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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 2. Reference Procedure for the Measurement of Catalytic Concentration of Creatine Kinase

[ATP: Creatine N-Phosphotransferase (CK), EC 2.7.3.2]

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This paper is the second 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 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 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-Glutamyltransferase, Lactate Dehydrogenase, Alanine Aminotransferase and Creatine Kinase at 37 °C.

A document describing the determination of preliminary reference values is also in preparation. The procedure described here is deduced from the previously

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described 30 °C IFCC reference method (1). Differences are tabulated and commented on in Appendix 3. Clin Chem Lab Med 2002; 40(6):635–642

Key words: IFCC reference procedure; Creatine kinase; Preliminary reference interval.

Abbreviations: ADP, adenosine 5′-diphosphate; CK, creatine kinase; AMP, adenosine 5′-monophosphate Ap_5A, P1, P5-di(adenosine-5′) pentaphosphate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; G-6-PD, glucose-6-phosphate dehydrogenase; HK, hexokinase; NAC, *N*-acetyl-L-cysteine; NADP, β-nicotinamide adenine dinucleotide 3′-phosphate; NADPH, β-nicotinamide adenine dinucleotide 3′-phosphate, reduced form.

Reaction Principle

Creatine phosphate + ADP —CK→ Creatine + ATP
ATP + Glucose —HK*→ ADP + Glucose-6-phosphate
Glucose-6-phosphate + $NADP^+$ —G-6-PD**→ Gluconate-6-phosphate + NADPH + H^+
*Hexokinase (HK; EC 2.7.1.1)
**Glucose-6-phosphate dehydrogenase (G-6-PD, EC 1.1.1.49)

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 CK.

Imidazole	100 mmol/l
pH (37 °C)	6.50 ± 0.05*
Creatine phosphate	30 mmol/l
ADP	2 mmol/l
EDTA	2 mmol/l
Magnesium acetate	10 mmol/l
N-Acetyl-L-cysteine	20 mmol/l
AMP	5 mmol/l
P^1, P^5-Di(adenosine-5′) pentaphosphate (P^1,P^5-diAP)	0.01 mmol/l
D-Glucose	20 mmol/l
NADP	2 mmol/l
Hexokinase (37 °C)	66.7 µkat/l (4000 U/l)
Glucose-6-phosphate dehydrogenase (37 °C)	46.7 µkat/l (2800 U/l)
Volume fraction of sample	0.0435 (1:23)

* expanded (k =2) combined uncertainty

Table 2 Conditions for the measurement of CK.

Temperature	37.0 °C ± 0.1 °C*
Wavelength	339 nm ± 1 nm
Band width	≤ 2 nm
Light path	10.00 mm ± 0.01* mm
Incubation time	180 s
Delay time	120 s
Measurement interval	120 s
Readings (measurement points)	≥ 6

* expanded (k =2) combined uncertainty

Reagents

1. Imidazole ($C_3H_4N_2$), M_r=68.08
2. Creatine phosphate, disodium salt, tetrahydrate ($C_4H_8N_3O_5PNa_2 \cdot 4\ H_2O$), M_r=327.2
3. Magnesium acetate, tetrahydrate ($C_4H_6O_4Mg \cdot 4\ H_2O$), M_r=214.5
4. D-Glucose, dehydrated ($C_6H_{12}O_6$), M_r=180.2
5. Ethylenediaminetetraacetic acid [EDTA] ($C_{10}H_{14}O_8N_2Na_2 \cdot 2\ H_2O$), disodium salt, dihydrate, M_r=372.2
6. Adenosine 5′-monophosphate (AMP), disodium salt, hexahydrate ($C_{10}H_{12}N_5O_7PNa_2 \cdot 6\ H_2O$), M_r= 499.2
7. Adenosine 5′-diphosphate (ADP), monopotassium salt, dihydrate ($C_{10}H_{13}N_5O_{10}P_2K \cdot 2\ H_2O$), M_r=501.3
8. *N*-Acetyl-L-cysteine (NAC) ($C_5H_9NO_3S$), M_r=163.2
9. β-Nicotinamide adenine dinucleotide phosphate (NADP), disodium salt ($C_{21}H_{26}N_7O_{17}P_3Na_2$), M_r=787.4
10. P^1, P^5-Di(adenosine-5′) pentaphosphate (Ap_5A), penta-lithium salt ($C_{20}H_{24}N_{10}O_{22}P_5Li_5$), M_r=946.0
11. Hexokinase (HK, EC 2.7.1.1), from yeast, lyophilized or in glycerol
12. Glucose-6-phosphate dehydrogenase (G-6-PD, EC 1.1.1.49), from yeast, lyophilized or in glycerol
13. Acetic acid ($C_2H_4O_2$), M_r=60.05, 1 mol/l
14. Sodium hydroxide solution (NaOH), M_r=40.00, 2 mol/l
15. Glycerol ($C_3H_8O_3$), M_r=92.10
16. 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.

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 actually measured temperature. The speed of agitation should be the same during the calibration, the control of the pH value 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	6.921	23.50	6.754	32.00	6.592
15.25	6.916	23.75	6.749	32.25	6.587
15.50	6.911	24.00	6.744	32.50	6.583
15.75	6.906	24.25	6.739	32.75	6.578
16.00	6.901	24.50	6.734	33.00	6.573
16.25	6.896	24.75	6.729	33.25	6.569
16.50	6.891	25.00	6.725	33.50	6.564
16.75	6.886	25.25	6.720	33.75	6.560
17.00	6.881	25.50	6.715	34.00	6.555
17.25	6.876	25.75	6.710	34.25	6.550
17.50	6.871	26.00	6.705	34.50	6.546
17.75	6.866	26.25	6.701	34.75	6.541
18.00	6.861	26.50	6.696	35.00	6.537
18.25	6.856	26.75	6.691	35.25	6.532
18.50	6.851	27.00	6.686	35.50	6.527
18.75	6.846	27.25	6.682	35.75	6.523
19.00	6.841	27.50	6.677	36.00	6.518
19.25	6.837	27.75	6.672	36.25	6.514
19.50	6.832	28.00	6.667	36.50	6.509
19.75	6.827	28.25	6.663	36.75	6.504
20.00	6.822	28.50	6.658	37.00	6.500
20.25	6.817	28.75	6.653	37.25	6.495
20.50	6.812	29.00	6.648	37.50	6.491
20.75	6.807	29.25	6.644	37.75	6.486
21.00	6.802	29.50	6.639	38.00	6.482
21.25	6.797	29.75	6.634	38.25	6.477
21.50	6.792	30.00	6.629	38.50	6.473
21.75	6.788	30.25	6.625	38.75	6.468
22.00	6.783	30.50	6.620	39.00	6.464
22.25	6.778	30.75	6.615	39.25	6.459
22.50	6.773	31.00	6.611	39.50	6.455
22.75	6.768	31.25	6.606	39.75	6.450
23.00	6.763	31.50	6.601	40.00	6.446
23.25	6.758	31.75	6.597		

Table 4 Dependence of the pH value of Solution 3 upon temperature.

Temperature (°C)	pH	Temperature (°C)	pH	Temperature (°C)	pH
15.00	6.945	23.50	6.774	32.00	6.602
15.25	6.940	23.75	6.769	32.25	6.597
15.50	6.935	24.00	6.764	32.50	6.592
15.75	6.930	24.25	6.759	32.75	6.587
16.00	6.925	24.50	6.754	33.00	6.581
16.25	6.920	24.75	6.749	33.25	6.576
16.50	6.915	25.00	6.744	33.50	6.571
16.75	6.910	25.25	6.739	33.75	6.566
17.00	6.905	25.50	6.733	34.00	6.561
17.25	6.900	25.75	6.728	34.25	6.556
17.50	6.895	26.00	6.723	34.50	6.551
17.75	6.890	26.25	6.718	34.75	6.546
18.00	6.885	26.50	6.713	35.00	6.541
18.25	6.880	26.75	6.708	35.25	6.536
18.50	6.875	27.00	6.703	35.50	6.531
18.75	6.870	27.25	6.698	35.75	6.526
19.00	6.865	27.50	6.693	36.00	6.520
19.25	6.860	27.75	6.688	36.25	6.515
19.50	6.855	28.00	6.683	36.50	6.510
19.75	6.849	28.25	6.678	36.75	6.505
20.00	6.844	28.50	6.673	37.00	6.500
20.25	6.839	28.75	6.668	37.25	6.495
20.50	6.834	29.00	6.663	37.50	6.490
20.75	6.829	29.25	6.658	37.75	6.485
21.00	6.824	29.50	6.653	38.00	6.480
21.25	6.819	29.75	6.647	38.25	6.475
21.50	6.814	30.00	6.642	38.50	6.469
21.75	6.809	30.25	6.637	38.75	6.464
22.00	6.804	30.50	6.632	39.00	6.459
22.25	6.799	30.75	6.627	39.25	6.454
22.50	6.794	31.00	6.622	39.50	6.449
22.75	6.789	31.25	6.617	39.75	6.444
23.00	6.784	31.50	6.612	40.00	6.439
23.25	6.779	31.75	6.607		

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%.

Solution 1

0.791 g (116.2 mmol/l) Imidazole
0.311 g (14.52 mmol/l) Magnesium acetate, tetrahydrate
0.229 g (2.904 mmol/l) NADP, disodium salt
0.523 g (29.04 mmol/l) Glucose
0.146 g (2.904 mmol/l) ADP, monopotassium salt, dihydrate
0.362 g (7.259 mmol/l) AMP, disodium salt, hexahydrate
0.00136 g (0.01452 mmol/l) Ap_5A, penta-lithium salt
Note: The small amount of Ap_5A is difficult to weigh. Alternatively, an amount of 1.5 mg–2.0 mg Ap_5A (m_{Ap5A}) in a small vial can be weighed and dissolved in

1.00 ml water. A pipette with adjustable volume must be used for pipetting the volume [V_{Ap5A} (ml)] of the Ap_5A solution.
V_{Ap5A} (ml)=1.36/ m_{Ap5A}
– Dissolve in about 80 ml water.
– Adjust pH (37 °C) 6.5 with acetic 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: 2 weeks
Note: The absorbance of solution 1 at 339 nm should be < 0.25.

Solution 2

HK [15.49 mkat/l (929.2 kU/l) at 37 °C] in glycerol/water (50:50 v/v)
G-6-PD [10.84 mkat/l (650.4 kU/l) at 37 °C] in glycerol/water (50:50 v/v)
– If HK is lyophilised, reconstitute it in the amount of diluent as prescribed by the supplier.
– If G-6-PD is lyophilised, reconstitute it in the amount of diluent as prescribed by the supplier.
– For the determination of the catalytic concentrations of the enzyme stock solutions see Appendix 1 and Appendix 2.
– Dilute HK with glycerol/water (50:50 v/v) so that the dilution reaches a catalytic HK concentration of 915.49 mkat/l (29.2 kU/l) at 37 °C.
– Dilute G-6-PD with glycerol/water (50:50 v/v) so that the dilution reaches a catalytic G-6-PD concentration of 10.84 mkat/l (650.4 kU/l) at37 °C.
– Mix both enzyme dilutions volume by volume (1 + 1) to obtain Solution 2.
Stability of all dilutions at –25 °C: 3 months

Solution 3

0.395 g (116.2 mmol/l) Imidazole
0.216 g (11.62 mmol/l) EDTA, disodium salt, dihydrate
0.948 g (116.2 mmol/l) *N*-Acetyl-L-cysteine
– Dissolve in about 40 ml water.
– Adjust pH (37 °C) 6.5 with sodium hydroxide solution (1 mol/l).
– Transfer to a 50 ml volumetric flask.
– Equilibrate the volumetric flask and water to 20 °C.
– Fill the water (20 °C) up to the mark at calibration temperature.
– Stability at 2 °C–8 °C: 1 week

Reaction solution

20 ml Solution 1
0.25 ml Solution 2
5 ml Solution 3
– Mix thoroughly
Stability at 2 °C–8 °C: 1 day

Start reagent solution

1.13 g (345.0 mmol/l) Creatine phosphate, disodium salt, tetrahydrate
– 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 month

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 CK.

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 should shall have reached 37.0 °C.*
0.200 ml	Start reagent solution *Mix thoroughly, wait 120 s and monitor time and absorbance for additional 120 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 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 reagent blank rate exceeds 2.5×10^{-5} s^{-1} (0.0015 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 CK in control sera and calibrators. In case that the value of the sample blank rate exceeds 1% of total CK, 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: The sample blank rate includes the interference by adenylate kinase. For clinical assessment of the catalytic concentrations of total CK and CK isoenzymes in human sera, the subtraction of a high sample blank rate may become necessary.

Upper limit of the measurement range

If the change of absorbance exceeds 0.0067 s^{-1} (0.4 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 for the dilution. Since the CK catalytic concentration is dependent upon the sample volume fraction, the dilution of the sample should be as low as possible.

Sources of error

The catalytic CK concentration depends on the volume fraction of the sample. The catalytic concentration increases in case of sample dilution.

Considerations for the Analysis of Control Materials

In some control materials, the catalytic CK concentration is strongly affected by the water temperature during reconstitution. Therefore, the reconstitution directions must be strictly followed. A decrease of the catalytic concentration due to the effect of daylight has been observed. Storage at low temperatures (< 4 °C) can cause a decrease in the CK concentration. In some control materials, CK is not completely reactivated after the incubation time and the delay time. In some lyophilised control materials, CK does not remain stable at room temperature. This must be taken into account when considering shipment of specimens.

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}(NADPH)= 630 m^2/mol)

The catalytic concentration of CK is calculated in µkat/l.
$\Delta A/\Delta t_{CK}$: change of absorbance (in s^{-1}) after correction of the reagent blank rate
b_{CK}: catalytic concentration of CK
$b_{CK}=3651 \cdot \Delta A/\Delta t_{CK}$
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=740) and women (n=738) (1).

Gender	Upper reference limit* (and 90% confidence interval)
Women	2.41 µkat/l (2.21 µkat/l – 2.55 µkat/l)
Men	2.85 µkat/l (2.76 µkat/l – 2.95 µkat/l)

Gender	Upper reference limit* (and 90% confidence interval)
Women	145 U/l (133 U/l–153 U/l)
Men	171 U/l (166 U/l–177 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 G-6-PD in the Enzyme Stock Solution

Additional reagents

β-D-Glucose-6-phosphate, monosodium salt ($C_6H_{12}O_9PNa$), M_r=282.1

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 G-6-PD.

Imidazole	100 mmol/l
pH (37 °C)	6.50 ± 0.05*
D-Glucose-6-phosphate	10 mmol/l
ADP	2 mmol/l
EDTA	2 mmol/l
Magnesium acetate	10 mmol/l
N-Acetyl-L-cysteine	20 mmol/l
AMP	5 mmol/l
P^1, P^5-Di(adenosine-5′) pentaphosphate (Ap_5A)	0.01 mmol/l
D-Glucose	20 mmol/l
NADP	2 mmol/l
Volume fraction of sample	0.0435 (1:23)

* expanded (k =2) combined uncertainty

Table 7 Conditions for the measurement of G-6-PD.

Temperature	37.0 °C ± 0.1 °C*
Wavelength	339 nm ± 1 nm*
Band width	≤ 2 nm
Light path	10.00 mm ± 0.01 mm*
Incubation time	180 s
Delay time	120 s
Measurement interval	120 s
Readings (measurement points)	≥ 6

* expanded (k =2) combined uncertainty

Reaction solution

10 ml Solution 1
2.5 ml Solution 3
0.125 ml water
– Mix thoroughly.
Stability at 2 °C–8 °C: 1 day

Start reagent solution

32.5 mg (115.0 mmol/l) β-D-Glucose-6-phosphate, monosodium salt
– Dissolve in 1 ml water.
Stability: Solution must be freshly prepared

Dilution of the enzyme stock solution

Add 10 µl of reconstituted G-6-PD enzyme stock solution to 20 ml glycerol/water (50:50 v/v) and mix thoroughly.
Note: The catalytic concentration of the reconstituted G-6-PD enzyme stock solution may necessitate other dilutions. The dilution factor must be changed corresponding to the dilution.

Measurement procedure

Equilibrate only an adequate volume (~ 0.4 ml) of the 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 8 one after another into the cuvette.

Table 8 Analytical system for the measurement of G-6-PD.

2.000 ml	Reaction solution *Equilibrate to 37.0 °C.*
0.100 ml	Diluted enzyme stock solution *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 120 s and monitor time and absorbance for additional 120 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 CK. The result is the catalytic G-6-PD concentration in the diluted enzyme stock solution. For calculation of the catalytic G-6-PD concentration in the enzyme stock solution (G-6-PD_{stock}) this result must be multiplied by the dilution factor:

$F_{dilution}$=2001

Calculation:

$\Delta A/\Delta t_{G\text{-}6\text{-}PD}$: change of absorbance (in s^{-1}) in the reaction mixture after subtraction of the reagent blank rate

$G\text{-}6\text{-}PD_{stock}=3651 \cdot 2001 \cdot \Delta A/\Delta t_{G\text{-}6\text{-}PD}$

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

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

Additional reagents

Adenosine-5′-triphosphate, disodium salt, trihydrate ($C_{10}H_{14}N_5O_{13}Na_2 \cdot 3\ H_2O$), M_r=605.1

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 HK.

Imidazole	100 mmol/l
pH (37 °C)	6.50 ± 0.05*
ATP	10 mmol/l
ADP	2 mmol/l
EDTA	2 mmol/l
Magnesium acetate	10 mmol/l
N-Acetyl-L-cysteine	20 mmol/l
AMP	5 mmol/l
P^1, P^5-Di(adenosine-5′) pentaphosphate (Ap_5A)	0.01 mmol/l
D-Glucose	20 mmol/l
NADP	2 mmol/l
Glucose-6-phosphate dehydrogenase (37 °C)	47 µkat/l (2800 U/l)
Volume fraction of sample	0.0435 (1:23)

* expanded (k =2) combined uncertainty

Table 10 Conditions for the measurement of HK.

Temperature	37.0 °C ± 0.1 °C*
Wavelength	339 nm ± 1 nm*
Band width	≤ 2 nm
Light path	10.00 mm ± 0.01 mm*
Incubation time	180 s
Delay time	120 s
Measurement interval	120 s
Readings (measurement points)	≥ 6

* expanded (k =2) combined uncertainty

Reaction solution

10 ml Solution 1
2.5 ml Solution 3
0.0625 ml water
0.0625 ml G-6-PD solution*
*Lyophilised G-6-PD reconstituted and diluted, see preparation of Solution 2 for CK
– Mix thoroughly.
Stability at 2 °C–8 °C: 1 day

Start reagent solution

69.6 mg (115.0 mmol/l) ATP, disodium salt, trihydrate
– Dissolve in 1 ml water.
Stability: Solution must be prepared freshly.

Dilution of the enzyme stock solution

Add 10 µl of reconstituted HK enzyme stock solution to 20 ml glycerol/water (50:50 v/v) and mix thoroughly.
Note: The catalytic concentration of the reconstituted HK enzyme stock solution may necessitate other dilutions. The dilution factor must be changed corresponding to the dilution.

Measurement procedure

Equilibrate only an adequate volume (~ 0.4 ml) of the 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 one after another into the cuvette as listed in Table 11.

Table 11 Analytical system for the measurement of HK.

2.000 ml	Reaction solution *Equilibrate to 37.0 °C.*
0.100 ml	Diluted enzyme stock solution *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 120 s and monitor time and absorbance for additional 120 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 CK. The result is the catalytic HK concentration in the diluted enzyme stock solution. For calculation of the catalytic HK concentration in the enzyme stock solution (HK_{stock}) this result must be multiplied by the dilution factor:
$F_{dilution}$=2001
Calculation:
$\Delta A/\Delta t_{HK}$: change of absorbance (in s^{-1}) in the reaction mixture after subtraction of the reagent blank rate
$HK_{stock}=3651 \cdot 2001 \cdot \Delta A/\Delta t_{HK}$

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 CK. 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 prispecimens and human sera	Human sera	The reference procedure will be used marily for the investigation of calibration materials and control specimens.
pH value		
The pH optimum is 6.50.	The pH optimum is 6.60.	The shift of the pH optimum with the temperature coincidentally agrees with the shift of the pK value of the buffer.
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.
Incubation time		
180 s	A minimum of 300 s	The time of 180 s is sufficient at 37 °C to reactivate CK with N-acetyl-L-cysteine.

Table 12 Continued.

37 °C Reference procedure	30 °C Reference method	Comment
Measurement time		
180 s	At least 60 s	The time of 60 s is too short for sufficiently precise measurements.
Catalytic HK- and G-6-PD concentration		
HK 66.7 µkat/l (4000 U/l) G-6-PD 46.7 µkat/l (2800 U/l)	HK 50 µkat/l (3000 U/l) G-6-PD 33 µkat/l (2000 U/l)	The same amounts of HK and G-6-PD for 30 °C and for 37 °C. The higher catalytic concentrations are due to the higher temperature.
Sample blank rate		
Not taken into account	Subtraction	Usually, sample blank rates are not subtracted in routine procedures. Therefore, assigned values in calibrators and control materials are only useful for routine methods if they contain the sample blank rate value.
Volumes and composition of the reagent solutions		
Reduction of the number of the solutions and reduction of the volumes	Fifteen different solutions with final volumes up to 1000 ml must be prepared.	There is no need to prepare large volumes of reagent solutions, because the reagent solutions have to be prepared freshly for each campaign. The preparation of the reaction solution (solution R) is simplified. EDTA in Solution 3 stabilizes NAC. The reagent solutions remain stable for one campaign.
Collection of data		
Number of readings ≥ 6	No information except the monitoring the change of absorbance as function of time	Modern spectrophotometers employ a digital data processing. Several readings ≥ 6 shall ensure sufficient precision of the measurement results. Devices for a continuous monitoring are no longer in use.
Determination of the slope (time vs. 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 ≤ 2.25 µkat/l (≤ 135 U/l) Men ≤ 2.92 µkat/l (≤ 175 U/l)	No reference values for the method	In a study, the reference values for women and men were investigated separately.

References

1. Hørder M, Elser RC, Gerhardt W, Mathieu M. Sampson EJ. International Federation of Clinical Chemistry, Committee on Enzymes. Approved recommendation on IFCC methods for the measurement of catalytic concentration for enzymes. Part 7. IFCC method for creatine kinase. Eur.J Clin Chem Clin Biochem 1991; 29:435-56.