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1 Nonstandard abbreviations: ERM, enzyme reference material; ICES, International Clinical Enzyme Scale; CRM, Certified Reference Material; GGT, γ-glutamyltransferase; BCR, Community Bureau of Reference; BSA, bovine serum albumin.

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4 ICES originally meant unification of routine enzyme data in terms of the IFCC Reference Method at the reference temperature of ballium, 29.77 °C; in this paper, we apply the term to mean the scale unification in terms of any Reference Method.
Materials and Methods

INSTRUMENTATION
We used a Model 7050 automated chemistry analyzer (Hitachi, Tokyo, Japan) to evaluate the candidate ERMs as ICES calibrators and to measure the kinetic parameters of the candidate ERMs. To assign Reference Method values to the materials, we used a double-beam spectrophotometer (Model UV-260; Shimadzu, Kyoto, Japan), together with the temperature-control unit, as described previously [2, 10].

CHEMICALS AND CANDIDATE ERMS
Chemicals used for assigning IFCC Reference Method [9] values at 37 °C to candidate ERMs were the same as those described previously [10]. The following materials were investigated as candidate ERMs: Seraclear-HE containing GGT obtained from a macrophase cell line in partially delipidated human serum, at two concentrations, Normal and Abnormal (lot QO-04HE from Nippon Shoji, Osaka, Japan); CRM 319 (BCR) containing GGT from pig kidney; Enzyme Reference (Wako Pure Chemicals, Osaka, Japan) containing the same enzyme analyte of human origin as are in Seraclear-HE, but in a bovine serum albumin (BSA) base; Precipath E (BSA base) and calibrator for automated systems (human serum base), containing GGT from pig kidney (Boehringer Mannheim, Mannheim, Germany); and Moni-Trol II (Dade Div., Baxter Healthcare Corp., Miami, FL), containing GGT from bovine kidney in human serum base. All of these are lyophilized products. A liquid control was also studied: Ortho Liquid Reference Serum II, containing GGT from bovine kidney in human serum base (Ortho Diagnostic Systems, Tokyo, Japan). For comparison, a human serum pool was also studied as candidate material in each run.

REAGENTS
A set of 4-nitroaniline solutions were purchased from Wako Pure Chemicals.

Seven commercial reagent kits (reagents 2–8 of Table 1) were selected. Although the L-γ-glutamyl-group acceptor substrate is glycylglycine in all the selected reagents, the donor substrate can be classified into five analogs of L-γ-glutamylaminilide, as shown in Table 2. For reagents 1–5, factors were used to calculate activity. For reagent 6, a standard human serum with a known activity constituted part of the kit. For reagent 7, a GGT standard specified for the kit was used. Calibration of reagent 8 was carried out with a 2-N,N-di-n-propylamino benzoic acid solution as specified for the kit. In addition, the IFCC-recommended method was adapted for the analyzer as a routine method to give the same reagent concentrations in the final reaction mixture. The measurement procedure for this reagent at 37 °C is designated as automated (or modified) IFCC Method at 37 °C.

ANALYTICAL PROCEDURES
Determination of enzyme kinetic properties. The apparent Michaelis constant (app K_m) and pH-activity profiles of Seraclear-HE were determined on the analyzer at 30 °C and 37 °C as described previously for the transaminases [2], based on the IFCC Reference Method. For determination of app K_m, the concentration

<table>
<thead>
<tr>
<th>Table 1. Routine methods (reagents) and their characteristics.</th>
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<tr>
<td>7</td>
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<td>8</td>
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</table>

* See Table 2 for the structures.

SR, secondary reaction; BMY, Boehringer Mannheim Yamanouchi (Tokyo, Japan); WK, Wako Pure Chemicals; NS, Nippon Shoji; DA, Dia-iatron (Tokyo, Japan); NB, Nittobo Chemicals (Tokyo, Japan); IR, International Reagents (Kobe, Japan); DI, Daiichi Pure Chemicals (Tokyo, Japan); SCC, Scandinavian Society for Clinical Chemistry; TOOS, N-Methyl-N(2-hydroxy-3-sulfopropyl)-m-toluidine; and BOD, bilirubin oxidase.

<table>
<thead>
<tr>
<th>Table 2. Structures for donor substrates.</th>
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<td>Substrate</td>
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<td>-----------</td>
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<tr>
<td>A</td>
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<td>B</td>
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<tr>
<td>C</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>E</td>
</tr>
</tbody>
</table>
tration of the donor was varied from 0.1 mmol/L to 6 mmol/L, and the concentration of the acceptor was varied from 3.0 mmol/L to 150 mmol/L.

**Determination of Reference Method values.** The IFCC’s manual procedure was followed as described previously [10], except that the measurement was carried out at 37 °C instead of 30 °C for 14 patients’ sera and the candidate ERMs to establish the relation between the manual and automated IFCC procedures. For the test specimens (44 patients’ sera), the values obtained by the automated IFCC method at 37 °C calibrated with each candidate ERM were used as the Reference Method values for the commutability study as described previously [2]. These values were essentially the same as those of the automated IFCC method at 37 °C.

**Measurements of enzyme activity by eight reagents and calibration of the data with the candidate ERMs.** For these determinations, the analyzer settings were made according to the directions of the package inserts, except for the calculation factors for reagents 1–5. We determined the factor for each of these reagents at 30 °C and 37 °C using the 4-nitroaniline (for reagents 3–5) or 5-aminobenzoate (for reagents 1 and 2) solutions.

The activities of a group of patients’ sera (n ~25) were measured together with the candidate ERMs by using the eight reagents on the analyzer as described previously [2], at 37 °C and 30 °C. Two separate runs were performed, and two sera were rejected from the test specimens. One was rejected because its activity was over the linearity range in one reagent, and the other showed an abnormally high activity ratio (activity 37 °C/ activity 30 °C). In total, 44 patients’ sera (the activity up to ~1000 U/L by the automated IFCC method at 37 °C) were used as test specimens. For each temperature, the between-reagent mean and variation were calculated for each patient’s serum and then the average values for the 44 sera (mean and CV) were determined. The raw data of the reagents were calibrated with each candidate ERM in terms of the Reference Method, and the above procedure was repeated to obtain the calibrated mean and CV for each candidate ERM.

**Evaluation of the commutability of the candidate ERMs.** We evaluated the candidate ERMs in a total of 44 test specimens in two runs at both temperatures, as described in part 2 of this series of articles [2]. We studied the commutability between the Reference Method at 37 °C and each of the seven commercial reagents at 37 °C, and also between the Reference Method (at 37 °C) and each of the seven commercial reagents plus the automated IFCC method at 30 °C. Thus, a total of 15 different procedures was investigated with the test specimens.

**Results**

**MODIFICATION OF THE IFCC REFERENCE METHOD FOR THE ANALYZER**

The modified method values (γ) for the 14 patients’ sera tested at 37 °C correlated well with the manual IFCC Reference Method values at 37 °C (α) with the following regression line: 

\[ y = 0.065 + 0.999x, \quad r = 0.9996, \quad S_{yy} = 1.96 \text{ U/L} \]

The data obtained by the two procedures differed by <2% for any of the candidate ERMs.

**KINETIC PARAMETERS**

App \( K_m \) values are listed for the candidate ERMs in Table 3, together with the average values for four patients’ sera. Our values, though apparent values, compare favorably with the 0.65 mmol/L and 18 mmol/L at 30 °C for the donor and the acceptor, respectively, reported by Shaw et al. [9], based on a ping-pong bi–bi mechanism for GGT in human sera. Schiele et al. [5] reported app \( K_m \) values of 1.12 mmol/L (for the donor) and 31.0 mmol/L (for the acceptor) for GGT in CRM 319 at 30 °C. For GGT in human serum pools, the same authors reported 0.8–0.9 mmol/L for the donor and 17–18 mmol/L for the acceptor. These data indicate that the app \( K_m \) values of GGT of animal origin for the donor are generally greater than those of GGT in human sera.

<table>
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<tr>
<th>Patients’ sera/Candidate ERM</th>
<th>Donor</th>
<th>Acceptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients’ sera (n = 4), Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 °C</td>
<td>37 °C</td>
</tr>
<tr>
<td></td>
<td>30 °C</td>
<td>37 °C</td>
</tr>
<tr>
<td>SHE (N)</td>
<td>0.94</td>
<td>0.68</td>
</tr>
<tr>
<td>SHE (A)</td>
<td>0.95</td>
<td>0.92</td>
</tr>
<tr>
<td>E-REF</td>
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<td>0.86</td>
</tr>
<tr>
<td>CRM-319</td>
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<td>1.09</td>
</tr>
<tr>
<td>PRE-E</td>
<td>1.21</td>
<td>1.32</td>
</tr>
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<td>1.19</td>
</tr>
<tr>
<td>MONIT</td>
<td>2.99</td>
<td>1.52</td>
</tr>
<tr>
<td>ORTHO-LR</td>
<td>1.76</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>14.7 ± 3.3</td>
<td>23.9 ± 2.2</td>
</tr>
</tbody>
</table>

* The kinetic parameter was calculated from Lineweaver-Burk plots for the concentration ranges of 0.1 to 2.0 mmol/L for the donor and 3 to 60 mmol/L for the acceptor.

SHE, Seraclear-HE (N) Normal and (A) Abnormal; E-REF, Enzyme Reference; PRE-E, Precipath E; CALIB, calibrator for automated systems (human serum base); MONIT, Moni-Trol II; ORTHO-LR, Ortho Liquid Reference II.
The pH-activity profiles at 37 °C are shown for some materials in Fig. 1. The activities are presented as relative values, taking the activities at pH 7.7 at 37 °C as 100%. Analogous patterns were observed at 30 °C. The pH values at 37 °C were 0.1–0.2 (≈0.14) less than those at 30 °C. The optimum pH was apparently higher for the materials containing GGTs of animal origin than that of the human serum, whereas the optimum pH of GGT in Seraclear-HE was slightly less than that of GGT in human sera. Schiele et al. [5] reported optimum pH values of 7.9 and 8.3 at 30 °C for GGT in human serum pools and in CRM 319, respectively.

The kinetic properties of the GGT of human origin are generally more similar to those of human sera than to those of animal origin.

ROUTINE METHOD EVALUATION
Each routine method was evaluated against the automated IFCC Method at 37 °C in two runs with sera from 44 patients. The routine method results (y) at both temperatures correlated well with the automated IFCC results (at 37 °C, x), with the correlation coefficients (r) being >0.999 in every case (Table 4). Therefore, all procedures, including those with reagents 6–8, which measure GGT under conditions considerably different from the conditions under which GGT was measured with reagents 1–5 (Table 1), are considered to be traceable to the Reference Method and to be satisfactory for evaluating the candidate ERMs.

EFFECT OF TEMPERATURE ON ENZYME ACTIVITY
The average activity ratio (activity 37 °C/activity 30 °C) for the 44 patients' sera showed a slight reagent-dependent pattern with a lower trend for reagents 6 and 7 and a higher trend for reagent 8 (Fig. 2). Seraclear-HE containing GGT of human origin followed a pattern similar to the pattern followed generally by the patients' sera, whereas candidate ERMs of animal origin deviated from the pattern of patients' sera in reagents 5–8. In particular, reagent 6 gave an anomalous activity ratio of less than unity for candidate ERMs containing GGT from pig kidney (CRM 319 and Precipath E).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>t, °C</th>
<th>A</th>
<th>B</th>
<th>r</th>
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<tr>
<td></td>
<td>37</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>-1.76</td>
<td>0.63</td>
<td>0.9996</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>3</td>
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<td>0.59</td>
<td>0.9999</td>
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<td></td>
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<td>-2.90</td>
<td>0.78</td>
<td>0.9999</td>
</tr>
<tr>
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<td>0.47</td>
<td>0.9997</td>
</tr>
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<td>-1.43</td>
<td>0.61</td>
<td>0.9995</td>
</tr>
<tr>
<td>6</td>
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<td>0.48</td>
<td>0.9998</td>
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<td>37</td>
<td>0.98</td>
<td>0.66</td>
<td>0.9993</td>
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</table>

* y = a + bx, x = the automated IFCC method value at 37 °C.

EVALUATION OF CANDIDATE ERMS AS ICES CALIBRATORS
The average intermethod variation (CV, %) for all reagents (Table 5, n = 8) of raw data for the 44 patients' sera was ≈20%, irrespective of the temperature at which the measurements were carried out. With Seraclear-HE as the common calibrator, the corresponding CV was reduced to ≈4%, which was higher than when the human serum pool was used as a calibrator (≈2.5%). Unlike the results of a similar investigation on the prototype [11], Enzyme Reference showed less satisfactory results, at both temperatures, for all eight reagents. For GGTs of animal origin, including CRM 319, the CVs were all ≈30% with no reduction after calibration (Table 5). When the routine reagents were restricted to those five with the 4-nitroanilide or 3-carboxy-4-nitroanilide analog as substrate (Table 5, n = 5), remarkable improvement was observed for candidate ERMs containing GGT from pig kidney. Those containing GGT of human origin

![Fig. 1. Profiles of pH-activity for GGT in various candidate materials at 37 °C.](image)

![Fig. 2. Comparison of activity ratios (37 °C/30 °C) of candidate ERMs with those of patients' sera in each of eight reagents.](image)
Table 5. Effect of calibration with candidate ERMs on the unification of data from routine methods.

<table>
<thead>
<tr>
<th>Candidate ERM</th>
<th>t, °C</th>
<th>RMV* 37 °C</th>
<th>Mean</th>
<th>CV, %</th>
<th>Mean</th>
<th>CV %</th>
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<td>172</td>
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<td>177</td>
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<td>262</td>
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</table>

*a RMV, Reference Method value.
b For all reagents (n = 8), and for reagents in which the substrate is either 4-nitroanilide or 3-carboxy-4-nitroanilide analog (n = 5).

For abbreviations, see Table 3.

showed some improvement as well for the restricted number of methods, particularly with Enzyme Reference. Table 5 also indicates that the materials with lower CV values give means close to those of the human serum pool; thus, both mean and CV together indicate an overall effect of calibration for a candidate ERM.

The results of evaluation of the commutability are presented in Fig. 3 as the average percent deviation of the calibrated values from those of the Reference Method values of the 44 test specimens. The results demonstrate in which procedures each candidate material is compatible and thus usable as the calibrator. At both temperatures of measurement, the candidate ERMs containing GGT's of animal origin, including CRM 319, were generally not commutable for as many reagents as those containing GGT's of human origin. In the animal-derived group, temperature-dependent commutability was most noticeable in

Fig. 3. Commutability of GGT in each candidate ERM shown as the average deviation (%) of the calibrated data of each routine method from the results of the Reference Method (IFCC-recommended method at 37 °C) for 44 test specimens.

■, Reagent 1; ■, reagent 2; □, reagent 3; □, reagent 4; ■, reagent 5; ■, reagent 6; □, reagent 7; □, reagent 8. The data obtained at 37 °C of the candidate ERMs containing GGT of human origin are shown enlarged at the top. HSP, human serum pool; for other abbreviations, see Table 3.
reagent 6. This was expected for this reagent since GGTs of animal origin, and in particular that from pig kidney, showed anomalous temperature-dependent change in activity (Fig. 2).

Among the three preparations containing the cell-cultured GGT of human origin, Seraclear-HE (Abnormal) gave the best pattern, its greatest deviation being for reagent 8 (−8.9% at 30 °C and −6.8% at 37 °C). In all the other methods, with the deviation being less than ±4%, this material may be considered commutable with GGT in serum. Enzyme Reference contains 100% cell-line-derived GGT in a defined matrix (3% BSA as the base matrix), whereas Seraclear-HE contains a mixture of the same cell-cultured GGT and endogenous serum GGT (−7.3, in Abnormal). Enzyme Reference was not compatible with reagent 7, whereas Seraclear-HE was. With reagent 7, Enzyme Reference gave a nonlinear absorbance change with time in the reaction monitor. This is most likely responsible for its poor commutability for this reagent.

Discussion

Development of ERMs has been most active in European communities [12–14]. However, many of those now available as CRMs are prepared with enzymes of animal origin. The first CRM made commercially available by BCR was CRM 319 for GGT. The average percent deviation of this material was reported to be no more than 4.8% [5] between the IFCC Reference Method and each of the recommended methods of the Scandinavian Society for Clinical Chemistry at 30 °C and 37 °C and of the French Society at 30 °C. In both of these recommended methods, the donor substrate is the 4-nitroanilide. Recently, from a study involving two laboratories, less satisfactory results were reported for CRM 319 for a kit in which the 4-nitroanilide was used as the donor substrate [15].

In our evaluation of CRM 319, commutabilities for reagents with either the 4-nitroanilide or 3-carboxy-4-nitroanilide as a donor substrate were generally much better at 37 °C than reagents with the other three donor substrates. Both Precipath E and Calibrator, also containing GGT from pig kidney, showed commutability patterns closely related to the pattern of CRM 319 (Fig. 3). Moni-Trol and Ortho Liquid Reference, which contain GGT from bovine kidney, showed almost identical patterns in commutability at both temperatures, but their patterns are different from those of preparations containing GGT from either pig kidney or human sources.

These observations indicate that the commutability of GGT in these preparations depends largely upon the species rather than upon the organ of origin, with other factors such as the matrix contributing to a lesser extent. In this connection, Tsukada et al. [16] reported that the reactivity of GGT to different donor substrates varied greatly, depending upon the mammalian species the GGT derived from, but that the reactivity of GGT was not so dependent upon the organ of origin within a species. Thus, using enzymes of human origin may be an important factor, and GGT derived from the human macrophage is more likely to be commutable with GGT in human serum in a wider range of reagents than is GGT of animal origin, although still not equivalent to human sera. These differences among GGTs of different origin are reflected in differences in kinetic properties such as app kcat, optimum pH, and the temperature effect on activity determined under the conditions of the IFCC Reference Method.

Because human origin is apparently an important feature of GGT ERMs, human hepatoma cell lines such as Hep G2 and PLC/PRF/5 have been investigated as a source for the preparation of GGT [17]. Also, human recombinant enzymes are considered a potential source of ERMs for enzymes of clinical importance such as GGT [18]. A stable transgenic V79 Chinese hamster cell line expressing human Hep G2 GGT [19] is also a source of GGT for secondary ERMs, which require commutability in many routine methods.

Although CRM 319 is unsuitable as a secondary ERM for calibration of routine methods in terms of a Reference Method in Japan, its usefulness as a primary ERM is not jeopardized as long as its use is restricted to the IFCC Reference Method and the methods closely related to it. To transfer a Reference Method value to a number of routine methods, the commutability of the ERM preparation must be verified for each of the methods, even if the origin of the enzyme is human, since interferences (and matrix effects) may intervene, as observed with Enzyme Reference for reagent 7 in the present study.

In summary, Seraclear-HE is more suitable than commercial preparations containing GGT of animal origin for unification of interlaboratory GGT data through the ICES approach [2]. Since the Japan Society of Clinical Chemistry has recently recommended a Reference Method [20] that is based upon the IFCC Reference Method, it is hoped that the society will initiate a reference system for activity measurement of GGT.

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References


