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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 3. Reference Procedure for the Measurement of Catalytic Concentration of Lactate Dehydrogenase

[L-Lactate: NAD^+ Oxidoreductase (LDH), EC 1.1.1.27]

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This paper is the third 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 4. Reference Procedure for the Measurement of Catalytic Concentration of Alanine Aminotransferase; Part 5. Reference Procedure for the Measurement of Catalytic Concentration of Aspartate Aminotransferase; Part 6. Reference Procedure for the Measurement of Catalytic Concentration of γ-Glutamyltransferase; Part 7. Certification of Four Reference Materials for the Determination of Enzymatic Activity of γ-Glu-

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tamyltransferase, 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(6):643–648

Key words: IFCC reference procedure; Lactate dehydrogenase; Preliminary reference interval.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; NAD, β-nicotinamide adenine dinucleotide; NADH, β-nicotinamide adenine dinucleotide, reduced form.

Reaction Principle

L-(+)-Lactate + NAD^+ –LDH→ Pyruvate + NADH + H^+

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 LDH.

N-Methyl-D-glucamine	325 mmol/l
pH (37 °C)	9.40 ± 0.05*
L-(+)-Lactate	50 mmol/l
β-NAD^+	10 mmol/l
(free acid 3.15 mmol/)	
(lithium salt 6.85 mmol/l)	
Volume fraction of sample	0.0435 (1:23)

* expanded (k=2) combined uncertainty

Table 2 Measurement conditions for the measurement of LDH.

Temperature	37.0 °C ± 0.1 °C*
Wave length	339 nm ± 1 nm
Band width	≤ 2 nm
Light path	10.00 mm ± 0.01 mm*
Incubation time	180 s
Delay time	90 s
Measurement interval	180 s
Readings (measurement points)	≥ 6

* expanded (k=2) combined uncertainty

Reagents

1. *N*-Methyl-D-glucamine, ($C_7H_{17}NO_5$), M_r=195.22
2. L(+)-Lactic acid, monolithium salt ($C_3H_5O_3Li$), M_r=96.01
3. β-Nicotinamide adenine dinucleotide (NAD), free acid ($C_{21}H_{27}N_7O_{14}P_2$), M_r=663.4
4. NAD, lithium salt, dihydrate ($C_{21}H_{26}N_7O_{14}P_2Li \cdot 2 H_2O$), M_r=705.4
5. Hydrochloric acid (HCl), M_r=36.46, 2 mol/l
6. Sodium chloride (NaCl), M_r=58.44

Note: NAD can contain inhibitors for LDH. The absence of inhibitors should be declared by the manufacturer.

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.

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 < 2µS/cm, pH 6–7, silicate < 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%.

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

Temperature (°C)	pH	Temperature (°C)	pH	Temperature (°C)	pH
15.00	9.976	23.50	9.741	32.00	9.522
15.25	9.969	23.75	9.734	32.25	9.516
15.50	9.962	24.00	9.728	32.50	9.509
15.75	9.955	24.25	9.721	32.75	9.503
16.00	9.948	24.50	9.714	33.00	9.497
16.25	9.940	24.75	9.708	33.25	9.491
16.50	9.933	25.00	9.701	33.50	9.485
16.75	9.926	25.25	9.695	33.75	9.479
17.00	9.919	25.50	9.688	34.00	9.472
17.25	9.912	25.75	9.681	34.25	9.466
17.50	9.905	26.00	9.675	34.50	9.460
17.75	9.898	26.25	9.668	34.75	9.454
18.00	9.891	26.50	9.662	35.00	9.448
18.25	9.884	26.75	9.655	35.25	9.442
18.50	9.877	27.00	9.649	35.50	9.436
18.75	9.870	27.25	9.642	35.75	9.430
19.00	9.864	27.50	9.636	36.00	9.424
19.25	9.857	27.75	9.629	36.25	9.418
19.50	9.850	28.00	9.623	36.50	9.412
19.75	9.843	28.25	9.617	36.75	9.406
20.00	9.836	28.50	9.610	37.00	9.400
20.25	9.829	28.75	9.604	37.25	9.394
20.50	9.822	29.00	9.597	37.50	9.388
20.75	9.815	29.25	9.591	37.75	9.382
21.00	9.809	29.50	9.585	38.00	9.376
21.25	9.802	29.75	9.578	38.25	9.371
21.50	9.795	30.00	9.572	38.50	9.365
21.75	9.788	30.25	9.566	38.75	9.359
22.00	9.781	30.50	9.559	39.00	9.353
22.25	9.775	30.75	9.553	39.25	9.347
22.50	9.768	31.00	9.547	39.50	9.341
22.75	9.761	31.25	9.540	39.75	9.335
23.00	9.754	31.50	9.534	40.00	9.330
23.25	9.748	31.75	9.528		

Reaction solution

7.30 g (373.8 mmol/l) *N*-Methyl-D-glucamine
0.552 g (57.50 mmol/l) Lactic acid, monolithium salt
– Dissolve in about 80 ml water.
– Adjust to pH (37 °C) 9.4 with 2 mol/l hydrochloric acid.
– 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: 1 month

Start reagent solution

0.240 g (36.23 mmol/l) NAD, free acid
0.556 g (78.78 mmol/l) NAD, lithium salt, dihydrate
Note: The use of NAD free acid without NAD lithium salt for the preparation of the start reagent solution (as stipulated for the IFCC 30 °C reference method) reasonably decreases the pH value of the final complete reaction mixture.

A start reagent solution containing a mixture of NAD free acid and NAD lithium salt has two advantages:

1. The pH value of the final complete reaction mixture does not decrease.
2. The absorbance of NAD depends strongly on the pH value but the change of the absorbance due to a change of the pH value occurs not spontaneously. Therefore, the reagent blank rate reaction remains non-linear for about 4–6 minutes if NAD free acid as the start reagent solution is added to the alkaline reaction solution. This effect is considerably reduced if the start reagent solution contains a mixture of NAD free acid and NAD lithium salt.

Note: The above recommended NAD free acid/NAD lithium salt mixture dissolves rather slowly. Raising the temperature (up to 40 °C) speeds up the dissolving process.
– Dissolve in about 6 ml water.
– Transfer to a 10 ml volumetric flask.
– Equilibrate the volumetric flask and water to 20 °C.
– Fill 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 (~ 0.4 ml) of start reagent solution at 37 °C in preparation of the measurement procedure. The remaining volume of the start reagent solution should be stored at 2 °C–8 °C.

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

Table 4 Analytical system for the measurement of the overall rate of conversion.

2.000 ml	Reaction solution *Equilibrate to 37.0 °C.*
0.100 ml	Sample *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.*
0.200 ml	Start reagent solution *Mix thoroughly, wait 90 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 wavelength adjustment.)

The expanded (k=2) combined uncertainty (normally distributed) of the volume fraction of the 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.

Sample blank rate

Due to the lactate content in the sample, it is not possible to determine the sample blank rate.

Upper limit of the measurement range

If the change of absorbance exceeds 0.00275 s^{-1} (0.165 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

If alanine aminotransferase (ALT) or aspartate aminotransferase (AST) have been examined in the cuvette prior to the LDH determination, a possible interference of displaced LDH from AST/ALT test mixtures with the measurements must be taken into account.

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=3651 (measurement at 339 nm, ε_{339}(NADH)= 630 m^2/mol).

The catalytic concentration of LDH is calculated in µkat/l.

$\Delta A/\Delta t_{LDH}$: change of absorbance (in s^{-1}) after correction of the reagent blank rate

b_{LDH}: catalytic concentration of LDH

$b_{LDH}=3651 \cdot \Delta A/\Delta t_{LDH}$

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

37 °C IFCC reference procedure	30 °C Reference method	Comment
Specimen of investigation		
Calibration material, control specimens and human sera	Human sera	The reference procedure will be used primarily for the investigation calibration materials and control specimens.
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.
Uncertainty of the pH value adjustment		
ΔpH ± 0.05	Not specified	
Delay time		
90 s	30 s	The reagent blank rate remains non-linear up to 90 s after the addition of the start reagent solution.

Table 5 Continued.

37 °C IFCC Reference procedure	Reference method 30 °C	Comment
Measurement time		
180 s	At least 240 s	The reaction rate is not linear and the non-linearity proportionally increases with the time and the catalytic LDH concentration. Shorter measurement time implies less non-linearity.
Pipetting volumes and volume fractions		
Solution R: 2000 µl Serum: 100 µl Start solution: 200 µl	Solution R: 2700 µl Serum: 150 µl Start solution: 300 µl	Volumes better suited for conventional pipetting systems were used. Consequently, the concentrations of the reagent solutions were adapted to the new volume fractions (the volume fraction of sample changes from 1:21 to 1:23).
Start reagent solution		
Aqueous mixture of NAD free acid and NAD lithium salt	Aqueous solution of NAD free acid	The use of the free acid as start solution changes the pH value of the reaction mixture. The mixture of free acid and lithium salt is more stable and the non-linearity of the reagent blank rate is reduced.
Buffer stock solution		
No preparation of a buffer stock solution	Preparation of a buffer stock solution	The preparation of a buffer stock solution is not necessary.
Temperature of the start reagent solution before use		
Start with substrate: before use the start reagent solution should be at 37 °C	No temperature equilibration of the start reagent solution is described	The use of start reagent solution with ambient temperature decreases the temperature in the cuvette.
Collection of data		
Number of readings ≥ 6	Monitoring of the increase in absorbance	Modern spectrophotometers 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 well-defined statistical method is necessary to ensure the reproducibility of the calculation of the slope.
Reference range		
Women and men ≤ 4.12 µkat/l (≤ 245 U/l)	No reference values for the method	The reference values for women and men were investigated separately.

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 separately for men (n=441) and women (n=438) (1).

Gender	Upper reference limit* (and 90% confidence interval)
Women	4.12 µkat/l (4.07 µkat/l–4.25 µkat/l)
Men	4.13 µkat/l (4.05 µkat/l–4.22 µkat/l)

Gender	Upper reference limit* (and 90% confidence interval)
Women	247 U/l (244 U/l–255 U/l)
Men	248 U/l (243 U/l–253 U/l)

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

Appendix 1: Changes in the IFCC 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 (1) which provides optimised conditions for the measurement of catalytic activity concentrations of LDH. 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 standardisation of the measurements, it is also described here.

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