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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 5. Reference Procedure for the Measurement of Catalytic Concentration of Aspartate Aminotransferase

[L-Aspartate: 2-Oxoglutarate-Aminotransferase (AST), EC 2.6.1.1]

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This paper is the fifth 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;

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Part 4. Reference Procedure for the Measurement of Catalytic Concentration of Alanine 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-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 3. Clin Chem Lab Med 2002; 40(7):725–733

Key words: IFCC reference procedure; Aspartate aminotransferase; Preliminary upper reference limit.

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

Reaction Principle

L-Aspartate + 2-Oxoglutarate – AST → Oxalacetate + L-Glutamate

Oxalacetate + NADH + H^+ – MDH* → L-Malate + NAD^+

*Malate dehydrogenase (MDH, EC 1.1.1.37)

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 AST.

Tris(hydroxymethyl)aminomethane	80 mmol/l
pH (37 °C)	7.65±0.05*
L-Aspartate	240 mmol/l
NADH	0.18 mmol/l
Pyridoxal-5′-phosphate	0.1 mmol/l
Malate dehydrogenase (37 °C)	10 µkat/l (600 U/l)
Lactate dehydrogenase (37 °C)	15 µkat/l (900 U/l)
2-Oxoglutarate	12 mmol/l
Volume fraction of sample	0.0833 (1:12)

*expanded (k=2) combined uncertainty

Table 2 Conditions for the measurement of AST.

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	300 s
Delay time	90 s
Measurement interval	180 s
Readings (measurement points)	≥6

*expanded (k=2) combined uncertainty

Reagents

1. Tris(hydroxymethyl)aminomethane (Tris) ($C_4H_{11}NO_3$), M_r=121.1
2. L(+)-Aspartic acid, free acid ($C_4H_7NO_4$), M_r=133.1
3. 2-Oxoglutaric acid, disodium salt, dihydrate ($C_5H_4O_5Na_2 \cdot 2\ H_2O$), M_r=226.1
4. β-Nicotinamide adenine dinucleotide, reduced form (NADH) ($C_{21}H_{27}N_7O_{14}P_2Na_2$), disodium salt, M_r=709.4
5. Pyridoxal-5′-phosphoric acid, monohydrate ($C_8H_{10}O_6NP \cdot H_2O$), M_r=265.2
6. Lactate dehydrogenase (LDH, EC 1.1.1.27), from pig skeletal muscle, in glycerol
7. Malate dehydrogenase (MDH, EC 1.1.1.37), from pig heart, in glycerol
8. Sodium azide (NaN_3), M_r=65.01
9. Hydrochloric acid (HCl), M_r=36.47, 1 mol/l
10. Sodium hydroxide solution (NaOH), M_r=40.00, 5 mol/l
11. Sodium chloride (NaCl), M_r=58.44
12. Bovine serum albumin, Fraction V, M_r=68 000

Note: Ammonium sulphate suspensions of the LDH and MDH reagent enzymes shall not be used due to glutamate dehydrogenase activity in the sample.
Note: The reagent enzyme preparations (LDH, MDH and bovine albumin) must be free from glutamate dehydrogenase and AST. The absence of these contaminants must be declared by the manufacturer or experimentally investigated in the reference laboratory.
Note: Contamination of 2-oxoglutarate by pyruvate leads to a consumption of NADH and decreases the initial absorbance of the final complete reaction mixture.
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 Tables 3 and 4, if necessary. The same applies to the adjustment of the temperature compensation of the pH meter.

Table 3 Dependence of the pH value of Solution 1 upon temperature.

Temperature (°C)	pH	Temperature (°C)	pH	Temperature (°C)	pH
15.00	8.336	23.50	8.066	32.00	7.802
15.25	8.328	23.75	8.059	32.25	7.795
15.50	8.320	24.00	8.051	32.50	7.787
15.75	8.312	24.25	8.043	32.75	7.779
16.00	8.304	24.50	8.035	33.00	7.772
16.25	8.296	24.75	8.027	33.25	7.764
16.50	8.288	25.00	8.019	33.50	7.756
16.75	8.280	25.25	8.012	33.75	7.749
17.00	8.272	25.50	8.004	34.00	7.741
17.25	8.264	25.75	7.996	34.25	7.733
17.50	8.256	26.00	7.988	34.50	7.726
17.75	8.248	26.25	7.980	34.75	7.718
18.00	8.240	26.50	7.972	35.00	7.710
18.25	8.232	26.75	7.965	35.25	7.703
18.50	8.224	27.00	7.957	35.50	7.695
18.75	8.216	27.25	7.949	35.75	7.688
19.00	8.208	27.50	7.941	36.00	7.680
19.25	8.201	27.75	7.934	36.25	7.672
19.50	8.193	28.00	7.926	36.50	7.665
19.75	8.185	28.25	7.918	36.75	7.657
20.00	8.177	28.50	7.910	37.00	7.650
20.25	8.169	28.75	7.903	37.25	7.642
20.50	8.161	29.00	7.895	37.50	7.634
20.75	8.153	29.25	7.887	37.75	7.627
21.00	8.145	29.50	7.879	38.00	7.619
21.25	8.137	29.75	7.872	38.25	7.612
21.50	8.129	30.00	7.864	38.50	7.604
21.75	8.121	30.25	7.856	38.75	7.597
22.00	8.114	30.50	7.848	39.00	7.589
22.25	8.106	30.75	7.841	39.25	7.581
22.50	8.098	31.00	7.833	39.50	7.574
22.75	8.090	31.25	7.825	39.75	7.566
23.00	8.082	31.50	7.818	40.00	7.559
23.25	8.074	31.75	7.810		

Table 4 Dependence of the pH value of Solution 2 upon temperature.

Temperature (°C)	pH	Temperature (°C)	pH	Temperature (°C)	pH
15.00	8.274	23.50	8.007	32.00	7.773
15.25	8.266	23.75	8.000	32.25	7.766
15.50	8.258	24.00	7.993	32.50	7.760
15.75	8.249	24.25	7.985	32.75	7.753
16.00	8.241	24.50	7.978	33.00	7.747
16.25	8.233	24.75	7.971	33.25	7.741
16.50	8.225	25.00	7.963	33.50	7.734
16.75	8.217	25.25	7.956	33.75	7.728
17.00	8.208	25.50	7.949	34.00	7.722
17.25	8.200	25.75	7.942	34.25	7.716
17.50	8.192	26.00	7.935	34.50	7.710
17.75	8.184	26.25	7.928	34.75	7.704
18.00	8.176	26.50	7.921	35.00	7.697
18.25	8.168	26.75	7.914	35.25	7.691
18.50	8.160	27.00	7.907	35.50	7.685
18.75	8.152	27.25	7.900	35.75	7.679
19.00	8.145	27.50	7.893	36.00	7.673
19.25	8.137	27.75	7.886	36.25	7.667
19.50	8.129	28.00	7.879	36.50	7.661
19.75	8.121	28.25	7.872	36.75	7.655
20.00	8.113	28.50	7.865	37.00	7.650
20.25	8.105	28.75	7.858	37.25	7.644
20.50	8.098	29.00	7.852	37.50	7.638
20.75	8.090	29.25	7.845	37.75	7.632
21.00	8.082	29.50	7.838	38.00	7.626
21.25	8.075	29.75	7.832	38.25	7.621
21.50	8.067	30.00	7.825	38.50	7.615
21.75	8.060	30.25	7.818	38.75	7.609
22.00	8.052	30.50	7.812	39.00	7.604
22.25	8.044	30.75	7.805	39.25	7.598
22.50	8.037	31.00	7.798	39.50	7.592
22.75	8.029	31.25	7.792	39.75	7.587
23.00	8.022	31.50	7.785	40.00	7.581
23.25	8.015	31.75	7.779		

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, 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%.

Solution 1

1.17 g (96.92 mmol/l) Tris
4.02 (302.4 mmol/l) Aspartic acid, free acid
0.052 g (8.00 mmol/l) Sodium azide

- Dissolve in about 80 ml water.
- Add about 80 ml water.
- Add 4.2 ml to 4.4 ml 5.00 mol/l sodium hydroxide solution.
- Stir until the reagents are dissolved completely. The pH value of the mixture should be lower (about 0.3 to 0.9 pH units) than the target pH value.
- Adjust pH (37 °C) 7.65 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: 3 months

Note: Aspartic acid does not dissolve after the addition of water. Sodium hydroxide solution must be added first. The concentration of 5 mol/l instead of 2 mol/l decreases the required volume. For the exact adjustment of the pH value sodium hydroxide solution of 2 mol/l should be used.

Solution 2

1.17 g (96.92 mmol/l) Tris
0.052 g (8.00 mmol/l) Sodium azide
– Dissolve in about 80 ml water.
– Adjust pH (37 °C) 7.65 with hydrochloric acid (1 mol/l).
– 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: 3 months

Solution 3

16.7 mg (6.300 mmol/l) Pyridoxal-5'-phosphoric acid, monohydrate
– Dissolve in about 6 ml solution 2.
– Transfer to a 10 ml volumetric flask.
– Equilibrate the volumetric flask and Solution 2 to 20 °C.
– Fill up the Solution 2 (20 °C) up to the calibration mark of the volumetric flask.
– Store protected from light (*e.g.* in a brown bottle).

Stability at 2 °C – 8 °C: 1 week

Solution 4

16.1 mg (11.34 mmol/l) NADH, disodium salt
– Dissolve in 2.00 ml Solution 2.
– Store protected from light (*e.g.* in a brown bottle).

Stability at 2 °C – 8 °C: 1 week

Diluent for reagent enzymes

1.20 g Bovine serum albumin
0.90 g (154 mmol/l) NaCl
– Dissolve in about 80 ml water.
– 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 at least 1 month

Solution 5

Lactate dehydrogenase solution [1.89 mkat/l (113.4 kU/l) at 37 °C]
Malate dehydrogenase solution [1.26 mkat/l (75.6 kU/l) at 37 °C]
– Dilute the stock lactate dehydrogenase solution with diluent for reagent enzymes so that the dilution exhibits a catalytic LDH concentration of 3.78 mkat/l (226.8 kU/l) at 37 °C.

Example: LDH_{stock}: catalytic LDH concentration of the enzyme stock solution in mkat/l, see Appendix 1; $V_{diluent}$: volume of diluent for reagent enzymes for the dilution of the stock LDH solution. $V_{diluent}=0.1 \cdot (LD_{stock} -3.78)/3.78$. Add the volume (ml) of $V_{diluent}$ to 0.1 ml enzyme stock solution.
– Dilute the stock malate dehydrogenase solution with diluent for reagent enzymes so that the dilution exhibits a catalytic MDH concentration of 2.52 mkat/l (151.2 kU/l) at 37 °C.

Example: see LDH.
– Mix both enzyme dilutions volume by volume (1 + 1) to obtain Solution 5.

Stability at 2 °C – 8 °C: 2 days

Reaction solution

10.0 ml Solution 1
0.200 ml Solution 3
0.200 ml Solution 4
0.100 ml Solution 5
– Mix thoroughly and store protected from light.

Stability at 2 °C – 8 °C: 1 day

Start reagent solution

0.326 g (144.0 mmol/l) 2-Oxoglutaric acid, disodium salt, dihydrate
– Dissolve in about 6 ml water.
– Transfer to a 10 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 (~0.4 ml) of the 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.

Pipette the following volumes one after another into the cuvette as listed in Table 5.

Table 5 Analytical system for the measurement of AST.

2.000 ml	Reaction solution *Equilibrate to 37.0 °C.*
0.200 ml	Sample *Mix thoroughly and incubate for 300 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 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 sample is replaced by 9 g/l (154 mmol/l) sodium chloride solution. The measurement procedure is then carried out as described above. If the absolute reagent blank rate exceeds 3.3×10^{-5} s^{-1} (0.002 min^{-1}) or has a reverse direction, the measurements must be repeated and if necessary the reaction 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 AST in control sera and calibrators. In case that the value of the sample blank rate exceeds 1% of total AST, 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 sodium chloride solution.

Upper limit of the measurement range

If the change of absorbance exceeds 0.0022 s^{-1} (0.13 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 sample. The obtained value must then be multiplied by the corresponding factor of the dilution.

Sources of error

High pyruvate concentrations in the sample lead to high NADH consumption during the incubation period. This can reduce the upper limit of the measurement range and considerably lower the results of analyses.

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=1905 (measurement at 339 nm, ε_{339}(NADH)= 630 m^2/mol)

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

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

b_{AST}: catalytic concentration of AST

$b_{AST}=1905\ \Delta A/\Delta t_{AST}$

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=418) and women (n=419).

Gender	Upper reference limit* (and 90% confidence interval)
Women	0.52 µkat/l (0.48 µkat/l – 0.57 µkat/l)
Men	0.58 µkat/l (0.52 µkat/l – 0.59 µkat/l)

Gender	Reference limit* (and 90% confidence interval)
Women	31 U/l (29 U/l – 34 U/l)
Men	35 U/l (31 U/l – 35 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: Determination of the Catalytic Concentration of LDH in the Enzyme Stock Solution

Additional reagents

Pyruvic acid, monosodium salt ($C_3H_3O_3Na$), crystallised, M_r=110.0

Measurement conditions

Concentrations in the reaction mixture and measurement conditions are listed in Tables 6 and 7.

Table 6 Concentrations in the final complete reaction mixture for the measurement of LDH.

Tris(hydroxymethyl)aminomethane	80 mmol/l
pH (37 °C)	7.65±0.05*
L-Aspartate	240 mmol/l
NADH	0.18 mmol/l
Pyridoxal-5'-phosphate	0.1 mmol/l
Pyruvate	3 mmol/l
Volume fraction of sample	0.0833 (1:12)

*expanded (k=2) combined uncertainty

Table 7 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	30 s
Delay time	30 s
Measurement interval	90 s
Readings (measurement points)	≥6

*expanded (k=2) combined uncertainty

Reaction solution

10.0 ml Solution 1
0.200 ml Solution 3
0.200 ml Solution 4
0.100 ml Water
– Mix thoroughly and store protected from light.
Stability at 2 °C – 8 °C: 1 day

Start reagent solution

0.0990 g (36.00 mmol/l) Pyruvic acid, monosodium salt
– Dissolve in about 6 ml water.
– Transfer to a 25 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 day

Dilution of the enzyme stock solution (immediately before use)

Step 1: Add 0.050 ml enzyme stock solution to 10.0 ml diluent for reagent enzymes and mix thoroughly. Step 2: Add 0.050 ml of the final solution from Step 1 to 10.0 ml diluent for reagent enzymes and mix thoroughly.
Note: The catalytic concentration of the stock LDH enzyme solution may necessitate dilutions different from the above described procedure. This requires respective modification of the dilution factor ($F_{dilution}$).

Measurement procedure

Equilibrate only an adequate volume (~0.4 ml) of the 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.

Pipette the volumes one after another into the cuvette as listed in Table 8.

Table 8 Analytical system for the measurement of LDH.

2.000 ml	Reaction solution *Equilibrate to 37.0 °C.*
0.200 ml	LDH solution Step 2 *Mix thoroughly and incubate for 30 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 30 s and monitor time and absorbance for additional 90 s.*

To determine the reagent blank rate, the volume of the diluted enzyme stock solution is replaced by 9 g/l (154 mmol/l) sodium chloride solution. The measurement procedure is then carried out as described above.

Calculation

The calculation is the same as the calculation for the catalytic concentration of AST. The result is the catalytic LDH concentration in the final solution of Step 2.

For calculation of the catalytic LDH concentration in the enzyme stock solution (LDH_{stock}) this result must be multiplied by the dilution factor:

$F_{dilution}$=40401.

Calculation:

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

$LDH_{stock}=2302 \cdot 40401 \cdot \Delta A/\Delta t_{LD}$

The catalytic concentration in µkat/l can be converted to kU/l by multiplication by the factor f=0.06. Convert from U/l to kU/l by division by 1000.

Appendix 2: Determination of the Catalytic Concentration of MDH in the Enzyme Stock Solution

Additional reagents

Oxalacetic acid, free acid ($C_4H_4O_5$), M_r=132.1

Measurement conditions

Concentrations in the reaction mixture and measurement conditions are listed in Tables 9 and 10.

Table 9 Concentrations in the final complete reaction mixture for the measurement of MDH.

Tris(hydroxymethyl)aminomethane	80 mmol/l
pH (37 °C)	7.65±0.05*
L-Aspartate	240 mmol/l
NADH	0.18 mmol/l
Pyridoxal-5'-phosphate	0.1 mmol/l
Oxalacetate	0.4 mmol/l
Volume fraction of sample	0.0833 (1:12)

*expanded (k=2) combined uncertainty

Table 10 Conditions for the measurement of MDH.

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	30 s
Delay time	30 s
Measurement interval	90 s
Readings (measurement points)	≥6

*expanded (k=2) combined uncertainty

Reaction solution

10.0 ml Solution 1
0.200 ml Solution 3
0.200 ml Solution 4
0.100 ml Water
– Mix thoroughly and store protected from light.
Stability at 2 °C – 8 °C: 1 day

Start reagent solution

0.0317 g (4.800 mmol/l) Oxalacetic acid, free acid
– Dissolve in about 40 ml water.

– Transfer to a 50 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.
– Cool the solution on ice.

Stability at 2 °C – 8 °C: 30 min

Dilution of the enzyme stock solution (immediately before use)

Step 1: Add 0.050 ml enzyme stock solution to 10.0 ml diluent for reagent enzymes and mix thoroughly. Step 2: Add 0.050 ml of the final solution from Step 1 to 10.0 ml diluent for reagent enzymes and mix thoroughly.

Note: The catalytic concentration of the stock MDH enzyme solution may necessitate dilutions different from the above described procedure. This requires respective modification of the dilution factor ($F_{dilution}$).

Measurement procedure

Equilibrate only an adequate volume (~0.4 ml) of the start reagent solution at 37 °C in preparation for the measurement procedure. The remaining volume of the start reagent solution should be stored on ice.

Pipette the volumes one after another into the cuvette as listed in Table 11.

Table 11 Analytical system for the measurement of MDH.

2.000 ml	Reaction solution *Equilibrate to 37.0 °C.*
0.200 ml	MDH solution Step 2 *Mix thoroughly and incubate for 30 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 30 s and monitor time and absorbance for additional 90 s.*

To determine the reagent blank rate, the volume of the diluted enzyme stock solution is replaced by 9 g/l (154 mmol/l) sodium chloride solution. The measurement procedure is then carried out as described above.

Calculation

The calculation is the same as the calculation for the catalytic concentration of AST. The result is the catalytic MDH concentration in the final solution of Step 2. For calculation of the catalytic MDH concentration in the enzyme stock solution (MDH_{stock}) this result must be multiplied by the dilution factor:

$F_{dilution}$=40401.

Calculation:

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

$MDH_{stock}=2302 \cdot 40401 \cdot \Delta A/\Delta t_{MD}$.

The catalytic concentration in µkat/l can be converted to kU/l by multiplication by the factor f=0.06.

Appendix 3: 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 (1) which provides optimised conditions for the measurement of catalytic activity concentrations of AST. 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 12. 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 12 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 7.65	The pH optimum is 7.8	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.
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.

Table 12 Continued.

37 °C Reference procedure	30 °C reference method	Comment
Incubation time		
300 s	At least 600 s	A time interval of 300 s is sufficient to saturate the AST with pyridoxal phosphate at 37 °C.
Delay time		
90 s	No information	Studies of the kinetics of the conversion rate of the substrate show that a lag phase up to 90 s can occur.
Measurement interval		
180 s	300 s	The available concentration of indicator for the measurement time decreases due to the consumption of NADH during the delay time. Higher signals at 37 °C allow shortening of the measurement time without enlarging the imprecision.
Catalytic MDH and LDH concentration		
15 µkat/l (900 U/l) 10 µkat/l (600 U/l)	LDH 10 µkat/l MDH 7 µkat/l	Same amounts of LDH and MDH for 30 °C and for 37 °C. The higher catalytic concentrations are due to the higher temperature.
Initial reagent solution		
pH value adjustment is not necessary	pH value adjustment with hydrochloric acid (5 mol/l)	The aqueous disodium salt solution has a pH(25 °C) of about 7.5 and practically no buffer capacity. A pH value adjustment with 5 mol/l hydrochloric acid is unnecessary and would be problematic.
Diluent for the reagent enzyme		
Bovine serum albumin and sodium chloride in water	Glycerol/water	Instability of LDH and MDH was observed in some glycerol-water mixtures
Sample blank rate		
Not taken into account	Subtraction	Sample blank rates usually are not subtracted in routine procedures. Therefore, the assigned values in calibrators and control materials are only useful for routine methods if they contain the sample blank rate value.
Volumes of the reagent solutions		
10.5 ml Reaction solution	105 ml Reaction solution	There is no need to prepare large volumes of reagent solutions, because the reagent solutions were prepared freshly for each campaign.
Temperature of the initial reagent solution before use		
Before use the start reagent solution should have 37 °C	No information about the temperature	The use of the 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. Analogue recorders for a continuously monitoring are no longer in use.
Determination of the slope (time vs. 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 limit of measurement range		
0.0022 s^{-1}	0.0025 s^{-1}	The upper limit of the measurement range was reinvestigated under the changed conditions (volume fraction of sample, delay time and measurement time).
Reference range		
Women ≤0.52 µkat/l (≤31 U/l) Men ≤0.58 µkat/l (≤35 U/l)	Healthy individuals 0.080–0.500 µkat/l and 0.060–0.500 µkat/l	The reference values for women and men were investigated separately.

References

1. Bergmeyer HU, Hørder M, Rej R. International Federation of Clinical Chemistry (IFCC). Approved recommendation (1985) on IFCC methods for the measurement of catalytic concentrations of enzymes. Part 2. IFCC method for aspartate aminotransferase. J Clin Chem. Clin Biochem 1986; 24:497–510.