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IFCC Primary Reference Procedures for the Measurement of Catalytic Activity Concentrations of Enzymes at 37 °C

International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)[1)2)]

Scientific Division
Committee on Reference Systems for Enzymes (C-RSE)[3)]

Part 6. Reference Procedure for the Measurement of Catalytic Concentration of γ-Glutamyltransferase

[(γ-Glutamyl)-Peptide: Amino Acid γ-Glutamyltransferase (GGT), EC 2.3.2.2]

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This paper is the sixth in a series dealing with reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 °C and the certification of reference preparations. Other parts deal with:

Part 1. The Concept of Reference Procedures for the Measurement of Catalytic Activity Concentrations of Enzymes; Part 2. Reference Procedure for the Measurement of Catalytic Concentration of Creatine Kinase; Part 3. Reference Procedure for the Measurement of Catalytic Concentration of Lactate Dehydrogenase; Part 4. Reference Procedure for the Measurement of Catalytic Concentration of Alanine Aminotransferase; Part 5. Reference Procedure for the Measurement of

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Catalytic Concentration of Aspartate Aminotransferase; Part 7. Certification of Four Reference Materials for the Determination of Enzymatic Activity of γ-Glutamyltransferase, Lactate Dehydrogenase, Alanine Aminotransferase and Creatine Kinase at 37 °C

A document describing the determination of preliminary upper reference limits is also in preparation. The procedure described here is deduced from the previously described 30 °C IFCC reference method (1). Differences are tabulated and commented on in Appendix 1. Clin Chem Lab Med 2002; 40(7):734–738

Key words: IFCC reference procedure; γ-Glutamyltransferase; Preliminary upper reference limit.

Abbreviation: GGT, γ-Glutamyltransferase.

Reaction Principle

L-γ-Glutamyl-3-carboxy-4-nitroanilide + Glycylglycine –GGT→
5-Amino-2-nitrobenzoate + L-γ-Glutamyl-glycylglycine
An autotransfer reaction contributes with about 1% to the total substrate rate:
L-γ-Glutamyl-3-carboxy-4-nitroanilide + L-γ-Glutamyl-3-carboxy-4-nitroanilide –GGT→
5-Amino-2-nitrobenzoate + L-γ-Glutamyl-γ-glutamyl -3-carboxy-4-nitroanilide

Specimens

Calibration materials, control specimens and human sera.

Measurement Conditions

Concentrations in the final reaction mixture and the measurement conditions are listed in Tables 1 and 2.

Table 1 Concentrations in the final complete reaction mixture for the measurement of GGT.

Glycylglycine	150 mmol/l
pH (37 °C)	7.70±0.05*
L-γ-Glutamyl-3-carboxy-4-nitroanilide	6 mmol/l
Volume fraction of sample	0.0909 (1:11)

*expanded (k=2) combined uncertainty

Table 2 Conditions for the measurement of GGT.

Temperature	37.0 °C±0.1 °C*
Wave length	410 nm±1 nm
Band width	≤2 nm
Light path	10.00 mm±0.01 mm
Incubation time	180 s
Delay time	60 s
Measurement interval	180 s
Readings (measurement points)	≥6

*expanded (k=2) combined uncertainty

Reagents

1. *N*-Glycylglycine, free base ($C_4H_8N_2O_3$), M_r=132.1
2. L-γ-Glutamyl-3-carboxy-4-nitroanilide, monoammonium salt, monohydrate ($C_{12}H_{12}N_3O_7 \cdot NH_4 \cdot H_2O$), M_r=346.3
3. Sodium hydroxide solution (NaOH), M_r=40.00, 2 mol/l
4. Sodium chloride (NaCl), M_r=58.44

Reagents of the highest purity must be used. If a chemical is suspected of containing impurities affecting the catalytic activity of the analyte, further investigations must be performed, *e.g.* comparisons with products from different manufacturers and different lots. It is recommended to use reagents which have already been tested and approved in comparisons.
Note: According to the IFCC reference method (for 30 °C), the contamination of *N*-glycylglycine by glycine must be <0.001 (mass fraction). The contamination of L-γ-glutamyl-3-carboxy-4-nitroanilide by L-γ-glutamyl-3-carboxy-4-nitroanilide must be <0.005 (mass fraction), and the contamination of L-γ-glutamyl-3-carboxy-4-nitroanilide by 5-amino-2-nitrobenzoic acid must be <0.001 (mass fraction). If the supplier of *N*-glycylglycine and L-γ-glutamyl-3-carboxy-4-nitroanilide cannot provide respective information, the reagents have to be tested chromatographically according to the description for the reference method (IFCC, 30 °C).

Charts for the Adjustment and the Control of the pH Values (Procedure for the Adjustment of pH Values at Temperatures Diverging from 37 °C)

Both the thermometer and the pH electrode are suspended in the mixed solution simultaneously. The stirred solution is then titrated to the pH value listed in the chart for the currently measured temperature. The speed of agitation should be the same during the calibration, the control and the adjustment of the pH value. The pH electrode should be positioned in the centre of the stirred solution.

The fact that the temperature can change during the titration must be taken into account. For this reason, the temperature in the proximity of the target value should be controlled again and the target pH value corrected according to Table 3, if necessary. The same applies to the adjustment of the temperature compensation of the pH meter.

Preparation of Solutions

The given mass of the compounds for the preparation of solutions refers to 100% content. If the content of the reagent chemical employed is less (*e.g.* yz %), the amount equivalent to the given mass is calculated by the use of a factor: $F_{content}$=100/yz.

Highly purified water with a quality comparable to bi-distilled water (conductivity <2 µS/cm, pH 6–7, sili-

Table 3 Dependence of the pH value of the reaction solution upon temperature.

Temperature (°C)	pH	Temperature (°C)	pH	Temperature (°C)	pH
15.00	8.286	23.50	8.041	32.00	7.820
15.25	8.278	23.75	8.034	32.25	7.813
15.50	8.271	24.00	8.027	32.50	7.807
15.75	8.263	24.25	8.021	32.75	7.801
16.00	8.256	24.50	8.014	33.00	7.795
16.25	8.248	24.75	8.007	33.25	7.789
16.50	8.241	25.00	8.000	33.50	7.783
16.75	8.234	25.25	7.993	33.75	7.777
17.00	8.226	25.50	7.987	34.00	7.771
17.25	8.219	25.75	7.980	34.25	7.765
17.50	8.211	26.00	7.973	34.50	7.759
17.75	8.204	26.25	7.967	34.75	7.753
18.00	8.197	26.50	7.960	35.00	7.747
18.25	8.190	26.75	7.954	35.25	7.741
18.50	8.182	27.00	7.947	35.50	7.735
18.75	8.175	27.25	7.940	35.75	7.729
19.00	8.168	27.50	7.934	36.00	7.724
19.25	8.161	27.75	7.927	36.25	7.718
19.50	8.153	28.00	7.921	36.50	7.712
19.75	8.146	28.25	7.914	36.75	7.706
20.00	8.139	28.50	7.908	37.00	7.700
20.25	8.132	28.75	7.901	37.25	7.695
20.50	8.125	29.00	7.895	37.50	7.689
20.75	8.118	29.25	7.889	37.75	7.683
21.00	8.111	29.50	7.882	38.00	7.678
21.25	8.104	29.75	7.876	38.25	7.672
21.50	8.097	30.00	7.870	38.50	7.666
21.75	8.090	30.25	7.863	38.75	7.661
22.00	8.083	30.50	7.857	39.00	7.655
22.25	8.076	30.75	7.851	39.25	7.649
22.50	8.069	31.00	7.844	39.50	7.644
22.75	8.062	31.25	7.838	39.75	7.638
23.00	8.055	31.50	7.832	40.00	7.633
23.25	8.048	31.75	7.826		

cate <0.1 mg/l) shall be used for the preparation of the reagent solutions. The expanded (k=2) combined uncertainty (normally distributed) of each weighing procedure (including the uncertainty of the purity of the substance) shall be ≤1.5%.

Reaction solution

2.73 g (206.3 mmol/l) Glycylglycine, free base
- Dissolve in about 80 ml water.
- Adjust pH (37 °C) 7.7 with 2 mol/l sodium hydroxide solution.
- Transfer to a 100 ml volumetric flask.
- Equilibrate the volumetric flask and water to 20 °C.
- Fill the water (20 °C) up to the calibration mark of the volumetric flask.

Stability at 2 °C – 8 °C: 2 weeks

Start reagent solution

0.229 g (33.00 mmol/l) L-γ-Glutamyl-3-carboxy-4-nitroanilide, monoammonium salt, monohydrate
- Dissolve in about 15 ml water.
- Transfer to a 20 ml volumetric flask.
- Equilibrate the volumetric flask and water to 20 °C.
- Fill the water (20 °C) up to the calibration mark of the volumetric flask.

Stability at 2 °C – 8 °C: 1 week

Measurement Procedure

Equilibrate only an adequate volume (~0.8 ml) of start reagent solution at 37 °C in preparation for the measurement procedure. The remaining volume of the start reagent solution should be stored at 2 °C – 8 °C.

Due to the large volume fraction of the sample it is necessary to equilibrate an adequate portion of the specimen near to 37 °C immediately before the measurement.

Pipette the volumes one after another into the cuvette as listed in Table 4.

Table 4 Analytical system for the measurement of GGT.

2.000 ml	Reaction solution *Equilibrate to 37.0 °C.*
0.250 ml	Sample (equilibrated close to 37.0 °C) *Mix thoroughly and incubate for 180 s. At the end of the incubation time, the temperature of the solution in the cuvette shall have reached 37.0 °C.*
0.500 ml	Start reagent solution *Mix thoroughly, wait 60 s and monitor time and absorbance for additional 180 s.*

The expanded (k=2) combined uncertainty (normally distributed) of the kinetic photometric measurement shall not exceed 1%. (This uncertainty does not include the uncertainty of the wave length adjustment.)

The expanded (k=2) combined uncertainty (normally distributed) of the volume fraction of sample shall be ≤1%.

Reagent blank rate

To determine the reagent blank rate, the specimen is replaced by 9 g/l (154 mmol/l) sodium chloride solution. The measurement procedure is then carried out as described above. If the reagent blank rate exceeds 1.7×10^{-5} s^{-1} (0.001 min^{-1}) or has a reverse direction, the measurements must be repeated and if necessary the reagent solution must be discarded.

Sample blank rate

For the determination of the sample blank rate the start reagent solution is replaced by 9 g/l (154 mmol/l) sodium chloride solution. The measurement procedure is then carried out as described above.

Note: The sample blank rate is determined and documented but not taken into account for calculation of the catalytic concentration of GGT in control sera and calibrators. In case that the value of the sample blank rate exceeds 1% of total GGT, a warning that the respective material is not appropriate for calibration should be issued.
Note: The reagent blank rate for the sample blank rate is determined by replacing the start reagent solution **and** the sample by 9 g/l (154 mmol/l) sodium chloride solution.
Note: Effects of the matrix of the sample on the indicator reaction have not been considered due to the omission of γ-glutamyl-3-carboxy-4-nitroanilide from the reaction mixture.

Upper limit of the measurement range

If the change of absorbance exceeds 0.0033 s^{-1} (0.2 min^{-1}) in the measurement interval, an analytical portion of the sample must be diluted with 9 g/l (154 mmol/l) sodium chloride solution and the measurement procedure must be repeated with the diluted specimen. The obtained value must then be multiplied by the corresponding factor of the dilution.

Sources of error

Due to the high volume fractions of serum and start reagent solution, the measuring temperature at the start of the measuring interval is only achieved if sample and substrate solution are tempered.

Higher catalytic GGT concentrations have been measured in some control specimens when the material had been diluted prior to the measurement. The information whether or not predilution has been performed should be declared.

Calculation

The temporal change of absorbance (s^{-1}) is calculated with the analysis of regression (method of the least squares). After subtraction of the reagent blank rate the corrected change of absorbance is multiplied by the factor

F=1382 (measurement at 410 nm, ε_{410}(5-amino-2-nitrobenzoate)=796 m^2/mol)

The catalytic concentration of GGT is calculated in μkat/l.
$\Delta A/\Delta t_{GGT}$: change of absorbance (in s^{-1}) after correction of the reagent blank rate
b_{GGT}: catalytic concentration of GGT
$b_{GGT}=1382 \cdot \Delta A/\Delta t_{GGT}$
The catalytic concentration in μkat/l can be converted to U/l by multiplication by the factor f=60.

Preliminary Upper Reference Limits

The preliminary upper reference limits for adults (≥17 years) were investigated seperately for men (n=407) and women (n=420).

Gender	Upper reference limit* (and 90% confidence interval)
Women	0.63 μkat/l (0.62 μkat/l – 0.65 μkat/l)
Men	0.92 μkat/l (0.89 μkat/l – 0.96 μkat/l)

Gender	Upper reference limit* (and 90% confidence interval)
Women	38 U/l (37 U/l – 39 U/l)
Men	55 U/l (53 U/l – 58 U/l)

*The upper reference limits are the 97.5th percentiles of the reference collectives. Inside brackets are the 90% confidence intervals of the 97.5th percentiles.

Appendix 1: Changes in the Reference Procedure for Measurements at 37 °C Compared with the Reference Method for Measurements at 30 °C as Described in the Original IFCC Document

The primary reference procedure is deduced from the IFCC reference method (2) which provides optimised conditions for the measurement of catalytic activity concentrations of GGT. The measurement temperature of 37 °C instead of 30 °C requires only minimal changes of certain measurement parameters to retain the optimum measurement conditions. The modifications are listed and commented on in Table 5. Furthermore, if in comparison to the 30 °C reference method a more accurate specification has become necessary for improving the high standardization of the measurements, it is also described here.

Table 5 Comparison of the IFCC methods for the measurement temperatures of 30 °C and 37 °C.

37 °C Reference procedure	30 °C Reference method	Comment
Specimen of investigation		
Calibration materials, control specimens and human sera	Human sera	The reference procedure will be used primarily for the investigation of calibration materials and control specimens.
pH value		
The pH optimum is 7.70	The pH optimum is 7.90	The shift of the pH optimum with the temperature coincidentally agrees with the shift of the pK value of the buffer. Therefore, the same reagent solution can be used at 30 °C and 37 °C.

Table 5 Continued.

37 °C Reference procedure	30 °C Reference method	Comment
Uncertainty of the pH value adjustment		
ΔpH±0.05	Not specified	
Uncertainty of the measurement temperature adjustment		
Uncertainty ≤0.1 °C (k=2)	Bias: less ± 0.05 °C Imprecision: less ± 0.1 °C	High quality spectrophotometer with devices for temperature adjustment and control provide an uncertainty (k=2) of the temperature ≤0.1 °C.
Start of the reaction		
Start with substrate	Start with serum	Start with substrate allows an incubation time for the equilibration of temperature in the cuvette.
Incubation time		
180 s	No incubation time, start with serum	Due to the large volume fraction of sample, 180 s are needed for the equilibration of temperature in the cuvette.
Delay time		
60 s	No delay time prescribed	The delay time is used for further temperature equilibration and to achieve a linear reaction rate.
Measurement interval		
180 s	Up to 300 s	At higher temperature the catalytic concentration of enzymes and with it the photometric signal becomes higher. Therefore a shorter measurement interval can be used.
Volumes of the reagent solutions		
100 ml *N*-glycylglycine buffer solution 20 ml L-γ-glutamyl-3-carb-oxy-4-nitroanilide solution	1000 ml *N*-glycylglycine buffer solution 100 ml L-γ-glutamyl-3-carboxy-4-nitroanilide solution	There is no need to prepare large volumes of reagent solutions, because the reagent solutions are prepared freshly for each campaign.
Temperature of the initial reagent solution before use		
Start with substrate: before use, the start reagent solution should have 37 °C	Start with serum: no temperature equilibation of the serum is described	The use of the start reagent solution with ambient temperature decreases the temperature in the cuvette.
Collection of data		
Number of readings ≥6	Recording the change of absorbance	Modern spectrophotometer employ digital data processing. Several readings ≥6 should ensure a sufficient precision of the measurement results. Devices for a continuous monitoring are no longer in use.
Determination of the slope (time versus absorbance)		
Regression analysis of the method of least squares	No information	A definitive statistical method ensures the reproducibility of the calculation and allows for the estimation of uncertainty.
Upper reference limits		
Women ≤0.63 µkat/l (≤38 U/l) Men ≤0.92 µkat/l (≤55 U/l)	No reference values for the method	The reference values for women and men. were investigated separately.

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