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Klinisk kemi i Norden

Tidskrift för Nordisk Förening för Klinisk Kemi

ECCLS

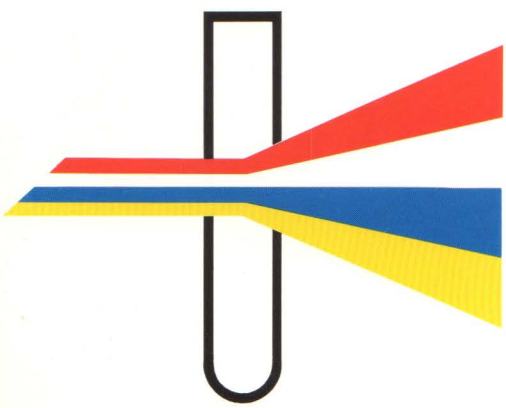
Standards for Enzyme Determination

CREATINE KINASE

ASPARTATE AMINO-TRANSFERASE

ALANINE AMINO-TRANSFERASE

GAMMA-GLUTAMYL-TRANSFERASE



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Supplement
1990

INNEHÅLLSFÖRTECKNING

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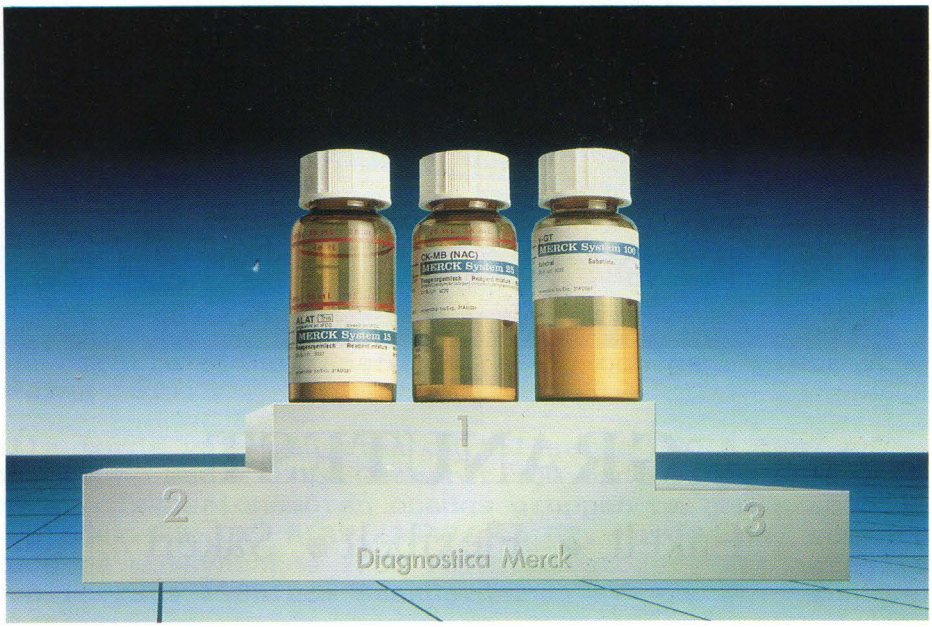

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GT

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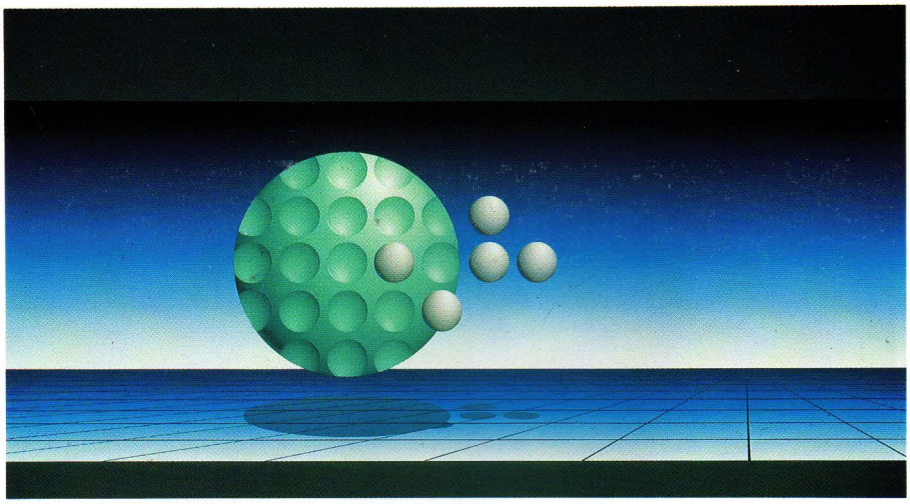

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KLINISK KEMI I NORDEN

Årgång 2, supplement, 1990

Redaktionellt

Här kommer så årets fjärde nummer av Klinisk Kemi i Norden. Emellertid har vi kallat det ett supplement, eftersom det är ett specialnummer. Det upptar enbart artiklar rörande övergången till de nya ECCLS-metoderna för analys av kreatinkinas (CK), alaninaminotransferas (ALAT), aspartataminotransferas (ASAT) och gammaglutamyltransferas (GT).

Som alla torde vara medvetna om tillsatte ECCLS 1987 en ad-hoc grupp, med Willie Gerhardt som ordförande. Efter ett antal diskussionsmöten enades gruppen om gemensamma enzymmetoder för CK, ALAT, ASAT och GT. Metoderna, som är baserade på motsvarande IFCC referensmetoder, har antagits och publicerats 1988 som Standards.

Table 1.
Preliminary reference ranges of the ECCLS methods.
Estimates for groups of healthy individuals. U/L and **µkat/L (bold)**

Reference group	S-ASAT	S-ALAT	S- GT	S-CK*
Adult females	10 - 35	10 - 35	5 - 50	< 150
	0.20 - 0.60	**0.20 - 0.60**	**0.10 - 0.80**	**< 2.50**
Adult males	10 - 50	10 - 50	5 - 80	< 270
	0.20 - 0.80	**0.20 - 0.80**	**0.10 - 1.30**	**<4.50**

* Estimates of upper refererence limits for S-CK from SCE: Experiences with the SCE methods. Scand. J. Clin Lab Invest 1981; 41: 107 - 116.

De stora skillnaderna jämfört med Skandinviska Enzymkommittens (SCE:s) tidigare metoder är att ALAT och ASAT skall aktiveras genom fem minuters förinkubering med pyridoxalfosfat samt att GT analyseras med det karboxylerade, vattenlösliga substratet L-gamma-glutamyl--3-carboxy-4-nitroanilide använt i en optimerad metod.

Under 1989 skickade SCE ett förslag till NFKK om hur en övergång till de nya metoderna skulle kunna ske i Norden. NFKK bad SCE att leda övergången till de nya metoderna. SCE har alltså fortsatt sitt tidigare arbete (trots att kommitten ju egentligen är avskaffad) och kunde vid den nordiska kongressen i klinisk kemi i Trondheim i juni 1990 presentera de förändrade referensintervallen, som de nya metoderna medför (se Tabell 1). På sid. 5 återges resultat av några av SCE:s prövningar av fyra resp. fem kommersiellt tillgängliga reagens för ALAT och ASAT.

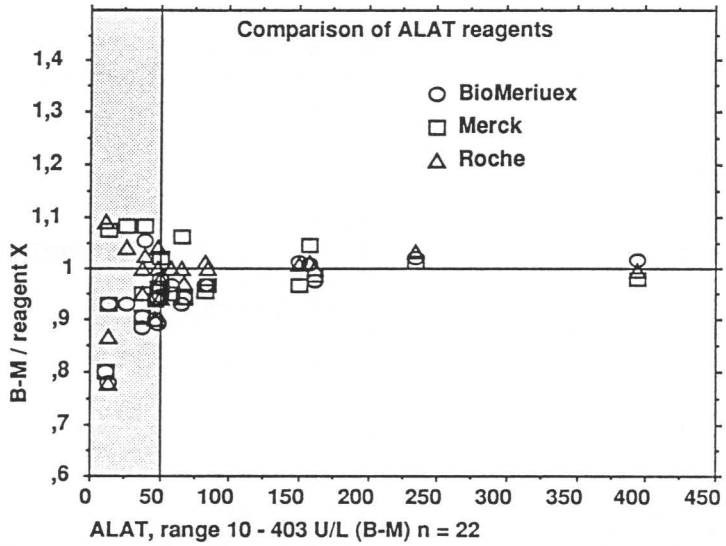
Styrelsen för NFKK har rekommenderat att övergången bör ske under så kort tidsperiod som möjligt och inom perioden: slutet av 1990 - början av 1991.

ECCLS-standarden (Dokument 3-4:1988) publicerades redan 1988. Eftersom den inte alltid är så lätt att få tag på återger vi den i sin helhet i detta nummer av Klinisk Kemi i Norden.

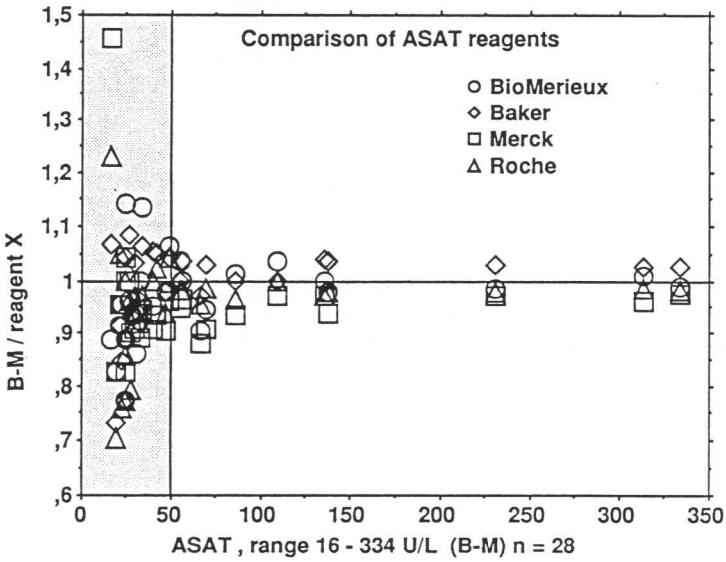
Jag vill samtidigt passa på att tacka den Skandinaviska Enzymkommitten för allt det värdefulla arbete de utfört genom åren. Enzymkommitten, som i sig upptar flera medlemmar i IFCC:s expertgrupp för enzymmetoder, har kommit att bli en mycket känd exponent för nordisk klinisk kemi. Deras arbeten har resulterat i några av de mest betydelsefulla verk, som kommit ut av det samlade nordiska samarbetet.

Er
Kristoffer Hellsing

Preliminary comparison of the performance of three commercial reagents relative to Boehringer-Mannheim (B-M) carried out on 28 random patient sera.
Fraction B-M reagent/reagent X **(Ordinate)** plotted as a function of S-ASAT, resp. S-ALAT (B-M) values U/L **(abscissa).**
The stippled areas show the preliminary reference ranges below 50 U/L. As expected the largest relative scatter (caused by small deviations of catalytic concentrations in absolute terms) occurs within the lower part of the reference range. The scatter decreases considerably with increasing values above the upper reference values of 50 U/L.



A. The median fraction B-M/all reagents = 0.97.
The median fraction B-M/SCE (Hitachi and Cobas Bio) = 1.25.



B. The median fraction B-M/all reagents = 0.97, median fraction B-M/SCE = 1.33.
Data by Strømme and Løhne, Ullevål, Oslo.

*1 – 905050

These standards have been drafted by an ad hoc Committee, set up by the Board of ECCLS and consisting of:

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Preface

The ECCLS subcommittee on enzymes was established in early 1987. In order to have as wide a European basis as possible 10 professionals, 2 industry advisers, and 2 health authority representatives were selected from 10 European nations.

The work was carried out in close co-operation with the ECCLS subcommittee on Enzyme Reference Materials (Chairman D.W. Moss) and the IFCC Expert Panel on Enzymes (Chairman R. Rej, Albany, U.S.A.

The first draft was produced through a number of working group meetings during the spring of 1987. The great amount of experimental work already laid down by the German Commission on Enzymes proved extremely helpful.

Draft 1 was approved at the first full meeting of the subcommittee and the consultants during the ECCLS 8th annual Conference, Salzburg, June 18, 1987.

Draft 1 was circulated to and commented by the ECCLS membership. A number of comments were received and inserted where appropriate.

Draft 1 was revised and approved as draft 2 by the ECCLS subcommittees on Enzyme and Reference Materials and by the IFCC Expert Panel on Enzymes during the I.S.C.E. Congress, Hannover, September 15, 1987.

Draft 2, version 1, was presented and discussed at the Second Meeting of European Clinical Enzymologists, Frankfurt, February 6, 1988, which was attended by 34 specialists in the field.

The present standard was finally revised according to comments received during and after the Frankfurt meeting.

The chairman wants to thank the subcommittee members and advisers for their dedicated work.

Special thanks are directed to A. Burlina, Universitá di Padova, J.C.M. Hafkenscheid, Sint Radboudziekenhuis, Nijmegen, and W. Stein, Medizinische Universitätsklinik IV, Tübingen who - with their specialist knowledge - have given a great contribution to the present standards.

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Related Publications from Standard Setting Organizations

ECCLS Guidelines for Temperature Control in Clinical Chemistry.
1st Draft (To be published)

ECCLS Standard for Enzyme Calibration Materials and Control Materials.
ECCLS Document ISSN 1011-6265; 1988:5

IFCC Expert Panel on Enzymes. Approved recommendation (1978) on IFCC Methods for the Measurement of Catalytic Concentration of Enzymes. Part 1. General Considerations Concerning the Determination of Catalytic Concentration of an Enzyme in the Blood Serum or Plasma of Man.
Clin Chim Acta 1979; 98: 163F - 174F

IFCC Expert Panel on Enzymes. IFCC Method for Gamma-glutamyltransferase,
EC 2.3.2.2, Stage 2, Draft 2, 1983.
J Clin Chem Clin Biochem 1983; 21:633-646

IFCC Panel on Instrumentation and Expert Panel on Enzymes. Guidelines for Classification , Calculation and Evaluation of Conversion Rates in Clinical Chemistry.
J Clin Chem Clin Biochem 1985; 23: 493 - 503

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J Clin Chem Clin Biochem 1986; 24: 497 - 510

IFCC Expert Panel on Enzymes. Part 3. IFCC Method for Alanine Aminotransferase (EC 2.6.1.2). Approved recommendation (1985) J Clin Chem Clin Biochem 1986; 24: 481 - 495

IFCC Expert Panel on Enzymes. IFCC Methods for Measurement of Enzymes. Part 6. Development of Methods and the Role of Reference Materials for Enzyme Measurements.
J Clin Chem Clin Biochem (in preparation)

IFCC Expert Panel on Enzymes. IFCC Methods of Measurement of Catalytic Concentration of Enzymes. Part 7. IFCC Method for Creatine Kinase (EC 2.7.3.2) Stage 2, Draft 2
J Clin Chem Clin Biochem (in press)

NCCLS Proposed Guideline: User Comparison of Quantitative Clinical Laboratory Methods using Patient Samples.
NCCLS Document 1986, EP9-P

NCCLS Methodological Principles for Selected Analytes: Enzymes; Proposed Guideline
NCCLS Document 1987, C5-P2

In NCCLS/NRSCL: Hørder M. Enzyme Reference Materials - Current Status. A Reference System for Clinical Enzymology (eds.: Bowers G N, Edwards G C and Rand R N) 1985

General Introduction

The results of measurements of the catalytic activity concentration of enzymes used in clinical chemistry are method dependent. Consequently, different methodologies yield not only numerically different values but vary in their analytical bias for different isoenzyme composition of samples (example: alkaline phosphatases, EC 3.1.3.1, lactate dehydrogenases, EC 1.1.1.27, and amylases, EC 3.2.1.1), activation of enzymes and suppression of interfering enzyme reactions (example: creatine kinase, EC 2.7.3.2), measurement of preformed holoenzyme or of all preformed and activated apoenzymes (examples: aminotransferases, EC 2.6.1.1 and 2.6.1.2.)

This dependence of results on methods has long been recognized as a barrier to the optimal clinical use of enzyme assays.
The present profusion of different enzyme methods has led to a bewildering variety of reference ranges for one and the same enzyme and therefore to difficulties in the interpretation of results, a less efficient use of the literature of clinical enzymology, and difficulties in external quality assessment programmes.

The problem has been alleviated to a large extent by the introduction of agreed or recommended methods, at National and International levels. These initiatives have demonstrated both the acceptability and the effectiveness of recommended methods.

However, the user is faced with a choice of different recommendations, although their differences in performance may, in many cases, be clinically insignificant.

Furthermore, no clear guidance exists as to how far defined methods may be modified in the interest of analytical convenience (e.g. in such aspects as relative sample and reagent

volumes, timing of reactivation and of measurements) while still producing comparable results.

Scope of ECCLS Standards for Enzyme Determinations in clinical laboratories.

The **ECCLS** Standards for enzyme determinations are established to ensure day-to-day intra- and inter-laboratory consistency of results leading to clinical information of improved value, more clearly defined reference ranges and greater comparability among laboratories and external quality assessment programmes.

The **ECCLS** Standards are intended as consensus standards detailing reaction conditions for measurement of the catalytic activity concentration of creatine kinase (CK), aspartate and alanine aminotranferases (ASAT and ALAT), and gamma-glutamyl transferase (GT) in human serum, and are intended for the assistance of manufacturers, professional societies and individual professionals in laboratory medicine, health agencies, and organizers of external quality assessment programmes.

Successful implementation of this Standard requires the assistance of reagent and instrument manufacturers, and of health authorities, and the availability of enzyme reference preparations with properties conforming to the specifications of the ECCLS Subcommittee on Enzyme Reference Materials.

Direct comparability of results of measurements of catalytic activity concentration of enzymes depends primarily on agreement on the following:

1. Chemical Composition of the Reaction Mixture

2. Reaction Temperature (cf. Convertibility, below).

3. Units of Catalytic Activity Concentration.

1. In clinical enzymology, the rigorously defined **IFCC Reference Methods** take the place of the primary reference materials used in the determination of non-enzyme analytes.

Whereas the procedures of the IFCC reference methods themselves considerably restrict their use as routine methods, it is possible to describe a **Simplified Standard Adaptation** of each of these methods that readily permits its implementation as an everyday routine method but still retains an "accuracy" and precision close to that of the unmodified IFCC Reference Method.

The present **ECCLS Standards for Enzyme Determination** do **not** represent a further set of "new" recommendations, but they are rather a codification of sound, practical analytical procedures based on existing knowledge and practice.

They draw on the existing body of knowledge on enzyme methods developed by the Expert Panel on Enzymes **(EPEnz)** of the IFCC and by the National Enzyme Committees. They specify allowable limits of procedural modifications in terms of performance specifications to provide routine enzyme assay methods which can readily be adopted in European clinical laboratories. Most of the procedural changes proposed represent minor variations that are already routine practice in most countries.

2. These proposed **ECCLS** Standards are based firmly on the reaction conditions of the corresponding IFCC Reference Methods. The main difference is the **acceptance of use of the widely used reaction temperatures of 37 °C and of 30 °C.**

In 1978 the **Council of the IFCC** approved by mail ballot the recommendation that, with as few exceptions as possible, the IFFC methods should be carried out at a uniform reaction temperature of 30 °C (1).
A reaction temperature of 30 °C has been officially recommended in **France** (2) and in **The Netherlands** (3).

In 1981 the **Expert Panel on Instrumentation** recommended to the Scientific Committee of the IFCC that 37 °C should be endorsed as the preferred incubation temperature. In 1982, an international survey of opinion of preferred reaction temperature for measurement of enzymes was carried out by the IFCC Expert Panels on Instrumentation and on Enzymes. This survey indicated a preference of 30 °C for reference, and 37 °C for routine methods (4).

At present (1987) a reaction temperature of 37 °C has been officially endorsed in the following countries: **Belgium, Denmark, Sweden, Norway, Finland** (5a-c), **Italy** (6), **Spain** (7), **Switzerland** (8), and in **Eastern Europe** from 1988 (9). The majority of laboratories in the **UK** measure at 37 °C (10 a,b). **The Federal Republic of Germany** intends to recommend 37 °C (11).

The list above is incomplete. Furthermore, even within the countries mentioned, **not all laboratories** comply with the National recommendations for reasons related to their available resources, e.g. instrumentation.

Consequently, it appears doubtful that European unification in the matter of reaction temperature can be obtained at present.

However, different practices with respect to temperature need not prevent unification of all other measurement conditions through the implementation of the present **ECCLS Standards for Enzyme Determination.**

In routine practice, results including those of external quality assessment should be reported at the reaction temperature at which they were obtained.

Although wide practical experience indicates that when the same methods are used, results obtained with clinical serum specimens at one of the two reaction temperatures, may indeed be converted to the other temperature without introducing clinically significant errors, routine use of temperature conversion factors cannot be recommended. As examples, the average temperature conversion factors 37 °C/ 30 °C presently used in Switzerland (CH) and in The Netherlands (NL) are: for CK 1.6 (CH and NL); see also macro CK, type II: about 2.8, p. 15; for ASAT: 1.50 (CH) and 1.58 (NL); for ALAT 1.50 (CH) and 1.42 (NL); for GT 1.35 (CH) and 1.28 (NL). The differences in some cases exceeds 5%. However, comparison among literature data from different countries may be useful provided data on calibration procedures and materials are given.

3. Throughout the description of this ECCLS Standard the SI coherent unit for catalytic activity concentration, **µkat/L** (12,13) is used. For convenience the equivalent value in **U/L** is also given.

1 µkat/L = 60 U/L

1 U/L = 16.7 nkat/L

Results should be reported in the IFCC format:

system - component, kind of quantity = numerical value x unit

Determination of the Catalytic Activity Concentration in Serum of Creatine Kinase (EC 2.7.3.2, CK)

Based on the Provisional IFCC CK Reference Method (14)

This ECCLS Standard is based on the corresponding IFCC reference Method for determination of CK, which has been adapted by this ECCLS Committee for routine use in European clinical chemistry laboratories. This Standard recommends reaction conditions and describes the tolerance limits for variations in measurement procedures that nevertheless allow routine determinations of the catalytic activity concentration of CK to be equivalent to those of the unmodified IFCC CK Reference Method.

For details not given in this Standard refer to the original IFCC document, CK, part 7 of the IFCC methods for measurement of catalytic concentration of enzymes (14).

1. REACTION PRINCIPLES

Creatine kinase (ATP:creatine N-phosphotransferase, EC 2.7.3.2, CK) catalyzes the dephosphorylation of creatine phosphate by Mg^{2+}-ADP complexes.
The method comprises three coupled enzyme reactions (15,16):

$$\text{Creatine phosphate} + \text{ADP} \xrightleftharpoons[\text{EC 2.7.3.2}]{\text{Creatine kinase}} \text{Creatine} + \text{ATP} \qquad \text{(I)}$$

$$\text{ATP} + \text{D-Glucose} \xrightleftharpoons[\text{EC 2.7.1.1}]{\text{Hexokinase}} \text{ADP} + \underline{\text{D}}\text{-Glucose-6-phosphate} \qquad \text{(II)}$$

$$\text{Glucose-6-phosphate} + \text{NADP}^{+} \xrightleftharpoons[\text{EC 1.1.1.49}]{\text{Glucose-6-phosphate-dehydrogenase}} \text{D-Glucono-lactone-6-phosphate} + \text{NADPH} + \text{H}^{+} \qquad \text{(III)}$$

The equilibrium of I favours the formation of creatine and ATP at pH values around 6 - 7 (14).The true substrates are the Mg^{2+}-ADP complex and free creatine phosphate (CP). For the sake of practicability concentrations are given below as total ADP, Mg, and creatine phosphate.

The primary reaction (I) is coupled through the auxiliary hexokinase reaction (II) to the NADPH-forming indicator reaction (III) catalyzed by glucose-6-phosphate dehydrogenase.
For increased precision at low catalytic activity concentrations an additional reaction IV has been proposed comprising 6-phosphoglucono lactonase (EC 3.1.1.31) and 6-phosphogluconate dehydrogenase (EC 1.1.1.43) (17).

1.1 ECCLS Measurement Procedure:

Substrate start, 37 °C and 30 °C.

The component concentrations in the final reaction mixture are the same as those of the **IFCC CK Reference Method**. The reaction system performs equally well at 37.0 °C and 30.0 °C. The standard reaction conditions have been defined at 37 °C in Table 1 below.

The imidazole buffer has a temperature dependence similar to that of the catalytic activity of the human CK isoenzymes MM, MB, and BB (5b, 18). Consequently, the same reaction system, adjusted to a pH of 6.60 at 30 °C will change to a pH of 6.5 0.02 at 37 °C supporting the reaction equally well at both temperatures (18).

The standard procedure involves two stages (Table 1). In the first, the sample is incubated with the reagent mixture without creatine phosphate (**reagent A**). CK molecules are activated during this phase. The catalyzed reaction is initiated by addition of creatine phosphate (**reagent B**).

The catalytic activity concentrations of the auxiliary enzymes are determined as described in the IFCC Reference CK Method at 30 °C and 37 °C. The average temperature conversion factors 37 °C/30 °C were found to be 1.30 for Hexokinase and 1.40 for Glucose-6-P-dehydrogenase (G-6-PDH) (19 a,b).

The values in Table 1 correspond to those obtained at 37 °C. It should be noted that most commercial reagents contain excess amounts of enzymes to ensure a realistic shelf-life.

Table 1. Concentrations are specified in the final reaction mixture at 37 °C, to ensure that maximal CK catalytic activity will be supported at both 37 °C and 30 °C.

Stage A. Incubation-activation with reagent A.			
Volume fraction in:			
incubation-activation stage	0.952		
complete reaction mixture	0.870		
			Example of volume (µL):
Imidazole pH 6.50	100.0	mmol/L	
EDTA	2.0	mmol/L	
N-acetylcysteine (NAC)	20.0	mmol/l	
Magnesium acetate	10.0	mmol/L	
ADP	2.0	mmol/L	1000 µL
AMP	5.0	mmol/L	
P1,P5-diAP	10.0	µmol/L	
D-Glucose	20.0	mmol/L	
NADP	2.0	mmol/L	
Hexokinase (4000 U/L)	≥ 65	µkat/L	
G-6-PHD (2800 U/L)	≥ 46	µkat/L	
Add sample.			
Volume fraction in reagent A:	0.0476		50 µL
Volume fraction in complete mixture	0.0435		
Mix carefully. Prior to start of the reaction the CK enzyme proteins are maximally activated by N-acetylcysteine within 180 s. Within this time thermal equilibration has to be reached.			
Stage B.			
Substrate start with reagent B:			
Creatine phosphate.			
Volume fraction	0.087		
Creatine phosphate	30 mmol/L		100 µL
Mix carefully. After a lag phase of up to 60 s a constant reaction rate is achieved that is monitored for the minimum time and number of data points to obtain acceptable precision around the upper reference limit.			

Wavelengths, pathlengths, final volume of reaction mixture: Instrument dependent, see below.
Reaction temperature: Strictly controlled at 37 °C or 30 °C with a maximal allowed deviation of ± 0.1 °C (20).

1.2 Specimen.

Serum is the preferred specimen. Use of EDTA plasma has been shown to give rise to unpredictable interferences (3,21,22). Hemolytic specimens may cause falsely increased values.

2. ACCEPTABLE VARIATIONS FROM ECCLS MEASUREMENT PROCEDURE

2.1 Performance Specifications

Deviations from the above measurement procedure may endanger the robustness of the method and should be kept as small as possible. However, for adaptation to routine use with different types of analyzers acceptable variations for sample and reagent volume fractions, component concentrations, substrate and serum start, activation, lag-phase, and measurement times will be described below.

Any changes in the reaction system must be documented by their proposers. The average total deviation of results must be less than 5% from those obtained with the IFCC Reference CK Method at the same temperature. This should be shown for sera with catalytic activity concentrations close to the upper reference limit and also for samples with elevated activities up to the limit of linearity of the method.

Individual sera with CK isoenzymes in clinically relevant proportions should be used as well as appropriate certified reference enzyme materials (ECCLS, BCR, NBS) when available. Evidence on the performance of the proposed methodology change should include the effects on sample and reagent blank rates.

NCCLS Guidelines on Method Comparison (23), the French Protocol for Validation of Methods (24) or the procedures according to Passing and Bablok (24 a) should be followed.

2.2 Measurement at 37 °C and 30 °C

The ECCLS Standard Method performs equally well at either reaction temperature. **In routine use and quality assessment results should be reported at the temperature at which the measurements were made.**

2.3 Temperature Conversion Factors

However, as discussed in the Introduction, conversion factors may be of use for comparison of literature data. In evaluating such data the choice of the calibration material and isoenzyme compositions must be considered. The different CK isoenzymes and MM variants all have different conversion factors 37 °C/30 °C (25-27):

	CK	MM1	MM2	MM3	MB	BB	macro type I	macro type II
37 °C/30 °C	1.82	1.70	1.55	1.68	1.74	1.52	2.78	

The prevalence of macro CK type II is about 3% of all hospitalized patients. Clinically, however, it is only 0.5% in sera with increased total CK catalytic activity concentration from patients suspected of an acute myocardial infarction (24).

As an example, the average conversion factors recommended in Switzerland and in The Netherlands are 1.60 and 1,59 respectively (8).

2.4 Concentrations of Components in the Reaction Mixture

Concentrations in the complete reaction mixture are dependent not only on the amounts in the solution(s), but also on the relative volumes of different solutions used to achieve the final reaction mixture, as well as on the relative volume of the sample.

An example of changes in relative reagent volumes in routine practice arises in the adaptation of some commercial reagents to automatic analyzers that add water to flush pipetting needles, thus diluting the reaction mixture at the incubation and/or reaction start stages. This causes dilution of the premade buffer solution and of the components therein. According to e.g. the DIN norm, followed by several manufacturers, the imidazole buffer solution contains Mg^{2+}, EDTA, and glucose. Dilution of the buffer with water causes corresponding dilution of these components, e. g. to 80% of the specified concentrations.

The relative volumes of reagents should be kept as close as possible to those of the ECCLS Standard (Table 1).

If, however, it can be documented that the resulting changes of reaction rates lie within the performance specification given in **2.1** such adaptation is acceptable. This should be documented by the manufacturer according to **2.1**.

In practice, the relative volumes of reagents A and B in the reaction mixture may be varied within certain limits. However, whatever volumes are chosen, the **concentrations** of individual reagents must be adjusted so that the final concentrations in the complete reaction mixture remain as close as possible to those of the Standard. Especially important are the concentrations of NAC, substrates, and auxiliary enzymes.

For example, several commercial lyophilized CK reagents are designed for a standard volume fraction of reagent A of 0.87. If the instrument uses a volume fraction of 0.70, correction is easily achieved by dissolving the lyophilized reagent A in the appropriate smaller volume, i. e. 0.70/0.87 x the solvent volume recommended by the manufacturer. This correction cannot be applied where reagents are supplied in the form of solutions. Therefore, evidence should be provided that the reagent still fulfills the performance specifications given in **2.1.**

2.5 Minimum Activation Time

The catalytic activity of CK enzyme proteins in serum decreases with time. Using the IFCC reagent (Table I), incubation of sample with N-acetylcysteine (23 mmol/L) in reagent A for about 180s during heating to the reaction temperature 30 °C or 37 °C activates CK catalytic activity completely in sera stored for two days at 4 °C (11, 26, 30).

In practice, activation proceeds during the cumulated times of pipetting of remaining samples, starting reagent and lag phase. By definition, both **activation and lag phase may be considered completed when constant rate is observed.**

The necessary activation time will vary with the **degree of prior inactivation** during storage of the sample. For instance, samples analyzed on the same day as they were taken, or after less than 2 days' storage at 4 °C require only a very short activation time. Activation during 180 s appears satisfactory for the majority of sera at both temperatures (26, 30).

Data with serum start indicate that the combined activation and lag-phase with such sera may be as short as 180 s at 37 °C when reaction conditions are otherwise kept within the specifications of the ECCLS Standard (26).

However, samples stored for **longer periods**, macro CK type 2, and certain lyophilized controls (30,31) may require a longer activation time, up to the full 300 s stated by the IFCC Reference Method (14).

2.6 Start of Reaction with Serum

Starting the reaction with serum has the following disadvantages:

1.The CK reaction proceeds at accelerating rate during the combined times of activation and lag phase accumulating inhibitory NADPH leading to high absorbance values. This decreases the analytical range of direct measurement of CK catalytic concentrations to about 15 µkat/L (900 U/L), i.e. about half of that which may be measured in the substrate start mode without dilution of samples.

2.When determining the residual catalytic concentration of CK MB after immunoinhibition of the CK M subunits (28,29):

a: The immunoinhibition of CK M-subunits may proceed more slowly and less completely in the presence of the substrate, requiring up to 10 min incubation in the presence of creatine phosphate compared to about half that time in its absence.

b: There is no possibility of measuring and substracting sample residual adenylate kinase (AK EC 2.7.4.3) blank reaction rates. Omission of this correction leads to falsely increased CK MB results in cases with increased liver AK or erythrocyte AK in hemolytic specimens (29).

3. Results of measurements made with serum start on patients' sera may be about 2% lower than those made with substrate start, and those made on certain control sera may be even lower (19 a, 31).

Nevertheless, some analyzers in routine use today cannot perform the recommended two-step method. For determination of the catalytic concentration of **total** CK, serum start may be considered acceptable, provided that the user is aware of the greater limitations in the direct measurable range of activities, and that the instrument's constant rate linearity finding programme is adequate.

2.7 Minimum Lag-Phase Time

At the IFCC and ECCLS standard sample volume fraction of 0.044 and under the standard reaction conditions a lag phase of up to 60 s appears satisfactory for the majority of samples at both reaction temperatures (26, 30).

2.8 Minimum Measurement Time

This is highly dependent on the catalytic activity concentration in the sample and the instrument. The only general guideline that can be given is that the reaction should be monitored for the time and number of data points required to obtain acceptable precision at the upper reference limit with the instrument's constant rate finding programme. Manual instruments may require longer measuring times at low catalytic activity concentrations.

Provided that the spectrometer is capable of accurate absorbance measurements of up to 2.000, increases of absorbance up to about 0.01 per second (about 40 μkat/L, 2400 U/L) may be measured. Beyond this rate samples must be diluted, with NaCl, 150 mmol/L.

2.9 Sample Volume Fraction

The effects of varying the volume fraction of sample are much more pronounced than variations in the relative volumes of reagents A and B. Only within a relatively narrow range is there proportionality between the serum volume fraction and the reaction rate (3, 5b-c, 11, 14, 21, 26).

We recommend that the sample volume fraction should remain within the limits specified in Table 2. Furthermore it must be remembered that any **predilution of sample** amounts to a major alteration of the sample volume fraction. The user should be aware that the dilution curves of human serum will be non-linear in several cases .

The sample volume fraction effects may vary for individual sera, and the values in Table 2 actually represent "the worst case". Sample volume fractions between 0.02 and 0.08 will lie within the ± 5% variation specified in **2.1 provided no other changes from the reaction conditions in Table 1 are made.** (3, 5b-c, 11, 14, 21, 26).

Table 2

Serum Volume Fraction	0.01	0.02	0.04	0.08	0.09	0.22	0.25
Relative Reaction Rate	1.10	1.05	1.00	0.95	0.90	0.80	0.70

3. REACTION BLANK RATES

3.1 Reagent Blank Rates

A reagent blank reaction rate higher than an absorbance increase at 340 nm of 0.001 per 60 s is considered unacceptable. In routine practice correction for reagent blank rate is therefore unnecessary.

3.2 Sample Blank Rates

A sample blank rate may be caused by adenylate kinase, which catalyzes the reversible reaction of ADP to ATP and AMP. AK in serum may come from the following sources:
erythrocytes, skeletal muscle, thrombocytes, and liver cells (2b,5b, 14, 18, 32, 33). The IFCC CK Reference Method describes the efficiency of the AK inhibitor combination of AMP, 5

mmol/L, and diadenosine-5′-penta-phosphate, 10 µmol/L (14, 18). A sample blank is not required for routine determination of **total** catalytic activity concentration of CK in the absence of gross hemolysis and acute liver damage.
However, if the method is varied in any way from that described, the validity of omitting sample blank corrections must be established. Cf. **2.1.**

4. CALCULATION OF CATALYTIC ACTIVITY CONCENTRATION

For routine determination of the catalytic activity concentration of CK in serum corrections for reagent blank and sample residual adenylate kinase blank rates may be omitted.

4.1 Molar Absorption Coefficient of the Monitored Product

The molar absorption coefficients of NADPH are: 6180 $m^2 \times mol^{-1}$ at 334 nm, 630 $m^2 \times mol^{-1}$ at 340 nm, and 350 $m^2 \times mol^{-1}$ at 365 nm.

4.2 Calculation at Constant Pathlength

Using measurement at **340 nm as an example**, the catalytic concentration of CK is calculated as follows:

Symbol	Type of Quantity	Units
b	Catalytic activity concentration	µkat/L
i	the increase in 340 nm absorbance per second	s^{-1}
ϵ	Molar 340 nm absorption coefficient of NADPH = 630	$m^2 \times mol^{-1}$
l	Light pathlength 0.01	m
v	Sample volume fraction: 0.04348 vol(L)/vol(L)	1

4.2.1 Calculations for a Pathlength of 0.01 m

$$b = \frac{i}{\epsilon \times l \times v} = \frac{i}{630 \times 0.01 \times 0.04348} \times \frac{s^{-1}}{m^2 \times mol^{-1} \times m}$$

$$b = \frac{i}{0.27392} = i \times 3.651 \times \frac{mol \times s^{-1}}{m^3}$$

$$b = i \times 3.651 \times kat/m^3$$

$$b = i \times 3651\ \mu kat/L$$

Calculated for a Measuring Time of 60 s:

$$b = i \times 3651 \times \mu mol \times (60s)^{-1} \times L^{-1}$$

$$b = i \times 3651\ U/L$$

$$b = i \times 60.85\ \mu kat/L$$

To convert from µkat/L to U/L multiply by 60.

To convert from U/L to µkat/L divide by 60.

4.2.2 Calculation for Other Pathlengths and for Other Volume Fractions:

Insert the pathlength (unit: meter) and the volume fraction (unit L) in the equation instead of the figures given above.

4.3 Calculation at Variable Pathlengths (longitudinal measurement)

Certain (centrifugal) analyzers measure longitudinally through the cuvettes. The light path thus becomes proportional to the total assay volume in the cuvette. Dilution of the assay medium in the cuvettes with an inactive fluid causes a corresponding elongation of the light path, and the absorbance remains constant. The net effect is that the calculation factor becomes proportional to the sample **volume** and independent of the sample volume **fraction** in the cuvette (34). In the example below the sample volume is 10 µL.

Symbols	Type of Quantity	Example	Unit
a	Area of cuvette end	0.25×10^{-4}	m^2
v	Sample volume	10×10^{-6}	L
i	equals increase in absorbance at 340 nm per s		s^{-1}
	Other symbols as given above.		

$$b = \frac{i \times a}{\varepsilon \times v} = \frac{i \times 0.25 \times 10^{-4}}{630 \times 10 \times 10^{-6}} \times \frac{s^{-1} \times m^2}{m^2 \times mol^{-1} \times L}$$

$$b = \frac{i \times 25}{6300} \times \frac{mol \times s^{-1}}{L}$$

$$b = i \times 0.003968 \times mol \times s^{-1} \times L^{-1}$$

$$b = i \times 0.003968 \text{ kat/L}$$

$$b = i \times 3968 \text{ µkat/L}$$

Calculated for a Measuring Time of 60 s:

$$b = i \times 3968 \text{ U/L}$$
$$b = i \times 66.13 \text{ µkat/L}$$

5. CALIBRATION BY REFERENCE ENZYME MATERIALS

Certain analyzers require the use of calibrators rather than calculation on the basis of the molar linear absorbance coefficient. Provided that the reagent and measurement procedures are in accordance with these ECCLS specifications calibration may be carried out using a reference enzyme preparation, e.g. BCR, ECCLS, NCCLS or equivalent fulfilling the performance criteria set for these materials (23, 24, 35-37). For details refer to ECCLS Reference Enzyme Materials, Draft 2, 1987 (35).

6. REFERENCE VALUES

Provided this standard is followed the variability of reference values should depend only on the individuals selected in different studies. Therefore, the population selection criteria, measurement temperature and, if temperature conversion factors have been used, detailed description of the calibration procedure and calibration material should be given to allow comparison.

7. WITHIN-LABORATORY IMPRECISION

This should be similar to that described for the IFCC CK Reference Method (14).

8. REAGENT SPECIFICATIONS

Refer to the IFCC CK Reference Method (14).

9. RELATIONSHIP TO OTHER METHOD PRINCIPLES

Instruments with specific instrument-dependent methods which do not fall within the tolerance limits of this ECCLS Standard Method may nevertheless be able to produce numerically equivalent results by a calibration process. In such cases the manufacturer should document that the methods fulfill the performance criteria given in 2.1 of these ECCLS Standards. The validation of calibration of enzyme methods is discussed in ECCLS Reference Enzyme Materials, Draft 2, 1987 (9).

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Determination of the Catalytic Activity Concentration in Serum of L-Aspartate Aminotransferase (EC 2.6.1.1, ASAT)

BASED ON THE IFCC ASAT REFERENCE METHOD (1)

This ECCLS Standard is based on the corresponding IFCC Reference Method for determination of ASAT, which has been adapted by this ECCLS Subcommittee for routine use in European clinical laboratories as described for CK in the General Introduction to the ECCLS Standards.

This Standard recommends reaction conditions and describes the tolerance limits for variations in measurement procedures that nevertheless allow routine determinations of the catalytic activity concentration of ASAT to be equivalent to those of the unmodified IFCC ASAT Reference Method.

For details not given in this Standard refer to the original IFCC document, ASAT, part 2 of the IFCC methods for measurement of catalytic concentration of enzymes (1).

Activation of apo-ASAT and apo-ALAT with pyridoxal-5′-phosphate

The IFCC ASAT and ALAT Reference Methods include activation of the apo-enzymes by pyridoxal-5′-phosphate (P-5′-P, 1,2). After careful consideration of the advantages and disadvantages of this procedure the ECCLS Subcommittee decided to include this step.

Addition of pyridoxal-5′-phosphate to serum activates the apo-enzymes and permits the measurement of **total** ASAT and ALAT catalytic activity concentrations in serum. Provided the saturation with P-5′-P is complete, this eliminates between-sample variations in the proportion of apo-enzyme. The average increases relative to measurements of ASAT and ALAT without activation are in the range 1.3 - 1.5 and 1.2 - 1.4, respectively (1 - 10 a,b).

However, measurement of P-5′-P activated ASAT and ALAT changes reference ranges previously established in the absence of P-5′-P, since some apo-enzyme is almost always present in healthy individuals. As ASAT and ALAT are activated to different degrees, clinically useful ratios between the ASAT, ALAT, and other enzymes are also changed. In the majority of cases clinical information is not significantly increased through the use of P-5′-P compared with that obtained by methods in which this coenzyme is not added. This applies also to patients suffering from the relative deficiencies in B-vitamins that may be encountered in Europe. However, the normal or abnormally low levels of ASAT observed in some severely ill patients in the absence of added P-5′-P may be converted to abnormally raised values in assays with the added coenzyme (4, 8, 9). Saturation of apo-ASAT and apo-ALAT in human sera may be obtained in three ways, and different procedures have been chosen in various National recommendations (7 - 16).

1. Incubation of sample in P-5′-P, 100 umol/L in reagent A. Start with 2-Oxoglutarate (1, 2, 5-13)

Advantage: Complete activation in the majority of sera. Can be automated.

Disadvantage: Prolongs incubation-activation time to 5 minutes.

2. Incubation of sample in complete reagent (8, 14, 15)

Advantage: Serum start may be used.

Disadvantage: Complete reactivation is not reached in all sera.

3. Addition of P-5′-P, 250 umol/L in TRIS buffer to sample (7-9, 16, 17)

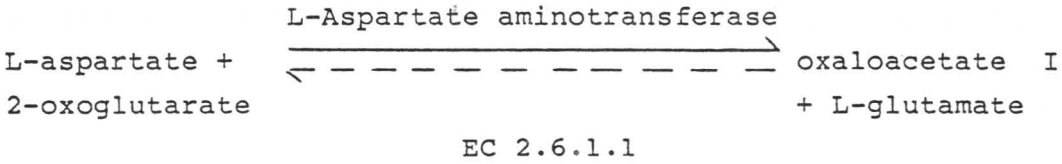
Advantage: Complete activation.
Serum start is possible.

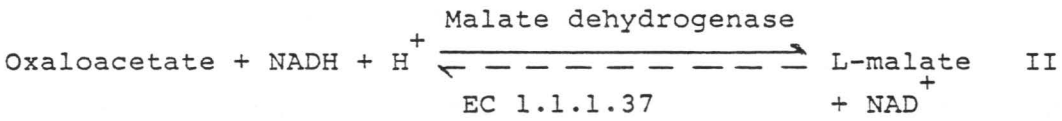
Disadvantage: Requires an additional, manual analytical step.
The activation time may be as long as 30 minutes, and the lag-phase increases to 5 minutes or more for some sera (5, 8, 9)
Increased absorbance at 405 - 410 nm.

The time required for complete activation varies considerably among individual sera. In the **IFCC ASAT and ALAT Reference Methods** procedure 1 and an activation time of 10 minutes were recommended. However, activation by procedure 1 will be complete for the majority of sera within 5 minutes. Accordingly, this activation time was selected for the ECCLS Standard Methods 2 - 3 for ASAT and ALAT determinations.

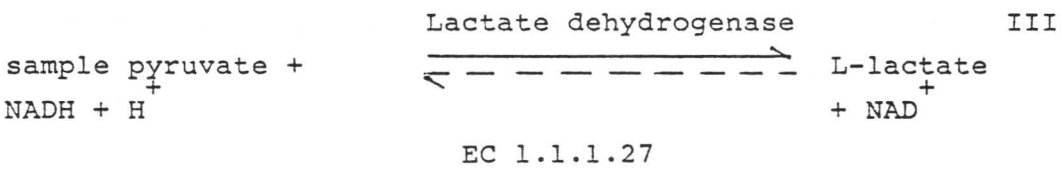
1. REACTION PRINCIPLES

The reactions catalyzed by ASAT (I) and the indicator enzyme (II) are as follow (1 - 18):





During the incubation-activation of sample with reagent A (see below):



The apoenzyme of ASAT is activated by addition of pyridoxal-5'-phosphate before measurement (1). The sample pyruvate + $NADH + H^+$ reaction catalyzed by sample lactate dehydrogenase (LDH) reaches completion during the incubation period through the inclusion of additional LDH in the incubation reagent (1, 7 - 16). The equilibrium of the indicator reaction (II) lies far toward the production of L-malate. Therefore, the equilibrium of the ASAT reaction (I) is not relevant, and the catalytic concentration of ASAT in the sample is determined by the conversion rate of NADH (1).

1.1 ECCLS Measurement Procedure:

Substrate start, 37 °C and 30 °C.

The component concentrations in the final reaction mixture are the same as those of the **IFCC ASAT Reference Method**. The standard reaction conditions have been defined at 37 °C in Table 1 below. The reaction system performs equally well at 30 °C. Optimum pH (30 °C) for ASAT in serum lies between pH 7.6 and 8.0 (1, 10).

The pH of the TRIS-HCl buffer decreases 0.15 for an increase in temperature from 30 °C to 37 °C (9). Consequently, the same reaction system, adjusted to 7.80 at 30 °C, will change to a pH of 7.65 ± 0.02 at 37 °C. However, due to the broad pH optimum described in the IFCC ASAT Reference Method (1, 10), this shift from optimum pH does not decrease the ASAT catalytic activity concentration by more than about 1%. Consequently, for routine use the same TRIS-HCl buffer adjusted to pH 7.80 (30 °C) will support the reaction with no significant loss at either reaction temperature.

The ECCLS Standard Procedure involves two stages (Table 1). In the first, the sample is incubated with **reagent A**, which contains pyridoxal-5′-phosphate but not 2-oxoglutarate. Apo-ASAT is reconstituted during this phase, and the LDH-catalyzed NADH reaction with sample pyruvate reaches completion.

In the second stage, the ASAT-catalyzed reaction is initiated by addition of 2-oxoglutarate (**reagent B**).

Table I. Concentrations apply to the complete reaction mixture and are specified at 37 °C, where conversion rates and Michaelis constants are higher, to ensure that maximal ASAT catalytic activity will be supported at both 37 °C and 30 °C.

A buffer adjusted to pH 7.80 (30 °C) may well be used. The catalytic activity concentration of the malate and lactate dehydrogenase have been determined as described in the IFCC ASAT Reference Method at 30 °C and 37 °C. The average temperature conversion factors are found to be 1,50 for Malate dehydrogenase and 1.35 for Lactate dehydrogenase (17). The values in Table I correspond to those obtained at 37 °C.

Stage A. Incubation-activation reagent A.		
volume fraction in: incubation-reactivation stage :	0.91	
complete reaction mixture	0.833	
		Example of volume (µL)
Tris(hydroxymethyl)aminomethane buffer pH 7.65	80 mmol/L	
L-Aspartate	240 mmol/L	
NADH	180 µmol/L	500 µL
Pyridoxal-5′-phosphate	100 µmol/L	
Malate dehydrogenase (600 U/L) >	10 µkat/L	
Lactate dehydrogenase (900 U/L) >	15 µkat/L	
Add Sample.		
Volume fraction in reagent A:	0.0909	50 µL
Volume fraction in complete mixture	0.0833	
Mix carefully. During an incubation period of 300 s the Apo-ASAT enzyme proteins become saturated with pyridoxal-5′-phosphate, the LDH-NADH reaction with sample pyruvate goes to completion, and the reaction temperature has to be reached.		
Stage B. Substrate start with reagent B: 2-oxoglutarate.		
Volume fraction:	0.0833	50 µL
2-Oxoglutarate	12 mmol/L	
Mix carefully. After a lag-phase of up to 60 s a constant rate is achieved that is monitored for the minimum time and number of data points to obtain an acceptable precision around the upper reference limit.		

Wavelengths, pathlengths, final volume of reaction mixture: Instrument dependent, see below. 340 nm has been used consistently in this description.
Reaction temperature: Strictly controlled at 37 °C or 30 °C with a maximal allowed deviation of ± 0.1 °C (Cf ref. 20, CK).

1.2 Specimen

Serum is the preferred specimen. Hemolytic samples will cause falsely increased values.

2. ACCEPTABLE VARIATIONS FROM ECCLS MEASUREMENT PROCEDURE

2.1 Performance specifications

Deviations from the above measurement procedure may endanger the robustness of the method and should be kept as small as possible. However, for adaptation to routine use with different types of analyzers acceptable variations for sample and reagent volume fractions, component concentrations, substrate and serum start, activation, lag-phase, and measurement times will be described below.

Any changes in the reaction system must be documented by their proposers. The average total deviation of results must be less than 5% from those obtained with the IFCC ASAT Reference Method at the same temperature. This should be shown for sera with catalytic activity concentrations close to the upper reference limit and also for samples with elevated activities up to the limit of linearity of the method.

Individual human sera with ASAT isoenzymes in clinically relevant proportions should be used as well as appropriate **certified (NBS, ECCLS, BCR)** reference enzyme material. Evidence on the performance of the proposed methological change should include the effect on sample and reagent blank rates.

NCCLS and equivalent guidelines on Method Comparison should be followed. (Cf refs 23 - 24a, CK).

2.2 Measurements at 37 °C and 30 °C

The ECCLS Standard Method performs equally well at either reaction temperature. **In routine use and quality assessments results should be reported at the temperature at which the measurements were made.**

2.3 Temperature Conversion Factors

However, as discussed in the General Introduction, conversion factors may be of use for comparison of literature data.

In evaluating such data the choice of the calibration material and isoenzyme composition must be considered. As an example, in Switzerland and in The Netherlands the average temperature conversion factors 37 °C/30 °C are 1.50 and 1.58, respectively. (13, 8).

2.4 Concentrations of Components in the Reaction Mixture

The concentrations in the complete reaction mixture depend not only on the amounts in the original solution(s) but also on the relative volumes of different solutions used to achieve the final reaction mixture, as well as on the relative volume of sample. Cf the discussion 2.4 in the CK Method.

2.5 Minimum Time of Saturation with Pyridoxal-5′-Phosphate

For the great majority of samples complete activation is reached through incubation of samples with P-5′-P for 5 minutes during the heating to the reaction temperature (7 - 13, 17 - 20).

2.6 Start of Reaction with Serum (Activated ASAT)

Certain analyzers in routine use today cannot perform the recommended two-stage method. It is possible to activate sample ASAT by incubation of serum samples at an excess concentration of P-5′-P in the reagent TRIS buffer, e.g. 250 µmol/L for 10 minutes at room temperature prior to analysis (7 - 9, 17 - 20). Other authors recommend 20 minutes at 30 °C at a concentration of 400 µmol/L of P-5′-P (18). Reactivation of ALAT is faster than for ASAT (19). This may be done either by adding the P-5′-P-TRIS solution to the sera or adding the sufficient amount of P-5′-P in substance, e.g. included in sample tubes, dosed into tubes, released from a stirring rod. The activated sample is then added to the complete ASAT reaction mixture. Achievement of constant reaction rates with some sera may require 5 minutes or more (6 - 9). The NADH consuming side reactions including that of LDH and pyruvate do not start until sample has been added to the NADH containing reagent A. It must be realized that addition of P-5′-P to sample increases the absorbance around 405 nm.

2.7 Minimum NADH Concentration for Measurement

During the incubation of sample with reagent A the endogenous sample pyruvate-LDH reaction oxidizes NADH. At exceptionally high sample pyruvate concentrations the remaining NADH concentration in the complete reaction mixture may be decreased to 80 µmol/L or less. If this occurs, the sample must be appropriately diluted. At the conditions of the ECCLS Standard procedure an absorbance value at 340 nm below 1.000 at the start of monitoring of reaction rate indicates that the NADH concentration is too low. (1).

2.8 Minimum Lag-phase Time

The lag-phase consists of two components: acceleration until reactions I and II attain an equilibrium, and a deceleration of the LDH catalyzed pyruvate to lactate reaction rate.

By definition, activation, sample pyruvate-LDH-NADH reaction, and the lag-phase may be considered completed when **constant reaction rates are achieved.** With certain sera and low catalytic concentrations of ASAT lag-phases of up to 60 s at 37 °C may occur. (7 -9, 20).

With serum start the lag-phase due to pyruvate is considerably prolonged with some sera (5, 7 - 9, 20). Attempts to decrease the activation time by increasing the P-5′-P concentration may lead to unpredictable lag-phases (7 - 9). A reduction of the LDH-catalyzed lag-phase reaction might conceivably be achieved by an increase of the amount of LDH, e.g. contained in the P-5′-P solution used for activation of the specimen (9). However, it must then be documented that this does not increase the reagent blank rate beyond the tolerance limit.

2.9 Minimum Measurement Time

This is highly dependent on the catalytic activity concentration in the sample, choice of substrate or serum start procedure, and on the instrument. The only general guideline that can be given is that the reaction should be monitored for the time and number of data points required to obtain an acceptable precision at the upper reference limit.

Under the described reaction conditions decreases of absorbance at 340 nm up to about 0.0025 per s (about 4 μkat/L,

240 U/L) remain constant over a measurement time of 300 s (1). However, for shorter measuring times catalytic activity concentrations up to about 15 μkat/L (900 U/L) may be measured before dilution with NaCl, 150 mmol/L becomes necessary (16).

2.10 Sample Volume Fraction

With the IFCC Reference Method a linear relationship has been documented to exist between sample volume fractions of about 0.015 to 0.08 and reaction rates and this relationship probably extends to about 0.10 (1).

Likewise, dilutions of samples with buffer or NaCl, 150 mmol/L do not result in significant errors (1).

3. REACTION BLANK RATES

3.1 Reagent Blank Rates

An LDH-MDH-2-oxoglutarate - NADH + H^+ reagent blank reaction is unavoidable.

Contamination of reagent enzymes, malate and lactate dehydrogenases with ASAT will cause a reagent blank rate. If reagent blank rates are higher than 60 nkat/L (about 4 U/L) corresponding to a decrease of absorbance at 340 nm of 0.002 per 60 s, the reagent should be discarded.

3.2 Sample Blank Rates

Sample blank rates may occur (1). In the presence of sample NH_4^+ concentrations compatible with life up to about 1% of high glutamate dehydrogenase catalytic activity concentrations may be measured. These conditions usually coincide with highly increased ASAT and ALAT values, and thus may be considered of no clinical importance.

Consequently, for routine use, correction for sample blank rates may be omitted (6 - 16).

4. CALCULATION OF CATALYTIC ACTIVITY CONCENTRATION

For routine determination of the catalytic activity concentration of ASAT in serum corrections for reagent and sample blank rates may be omitted.

4.1 Molar Absorption Coefficient of the Monitored Product

The molar absorption coefficients of NADH are:
618 $m^2 \times mol^{-1}$ at 334 nm, 630 $m^2 \times mol^{-1}$ at 340 nm,
and 340 $m^2 \times mol^{-1}$ at 365 nm.

4.2 Calculation at Constant Pathlength

Using measurement at **340 nm as an example**, the catalytic activity concentration is calculated as follows

Symbol	Type of quantity	Unit
b	Catalytic activity concentration	µkat/L
d	Decrease of (decadic) 340 nm absorbance per second	s^{-1}
ε	Molar 340 nm absorption coefficient of $NADH_2$ = 630	$m^2 \times mol^{-1}$
l	Light pathlength (0.01)	m
v	Sample volume fraction: 0.0833 vol(L)/vol(L)	l

4.2.1 Calculation for a Pathlength of 0.01 m

$$b = \frac{d}{\varepsilon \times l \times v} = \frac{d}{630 \times 0.01 \times 0.08333} \times \frac{s^{-1}}{m^2 \times mol^{-1} \times m}$$

$$b = \frac{d}{0.525} = d \times 1.905 \times \frac{mol \times s^{-1}}{m^3}$$

$$b = d \times 1.905 \times kat/m^3$$
$$b = d \times 1905 \times \mu kat/L$$

Calculated for a Measuring Time of 60 s:

$$b = d \times 1905 \times \mu mol \times (60s)^{-1} \times L^{-1}$$
$$b = d \times 1905 \ U/L$$
$$b = d \times 31.75 \ \mu kat/L$$

To convert from µkat/L to U/L multiply by 60.
To convert from U/L to µkat/L divide by 60.

4.2.2 Calculation for Other Pathlengths and for Other Volume Fractions

Insert the pathlength (unit: meter) and the volume fraction (unit L) in the equation instead of the figures given above.

4.3 Calculation at Variable Pathlengths (longitudinal measurement)

Certain (centrifugal) analyzers measure longitudinally through the cuvettes. The pathlength thus becomes proportional to the total assay volume in the cuvette. Dilution of the assay medium in the cuvettes with an inactive fluid causes a corresponding elongation of the pathlength, and the absorbance remains constant. The net effect is that the calculation factor becomes proportional to the sample **volume** and independent of the sample volume **fraction** in the cuvette. (Ref. 34, CK). In the example below the sample volume is 30 µL.

Symbols	Type of quantity	Example	Unit
a	Area of cuvette end	0.25×10^{-4}	m2
v	Sample volume	30×10^{-6}	L
d	Equals decrease in absorbance at 340 nm per second		s^{-1}
Other symbols as given above			

$$b = \frac{d \times a}{\varepsilon \times v} = \frac{d \times 0.25 \times 10^{-4}}{630 \times 30 \times 10^{-6}} \times \frac{s^{-1} \times m^2}{m^2 \times mol^{-1} \times L}$$

$$b = \frac{d \times 25}{189000} \times \frac{mol \times s^{-1}}{L}$$

$$b = d \times 0.001323 \times kat/L$$

$$b = d \times 1323\ \mu kat/L$$

Calculated for a Measuring Time of 60 s:

$$b = d \times 1323\ U/L$$

$$b = d \times 22.05\ \mu kat/L$$

5. CALIBRATION BY REFERENCE ENZYME MATERIALS

For details refer to the corresponding section and references in the ECCLS Standard for CK, above. A certified ASAT reference material from human erythrocytes has recently been produced (21).

6. REFERENCE VALUES

Provided this standard is followed the variability of reference values should depend only on the individuals selected in different studies. Therefore, the population selection criteria, measurement temperature and, if temperature conversion factors have been used, detailed description of the calibration procedure and calibration material should be given to allow comparison.

7. WITHIN-LABORATORY IMPRECISION

This should be similar to that described for the IFCC ASAT Reference Method (1).

8. REAGENT SPECIFICATIONS

Refer to the IFCC ASAT Reference Method (1).

9. RELATIONSHIP TO OTHER METHOD PRINCIPLES

Instruments with specific instrument-dependent methods which do not fall within the tolerance limits of this ECCLS Standard Method may nevertheless be able to produce numerically equivalent results by a calibration process. In such cases the manufacturer should document that the methods fulfill the performance criteria given in 2.1 of these ECCLS Standards. The validation of calibration of enzyme methods is discussed in ECCLS Reference Enzyme Materials, Draft 2, 1987 (9). It is not possible to relate methods without pyridoxal-5′-phosphate activation to the ECCLS Standards for ASAT and ALAT.

Determination of the Catalytic Activity Concentration in Serum of L-Alanine Aminotransferase (EC 2.6.1.2, ALAT)

BASED ON THE IFCC ALAT REFERENCE METHOD (2)

This ECCLS Standard is based on the corresponding IFCC Reference Method for determination of ALAT, which has been adapted by this ECCLS Subcommittee for routine use in European clinical laboratories, as described for CK in the General Introduction to the ECCLS Standard Method 1.

This Standard recommends reaction conditions and describes the tolerance limits for variations in measurement procedures that nevertheless allow routine determinations of the catalytic activity concentration of ALAT to be equivalent to those of the unmodified IFCC ALAT Reference Method.

For details not given in this Standard refer to the original IFCC document, ALAT, part 7 of the IFCC methods for measurement of catalytic concentration of enzymes (14).

The reference numbers refer to the reference list for the ASAT method.

Activation of apo-ALAT with pyridoxal-5′-phosphate

The IFCC, ASAT and ALAT Reference Methods include activation of the apo-enzymes by pyridoxal-5′-phosphate, (P-5′-P, 1,2). After careful consideration of the advantages and disadvantages of this procedure the ECCLS Subcommittee decided to include this step.

Addition of Pyridoxal-5′-phosphate to serum activates the apo-enzymes and permits the measurement of **total** ASAT and ALAT catalytic activity concentrations in serum. Provided the saturation with P-5′-P is complete, this eliminates between-sample variations in the proportion of apo-enzyme. The average increases relative to measurements of ASAT and ALAT without activation are in the range 1.3 - 1.5 and 1.2 - 1.4, respectively (1 - 10 a,b).

However, measurement of P-5′-P activated ASAT and ALAT changes reference ranges previously established in the absence of P-5′-P, since some apo-enzyme is almost always present in healthy individuals. As ASAT and ALAT are activated to different degrees, clinically useful ratios between the ASAT, ALAT, and other enzymes are also changed. In the majority of cases clinical information is not significantly increased through the use of P-5′-P compared with that obtained by methods in which this coenzyme is not added. This applies also to patients suffering from the relative deficiencies in B-vitamins that may be encountered in Europe. However, the normal or abnormally low levels of ALAT observed in some severely ill patients in the absence of added P-5′-P may be converted to abnormally raised values in assays with the added coenzyme (4, 8, 9). Saturation of apo-ASAT and apo-ALAT in human sera may be obtained in three ways, and different procedures have been chosen in various National recommendations (7 - 16).

1. Incubation of sample in P-5′-P, 100 µmol/L in reagent A. Start with 2-Oxoglutarate (1, 2, 5-13)

Advantage: Complete activation in the majority of sera.
Can be automated.

Disadvantage: Prolongs incubation-activation time to 5 minutes.

2. Incubation of sample in complete reagent (8, 14, 15)

Advantage: Serum start is used.

Disadvantage: Complete reactivation is not reached in all sera.

3. Addition of P-5′-P, 250 µmol/L in TRIS buffer to sample (7-9, 16, 17)

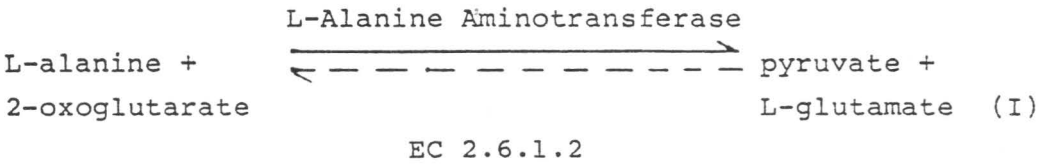
Advantage: Complete activation.
Serum start is possible.

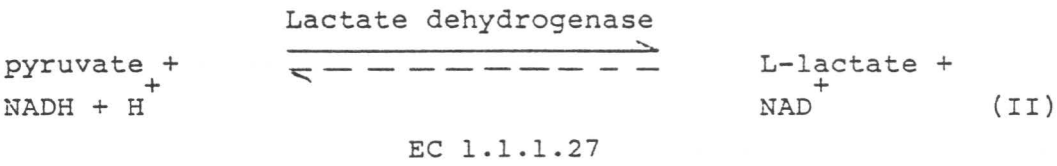
Disadvantage: Requires an additional, manual analytical step.
The activation time may be as long as 30 minutes, and the lag-phase increases to 5 minutes or more for some sera (5, 8, 9)
Increased absorbance at 405 - 410 nm.

The time required for complete activation varies considerably among individual sera. In the **IFCC ASAT** and **ALAT Reference Methods** procedure 1 and an activation time of 10 minutes were recommended. However, activation by procedure 1 will be complete for the majority of sera within 5 minutes. Accordingly, this activation time was selected for the ECCLS Standard Methods 2 - 3 for ASAT and ALAT determinations.

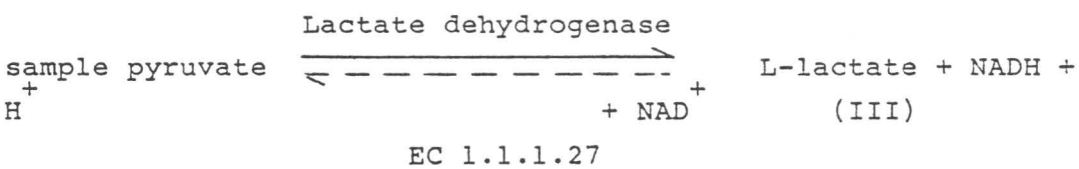
1. REACTION PRINCIPLES

The reactions catalyzed by ALAT (I) and the indicator enzyme (II) are as follow: (2 - 16)





During the incubation-activation of sample with reagent A (see below):



The apoenzyme of ALAT is activated by addition of pyridoxal-5′-phosphate before measurement. The sample pyruvate - NADH + H^+ reaction catalyzed by sample lactate dehydrogenase (LDH) reaches completion during the incubation period. (1, 7 - 16). The equilibrium of the indicator reaction (II) lies far toward the production of L-lactate. Therefore, the equilibrium of the ALAT reaction (I) is not relevant, and the catalytic active concentration of ALAT in the samples is determined by the conversion rate of NADH (2).

1.1 ECCLS Measurement Procedure:

Substrate start, 37 °C and 30 °C

The component concentrations in the final reaction mixture are the same as those of the **IFCC ALAT Reference Method** (2). The standard reaction conditions have been defined at 37 °C in Table 1 below. The reaction system performs equally well at 30 °C. Optimum pH (30 °C) for ALAT in serum lies in the interval 7.1 to 7.9, depending on the type of buffer with an optimum for TRIS-HCl buffer at 7.3 (2, 10 b).

The pH of the TRIS-HCl buffer decreases 0.15 for an increase in temperature from 30 °C to 37 °C (9). Consequently, the same reaction system, adjusted to 7.30 at 30 °C will change to a pH of 7.15 0.02 at 37 °C. However, due to the broad pH optimum described in the IFCC ALAT Reference Method (2, 10b) this shift from optimum pH does not decrease the ALAT catalytic activity concentration by more than about 1%. Consequently, for routine use, the same TRIS-HCl buffer adjusted to pH 7.30 (30 °C) will support the reaction with no significant loss at either reaction temperature.

The ECCLS Standard Procedure involves two stages (Table 1). In the first, the sample is incubated with **reagent A**, which contains pyridoxal-5′-phosphate but not 2-oxoglutarate. Apo-ALAT is reconstituted during this phase, and the LDH-catalyzed NADH reaction with sample pyruvate reaches completion.

In the second stage, the ALAT catalyzed reaction is initiated by addition of 2-oxoglutarate (**reagent B**).

Table I. Concentrations apply to the complete reaction mixture and are specified at 37 °C, where conversion rates and Michaelis constants are higher, to ensure that maximal ALAT catalytic activity will be supported at both 37 °C and 30 °C.

A buffer adjusted to pH 7.30 (30 °C) may well be used. The catalytic activity concentration of lactate dehydrogenase has been determined as described in the IFCC ALAT Reference Method at 30 °C and 37 °C. The average temperature conversion factor 37 °C/ 30 °C was found to be 1.35 (17). The value in Table I corresponds to that obtained at 37 °C.

Stage A Incubation-activation reagent A.		
Volume fraction in:		
incubation-reactivation stage	**0.91**	
complete reaction mixture	**0.833**	
		Example of volume (µL)
Tris(hydroxymethyl)aminomethane buffer pH 7.15	100 mmol/L	
L-alanine	500 mmol/L	500 µL
NADH	180 µmol/L	
Pyridoxal-5'-phosphate	100 µmol/L	
Lactate dehydrogenase (1700 U/L)	28 µkat/L	
Add sample		50 µL
Volume fraction in reagent A	0.0909	
Volume fraction in complete mixture	0.0833	
Mix carefully. During an incubation period of 300 s the Apo-ALAT enzyme proteins become saturated with pyridoxal-5'-phosphate, the LDH-NADH reaction value to sample pyruvate goes to completion, and the reaction temperature has to be reached.		
Stage B. Substrate start with reagent B: 2-oxoglutarate.		
Volume fraction:	**0.083**	**50 µL**
2-Oxoglutarate	12 mmol/L	
Mix carefully. After a lag-phase of up to 60 s a constant rate is achieved that is monitored for the minimum time and number of data points to obtain an acceptable precision around the upper reference limit.		

Wavelengths, pathlengths, final volume of reaction mixture: Instrument dependent, see below. 340 nm has been used consistently in this description.

Reaction temperature: Strictly controlled at 37 °C or 30 °C with a maximal allowed deviation of ± 0.1 °C (Cf ref. 20, CK).

1.2 Specimen

Serum is the preferred specimen.

2. ACCEPTABLE VARIATIONS FROM ECCLS MEASUREMENT PROCEDURE

2.1 Performance specifications

Deviations from the above measurement procedure may endanger the robustness of the method and should be kept as small as possible. However, for adaptation to routine use with different types of analyzers acceptable variations for sample and reagent volume fractions, component concentrations, substrate and serum start, activation, lag-phase, and measurement times will be described below.

Any changes in the reaction system must be documented by their proposers. The average total deviation of results must be less than 5% from those obtained with the IFCC ALAT Reference Method at the same temperature. This should be shown for sera with catalytic activity concentrations close to the upper reference limit and also for samples with elevated activities up to the limit of linearity of the method.

Individual human sera with ALAT isoenzymes in clinically relevant proportions should be used as well as appropriate **certified (NBS, ECCLS, BCR)** reference enzyme material. Evidence on the performance of the proposed methological change should include the effect on sample and reagent blank rates.

NCCLS and equivalent guidelines on Method Comparison should be followed. (Cf refs 23 - 24a, CK).

2.2 Measurements at 37 °C and 30 °C

The ECCLS Standard Method performs equally well at either reaction temperature. **In routine use and quality assessments results should be reported at the temperature at which the measurements were made.**

2.3 Temperature Conversion Factors

However, as discussed in the General Introduction, conversion factors may be of use for comparison of literature data.
In evaluating such data the choice of the calibration material and isoenzyme composition must be considered. As an example, in Switzerland and in The Netherlands the average temperature conversion factors 37 °C/37 °C are 1.50 and 1.42, respectively. (13, 8).

2.4 Concentrations of Components in the Reaction Mixture

The concentrations in the complete reaction mixture depend not only on the amounts in the original solution(s) but also on the relative volumes of different solutions used to achieve the final reaction mixture, as well as on the relative volume of sample. Cf the discussion 2.4 in the CK Method.

2.5 Minimum Time of Saturation with Pyridoxal-5′-Phosphate

For the great majority of samples complete activation is reached through incubation of samples with P-5′-P for 5 minutes during the heating to the reaction temperature (7 - 13, 17 - 20).

2.6 Start of Reaction with Serum (Activated ALAT)

Certain analyzers in routine use today cannot perform the recommended two-stage method. It is possible to activate sample ALAT by incubation of serum samples at an excess concentration of P-5′-P in the reagent TRIS buffer, e.g. 250 µmol/L for 10 minutes at room temperature prior to analysis (7 - 9, 17 - 20). Other authors recommend 20 minutes at 30 °C at a concentration of 400 µmol/L of P-5′-P (18). Reactivation of ALAT is faster than for ASAT (19). This may be done either by adding the P-5′-P-TRIS solution to the sera or adding the sufficient amount of P-5′-P in substance, e.g. included in sample tubes, dosed into tubes, released from a stirring rod. The activated sample is then added to the complete ALAT reaction mixture. Achievement of constant reaction rates with some sera may require 5 minutes or more (6 - 9). The NADH consuming side reactions including that of LDH and pyruvate do not start until sample has been added to the NADH containing reagent A. It must be realized that addition of P-5′-P to sample increases the absorbance at around 405 nm.

2.7 Minimum NADH Concentration

During the incubation of sample with reagent A the endogenous sample pyruvate-LDH reaction oxidizes NADH. At exceptionally high sample pyruvate concentrations the remaining NADH concentration in the complete reaction mixture may be decreased to 80 µmol/L or less. If this occurs, the sample must be appropriately diluted. At the conditions of the ECCLS Standard procedure an absorbance value at 340 nm below 1.000 at the start of monitoring of reaction rate indicates that the NADH concentration is too low. (1).

2.8 Minimum Lag-phase Time

The lag-phase consists of two components: acceleration until reactions I and II attain an equilibrium, and a deceleration of the LDH catalyzed pyruvate to lactate reaction rate.

By definition, activation, sample pyruvate-LDH-NADH reaction, and the lag-phase may be considered completed when **constant reaction rates are achieved.** With certain sera and low catalytic concentrations of ALAT lag-phases of up to 60 s at 37 °C may occur. (7 -9, 20).

With serum start the lag-phase due to pyruvate is considerably prolonged with some sera (5, 7 - 9, 20). Attempts to decrease the activation time by increasing the P-5′-P concentration may lead to unpredictable lag-phases (7 - 9). A reduction of the LDH-catalyzed lag-phase reaction might conceivably be achieved by an increase of the amount of LDH, e.g. contained in the P-5′-P solution used for activation of the specimen (9). However, it must then be documented that this does not increase the reagent blank rate beyond the tolerance limit.

2.9 Minimum Measurement Time

This is highly dependent on the catalytic activity concentration in the sample, choice of substrate or serum start procedure, and on the instrument. The only general guideline that can be given is that the reaction should be monitored for the time and number of data points required to obtain an acceptable precision at the upper reference limit .

Under the described reaction conditions decreases of absorbance at 340 nm up to about 0.0025 per s (about 4 μkat/L, 240 U/L) remain constant over a measurement time of 300 s (1).

However, for shorter measuring times catalytic activity concentrations up to about 15 μkat/L (900 U/L) may be measured before dilution with NaCl, 150 mmol/L becomes necessary (16).

2.10 Sample Volume Fraction

With the IFCC Reference Method a linear relationship has been documented to exist between sample volume fractions of about 0.015 to 0.08 and reaction rates and this relationship probably extends to about 0.10 (1).

Likewise, dilutions of samples with buffer or NaCl, 150 mmol/L do not result in significant errors (1).

3. REACTION BLANK RATES

3.1 Reagent Blank Rates

An LDH-2-oxoglutarate - NADH + H^+ reagent blank reaction is unavoidable.
Contamination of reagent enzymes, lactate dehydrogenases with ALAT will cause a reagent blank rate. If reagent blank rates are higher than 60 nkat/L (about 4 U/L) corresponding to a decrease of absorbance at 340 nm of 0.002 per 60 s, the reagent should be discarded.

3.2 Sample Blank Rates

Sample blank rates may occur (1). In the presence of sample NH_4^+ concentrations compatible with life up to about 1% of high glutamate dehydrogenase catalytic activity concentrations may be measured. These conditions usually coincide with highly increased ASAT and ALAT values, and thus may be considered of no clinical importance.

Consequently, for routine use, correction for sample blank rates may be omitted (6 - 16).

4. CALCULATION OF CATALYTIC ACTIVITY CONCENTRATION

For routine determination of the catalytic activity concentration of ALAT in serum corrections for reagent and sample blank rates may be omitted.

4.1 Molar absorption Coefficient of the Monitored Product

The molar absorption coefficients of NADH are:
618 $m^2 \times mol^{-1}$ at 334 nm, 630 $m^2 \times mol^{-1}$ at 340 nm,
and 340 $m^2 \times mol^{-1}$ at 365 nm.

4.2 Calculation at Constant Pathlength

Using measurement at **340 nm as an example**, the catalytic activity concentration of ALAT is calculated as follows:

Symbol	Type of quantity	Unit
b	Catalytic activity concentration	µkat/L
d	Decrease of (decadic) 340 nm absorbance per second	s^{-1}
ε	Molar 340 nm absorption coefficient of $NADH_2$ = 630	$m^2 \times mol^{-1}$
l	Light pathlength (0.01)	m
v	sample volume fraction: 0.0833 vol(L)/vol(L)	1

4.2.1 Calculation for a Pathlength of 0.01 m

$$b = \frac{d}{\varepsilon \times l \times v} = \frac{d}{630 \times 0.01 \times 0.08333} \times \frac{s^{-1}}{m^2 \times mol^{-1} \times m}$$

$$b = \frac{d}{0.525} = d \times 1.905 \times \frac{mol \times s^{-1}}{m^3}$$

$$b = d \times 1.905 \times kat/m^3$$
$$b = d \times 1905 \times \mu kat/L$$

Calculated for a Measuring Time of 60 s:
$b = d \times 1905 \times \mu mol \times (60s)^{-1} \times L^{-1}$
b = d x 1905 U/L
b = d x 31.75 µkat/L

To convert from µkat/l to U/L multiply by 60.
To convert from U/L to µkat/L divide by 60.

4.2.2 Calculation for Other Pathlengths and for Other Volume Fractions

Insert the pathlength (unit: meter) and the volume fraction (unit L) in the equation instead of the figures given above.

4.3 Calculation at Variable Pathlengths (longitudinal measurement)

Certain (centrifugal) analyzers measure longitudinally through the cuvettes. The pathlength thus becomes proportional to the total assay volume in the cuvette. Dilution of the assay medium in the cuvettes with an inactive fluid causes a corresponding elongation of the pathlength, and the absorbance remains constant. The net effect is that the calculation factor becomes proportional to the sample **volume** and independent of the sample volume **fraction** in the cuvette. (Ref. 34, CK) In the example below the sample volume is 30 uL.

Symbols	Type of quantity	Example	Unit
a	Area of cuvette end	0.25×10^{-4}	m^2
v	Sample volume	30×10^{-6}	L
d	Equals decrease in absorbance at 340 nm per second		s^{-1}
Other symbols as given above			

$$b = \frac{d \times a}{\varepsilon \times v} = \frac{d \times 0.25 \times 10^{-4}}{630 \times 30 \times 10^{-6}} \times \frac{s^{-1} \times m^2}{m^2 \times mol^{-1} \times L}$$

$$b = \frac{d \times 25}{189000} \times \frac{mol \times s^{-1}}{L}$$

$$b = d \times 0.001323 \times kat/L$$

$$b = d \times 1323\ \mu kat/L$$

Calculated for a Measuring Time of 60 s:

$$b = d \times 1323\ U/L$$

$$b = d \times 22.05\ \mu kat/L$$

5. CALIBRATION BY REFERENCE ENZYME MATERIALS

For details refer to the corresponding section and references in the ECCLS Standard for CK, above.

6. REFERENCE VALUES Provided this standard is followed the variability of reference values should depend only on the individuals selected in different studies. Therefore, the population selection criteria, measurement temperature and, if temperature conversion factors have been used, detailed description of the ca- libration procedure and calibration material should be given to allow comparison.

7. WITHIN-LABORATORY IMPRECISION

This should be similar to that described for the IFCC ALAT Reference Method (2).

8. REAGENT SPECIFICATIONS

Refer to the IFCC ALAT Reference Method (2).

9. RELATIONSHIP TO OTHER METHOD PRINCIPLES

Instruments with specific instrument-dependent methods which do not fall within the tolerance limits of this ECCLS Standard Method may nevertheless be able to produce numerically equivalent results by a calibration process. In such cases the manufacturer should document that the methods fulfill the performance criteria given in 2.1 of these ECCLS Standards. The validation of calibration of enzyme methods is discussed in ECCLS Reference Enzyme Materials, Draft 2, 1987 (9). **It is not possible to relate methods without pyridoxal-5′-phosphate activation to the ECCLS Standards for ASAT and ALAT.**

References concerning ASAT and ALAT

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Determination of the Catalytic Activity Concentration in Serum of Gamma-glutamyltransferase (EC 2.3.2.2, GT)

BASED ON THE PROVISIONAL IFCC REFERENCE METHOD (1)

AND ON THE NORDIC RECOMMENDED (SCE) METHOD

This ECCLS Standard is based on the corresponding IFCC Reference Method for determination of GT, which has been adapted by this ECCLS Subcommittee for routine use in European clinical chemistry laboratories. For reasons given below, an alternative (SCE) procedure using the non-patented substrate gamma-glutamyl-4-nitroanilide with calibration is also described.

This Standard recommends reaction conditions and describes the tolerance limits for variations in measurement that nevertheless allow routine determinations of the catalytic concentration of GT to be equivalent to those of the unmodified IFCC GT Reference Method.

For details not given in this standard refer to the original IFCC document, GT (Part 4, Stage 2, Draft 2, Provisional recommendation 1983) of the IFCC Reference Methods for measurement of catalytic concentration of enzymes (1).

Patented and alternative donor substrates.

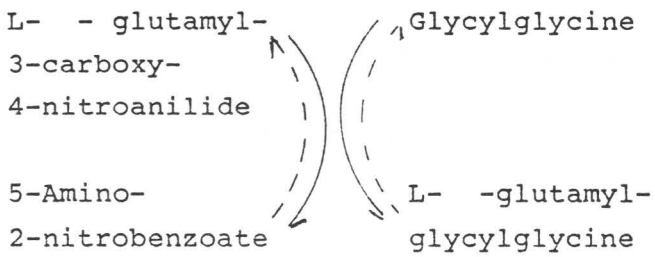
A particular problem for GT, however, is that the donor substrate, gamma-glutamyl-3-carboxy-4-nitroanilide (Glucana), in the IFCC GT Reference Method is patent-protected, and licenses for production of reagent kits for routine use are not at present universally available throughout Europe. For this reason, an alternative method employing the less-soluble non-carboxylated substrate is also presented as an acceptable

variant. The use of the BCR Reference Material for GT (8 a-c) as a calibrator ensures numerical comparability of results between the two methods.

This alternative is seen as a temporary measure only until the patent protection of the carboxylated substrate expires, i.e. from December 1990 (DE) to December 1993 (AT).

1. REACTION PRINCIPLES

L-gamma-glutamyl-3-carboxy-4-nitroanilide (L-Glucana) and glycylglycine (Glygly) are used as the donor and acceptor substrates, respectively.
The following reaction is catalyzed by human gamma-glutamyl-transferase (GT) in serum:



For details of the reaction kinetics refer to the IFCC document, ref.1.
Two hydrolysis and auto-transfer side reactions contribute less than 1% of the products (1).

1.1 ECCLS Measurement Procedure

Serum Start, 37 °C and 30 °C

The component concentrations in the final reaction mixture are the same as those of the **IFCC GT provisional Reference Method** (1). The standard reaction conditions have been defined at 37 °C in Table 1 below. The reaction system performs equally well at 37 °C and 30 °C (2-6). Optimum pH (30 °C) for GT in human serum lies between 7.8 and 8.0 (1). The pH of the glycylglycine-NaOH buffer decreases about 0.2 pH units by increasing the temperature from 30 °C to 37 °C. The same buffer, adjusted to a pH of 7.90 at 30 °C may be used for measurements at 37 °C (2-6). The effect on reaction rates is negligible.

The ECCLS Standard Procedure is a **one-stage** method in which the reaction is started by addition of serum (Table 1)

Table 1
Concentrations are specified in the complete reaction mixture at 37 °C, to ensure that maximal GT catalytic activity will be supported at both 37 °C and 30 °C.
A buffer adjusted to pH 7.90 (30 °C) may well be used.

Incubation-activation with reagent A. Volume fraction 0.909.		
		Example of volume (µL)
L-gamma-glutamyl-3-carboxy-4-nitroanilide	6 mmol/L	
Glycylglycine-NaOH buffer, pH 7.7 (37 °C)	150 mmol/L	500 µL
Add serum. **Volume fraction**	**0.091**	**50 µL**
Mix carefully. A constant reaction rate is achieved and monitored for the minimum time and number of data points to obtain acceptable precision around the upper reference limit.		

Wavelengths : 410 nm or 405nm.

Pathlengths, final volume of reaction mixture: Instrument dependent, see below.

Reaction temperature: Strictly controlled at 37 °C or 30 °C with a maximal, allowed deviation of ± 0.1 °C.

1.2 Specimen

Serum is the preferred sample. EDTA and citrate plasma give somewhat lower values (1,6). Heparin plasma may cause interfering turbidity effects, which can be counteracted through inclusion of NaCl 50 mmol/l in the final GT reagent (6) or starting the reaction with the substrate.
The **removal of lipids** may cause up to about 35% loss of GT and **should be avoided** (6a).

2. ACCEPTABLE VARIATIONS FROM ECCLS MEASUREMENT PROCEDURE

2.1 Performance Specifications

Deviations from the above measurement procedure may endanger the robustness of the method and should be kept as small as possible. However, for adaptation to routine use with different types of analyzers acceptable variations for sample and reagent volume fractions, component concentrations, substrate and serum start, activation, lag-phase, and measurement times will be described below.

Any changes in the reaction system must be documented by their proposers. The average total deviation of results must be less than 5% from those obtained with the IFCC GT Reference Method at the same temperature. This should be shown for sera with catalytic activity concentrations close to the upper reference limit and also for samples with elevated activities up to the limit of linearity of the method.

Individual human sera with GT variants in clinically relevant proportions should be used as well as appropriate **certified (ECCLS, BCR)** reference enzyme materials.

This has been studied in the case of the proposed alternative procedure (Section 2.7) using the non-patented gamma-glutamyl-4-nitroanilide as the substrate. In this particular case adequate documentation has proved the traceability to the IFCC Reference GT Method, and the calibration procedure ensures that the conditions described above are fulfilled.

2.2 Measurements at 30 °C and 37 °C

Refer to corresponding sections in the ECCLS Standard for CK.

2.3 Temperature Conversion Factors

As discussed in the General Introduction, conversion factors may be of use for comparison of literature data.
In evaluating such data the calibration material must be considered. As an example, in Switzerland and The Netherlands the temperature conversion factors 37 °C/37 °C have been established at 1.35 (2) and 1.28 (3a), respectively.

2.4 Concentrations of Components in the Reaction Mixture

Refer to the corresponding section for CK.

2.5 Acceptable Variation: Substrate Start

Due to the high solubility of the substrate L-gamma-glutamyl-3-carboxy-4-nitroanilide, substrate start with a concentrated solution is possible.

The relative volumes given below have been shown to give acceptable results in practice (1-7).

Table 2

Concentrations are specified in the final reaction mixture at 37 °C, to ensure that maximal GT catalytic activity will be supported at both 37 °C and 30 °C.

Stage A. Incubation with reagent A **Volume fraction:** **in incubation stage A** **in complete reaction mixture**	 **0.889** **0.727**	
Glycylglycine-NaOH buffer, pH 7.7	**150 mmol/L**	**Example of volume (uL)** **400 µL**
Add sample. (Final) Volume fraction	**0.091**	**50 µL**
Mix well. Incubate until the reaction temperature has been reached.		
Stage B. **Substrate Start with Reagent B:** **Volume fraction** **L-gamma-glutamyl-3-carboxy-4-nitroanilide**	 **0.182**	
L-Glucana	**6 mmol/L**	**100 µL**
Mix well. Monitor for the minimum time and number of data points to obtain acceptable precision around the upper reference limit.		

2.6 Acceptable Variation: Gamma-glutamyl-4-nitroanilide Substrate and Calibration using BCR Reference Material for GT

The analytical procedure is that recommended as a routine method in 1975 by the Scandinavian Society for Clinical Chemistry (7).

Due to the lower solubility of the non-carboxylated donor substrate gamma-glutamyl-4-nitroanilide, its final concentration was chosen to be 4 mmol/L. As a consequence of the lower donor substrate concentration, the acceptor substrate glycylglycine also had to be lowered (75 mmol/L), and TRIS was included to increase the buffer capacity. (7)

This gives a less optimized method than the IFCC method. The Nordic Recommended (SCE) method gives catalytic activity concentrations that on the average are about 20 per cent lower than those with the IFCC method, when patient's sera are assayed at 37 °C. Quality control materials based on some non-human enzymes give quite different ratios between the two methods due to species differences with respect to substrate specificity.

To obtain numerical comparability of the results with those obtained with the IFCC reference method or the ECCLS routine method described in 1.1 above, the variant procedure must be calibrated using an appropriate, certified enzyme reference material. The BCR Reference Material for GT (Product code CRM nr 319 (8 a-c) appears suitable for this purpose. The commutability of this preparation with clinical specimens when assayed with the IFCC reference and the Nordic recommended methods has been assessed, using procedures prescribed by the ECCLS Subcommittee on Enzyme Reference Materials (9 section II,2). This evaluation showed an average difference of < 2.5% between results obtained by the IFCC Reference Method at

30 °C, and the Nordic method at 37 °C after calibration with the BCR material (9 section II,2).

In conclusion, this alternative procedure is provided as a temporary measure only, since the procedure recommended in 1.1 cannot, at present, be freely applied by all reagent suppliers. The alternative procedure represents a combination of both a carefully studied and officially recommended method in wide use in clinical laboratories for many years with a well-characterized enzyme reference material. This combination has been demonstrated to satisfy the commutability criteria of the ECCLS Subcommittee on Enzyme Reference Materials (9) and of the NCCLS (10).
Other combinations may not be appropriate.

2.7 Minimum Lag-Phase Time
0 - 30 s. With most sera no lag-phase is observed (1-7).

2.8 Minimum Measurement Time
This is highly dependent on the catalytic activity concentration in the sample and on the instrument. The only general guideline that can be given is that the reaction should be monitored for the time and number of data points required to obtain an acceptable precision at the upper reference limit.

Reaction rates may remain constant for at least 120 and 180s, respectively, for increases of 410 nm absorbance around 0.325 and 0.220 per 60s (1-7). These correspond to about 7.5 and 5 μkat/l (450 and 300 U/L), respectively. At higher catalytic activity concentrations samples should be diluted with NaCl, 150 mmol/L (1-7).

2.9 Sample Volume Fraction
Serum volume fraction may be varied within the range of 0.01 to 0.25 without significant error (1).

3. REACTION BLANK RATES

3.1 Reagent Blank Rates

Autohydrolysis of L-gamma-glutamyl-3-carboxy-4-nitroanilide causes an apparent blank rate corresponding to an increase in 410 nm absorbance < 0.0005 per 60s (1,4,5). For routine use correction for reagent blank rate may be omitted (2-7).

4. CALCULATION OF CATALYTIC ACTIVITY CONCENTRATION

For routine determination of the catalytic activity concentration of GT in serum corrections for reagent blank and sample blank rates may be omitted.

4.1 Molar Absorption Coefficient of the Monitored Product

The apparent molar absorbance will vary markedly from one routine spectrometer to another, because the product has to be monitored under unfavourable photometric conditions. This is discussed in the original IFCC document (1) in which a procedure for the measurement of the absorption coefficient is also given.

Before calculation of the catalytic activity concentration is carried out the apparent molar absorption coefficient of the monitored product for the used type of spectrometer **must** be established (Cf. 4, above). An exception may be instruments using the Hg 405 nm emission line and one and the same, well-defined pathlength.

Under strictly defined conditions values in glycylglycine buffer, 150 mmol/L pH 7.90 (30 °C) at 410 nm of 790.8 and 796 $m^2 \times mol^{-1}$ have been found (1,11). In the **example** below a value of 790.8 has been used. At 405 nm values between 944.8 and 949.0 $m^2 \times mol^{-1}$ have been found (8,12)

4.2 Calculation at Constant Pathlength

Symbol	Type of Quantity	Units
b	Catalytic activity concentration	μkat/L
i	Increase of 410nm absorbance per second	s^{-1}
ε	Molar 410 nm absorption of 5-amino-2-nitrobenzoate = 790.8 **(example)**	$m^2 \times mol^{-1}$
l	Light pathlength (0.01)	m
v	Sample volume fraction: 0.0909 vol(L)/vol(L)	1

4.2.1 Calculation for a Pathlength of 0.01 m

$$b = \frac{i}{\epsilon \times l \times v} = \frac{i}{790.8 \times 0.01 \times 0.091} \times \frac{s^{-1}}{m^2 \times mol^{-1} \times m}$$

$$b = \frac{i}{0.7189} = 1.391 \times \frac{mol \times s^{-1}}{m^3}$$

$$b = i \times 1.391 \times kat/m^3$$

$$b = i \times 1391 \times \mu kat/L$$

Calculated for a measuring time of 60s:

$$b = i \times 1391 \times \mu mol \times (60s)^{-1} \times L^{-1}$$

$$b = i \times 1391 \; U/L$$

$$b = i \times 23.18 \; \mu kat/L$$

To convert from μkat/L to U/L multiply by 60.
To convert from U/L to μkat/L divide by 60.

4.2.2 Calculation for Other Pathlengths and for Other Volume Fractions

Insert the pathlength (unit: meter) and the volume fraction in the equation instead of the figures given above.

4.3 Calculation at Variable Pathlengths (longitudinal measurement)

Insert as previously described for CK and ASAT.

5. CALIBRATION BY REFERENCE ENZYME MATERIALS

Certain analyzers require the use of calibrators rather than calculation on the basis of molar linear absorbance. Provided that the reagent and measurement procedures are in accordance with these ECCLS specifications, calibration may be carried out using a certified calibration material or reference enzyme preparation, e.g. ECCLS, BCR (8), NCCLS or equivalent fulfilling the performance criteria set for these materials (9-10). For details refer to the ECCLS Reference Enzyme Materials, Draft 2, 1987 (9).

6. REFERENCE VALUES

Provided this standard is followed the variability of reference values should depend only on the individuals selected in different studies. Therefore, the population selection criteria, measurement temperature and, if temperature conversion factors have been used, detailed description of the calibration procedure and calibration material should be given to allow comparison.

7. WITHIN-LABORATORY IMPRECISION

This should be comparable to that described for the IFCC GT Reference Method (1).

8. REAGENT SPECIFICATIONS

Refer to the IFCC provisional Reference GT Method (1)

9. RELATIONSHIP TO OTHER METHOD PRINCIPLES

Instruments with specific instrument-dependent methods which do not fall within the tolerance limits of this ECCLS Standard Method may nevertheless be able to produce numerically equivalent results by a calibration process.In such cases the manufacturer should document that the methods fulfill the performance criteria given.in 2.1 of these ECCLS Standards. The validation of calibration of enzyme methods is discussed in ECCLS Reference Enzyme Materials, Draft 2, 1987 (9).

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Nordisk Förening för Klinisk Kemi (NFKK)

NFKK har som syfte att verka för utvecklingen av klinisk kemi i Norden. Den består av medlemmarna i de vetenskapliga föreningarna för klinisk kemi i Danmark, Finland, Island, Norge och Sverige. Verksamheten i NFKK bedrivs bl. a. genom de två huvudkommittéerna, Metodkommittén och Klinikkommittén och ett antal arbetsgrupper. Föreningen står dessutom för arrangerandet av de nordiska kongresserna i klinisk kemi.

Styrelsen består av från: **Danmark:** Ove Lauritsen, Egil Raabo, Sten Stender; **Finland:** Aimo Ruokonen, Reijo Vihko, Lasse Viinikka; **Island:** Leifur Franzson (sekreterare), Eggert Johansson, Veigar Gudmundsson (ordförande); **Norge:** Ludvig Daae, Sverre Landaas, Inge Romslo; **Sverige:** Jan Börjeson, Arne Hamfelt, Carl-Henric de Verdier.

Nordiskt Samprojekt för Klinisk Kemi (Nordkem)

Nordkem inrättades år 1977 i syfte att främja samarbetet mellan sjukhuslaboratorierna i Norden. Till och med 1982 bedrevs verksamheten på försöksbasis och med finansiering från Nordiska ministerrådet. Därefter övergick verksamheten till reguljära former, varvid finansieringsansvaret överfördes till hälsovårdsmyndigheter och sjukvårdshuvudmän i de fem länderna.

Nordkems högsta beslutande organ, dess styrelse, är trepartsammansatt och består år 1990 av: för **hälsovårdsmyndigheterna:** Erik Magid (DK), Jarmo Pikkarainen (SF), Eggert Johansson (IS), Pål Bjørnstad (N), Kurt Roos (S); för **laboratoriemedicinen:** Mogens Hørder (DK, ordförande), Raimo Tenhunen (SF), Veigar Gudmundsson (IS), Inge Romslo (N), Carl-Henric de Verdier (S); för **sjukvårdshuvudmännen:** Johanne Louise Dithmer (DK), Arvo Relander (SF), Ragnar Akre-Aas (N), Thorsten Thor (S, vice ordförande).

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Bidrag till KLINISK KEMI I NORDEN sändes i två exemplar till den nationella redaktören, som finns angiven på omslagets andra sida. Manuskripten skall vara maskinskrivna och följa de instruktioner som angetts i Vancouver-avtalet (Nordisk Medicin 1988; 103: 93–6). Språket skall vara nordiskt.

Meddelanden och korta inlägg skrives helst fortlöpande, medan längre artiklar med fördel delas i avsnitt med en kort överskrift.

Tabeller skrives på särskilda ark tillsammans med en text, som gör tabellen självförklarande.

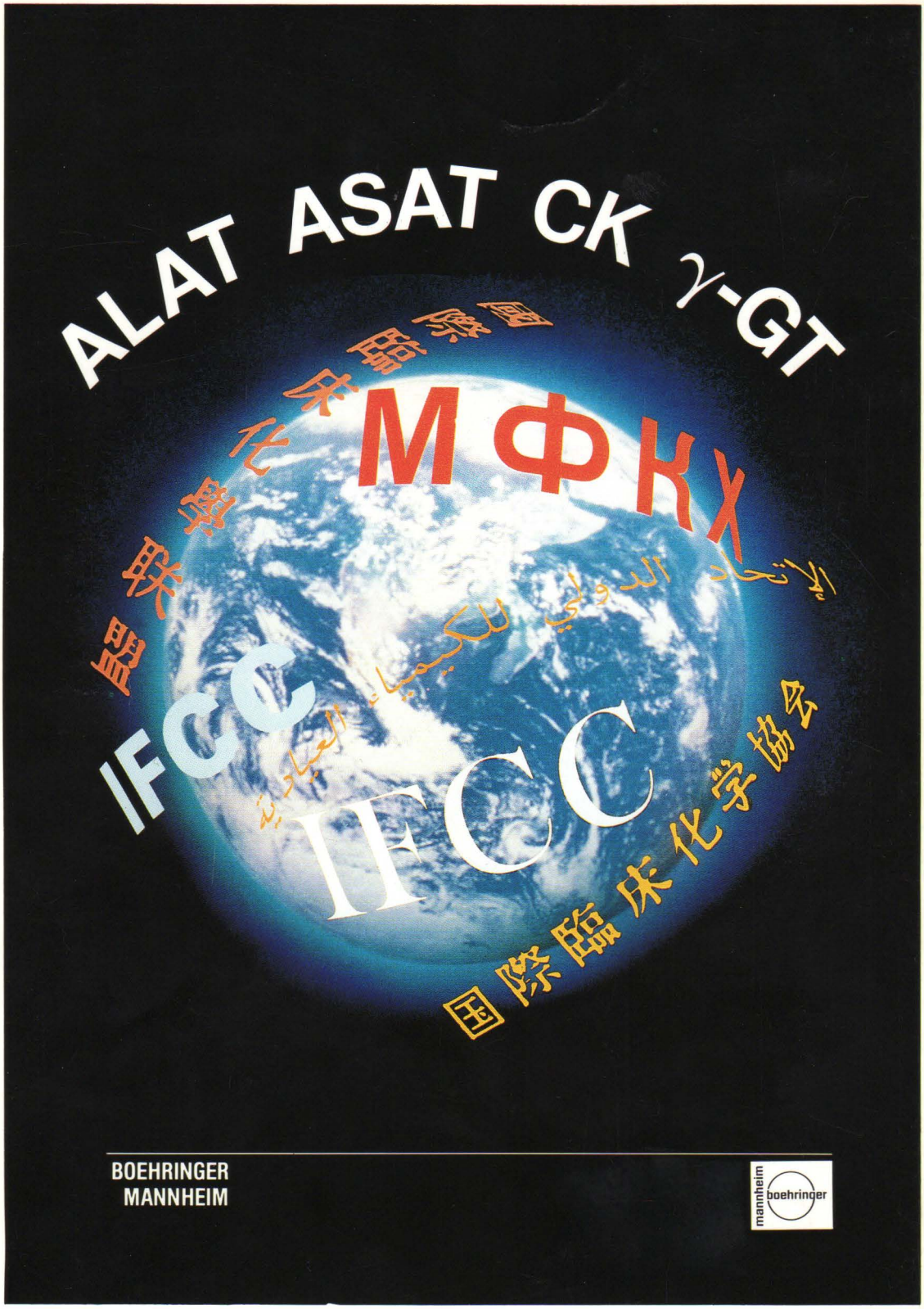
Figurer måste vara av tekniskt god kvalitet med text och symboler tillräckligt stora för att tåla förminskning. Till varje figur skrives en förklarande text.

Litteraturhänvisningar numreras i den ordning de anges i texten och skrives som i följande exempel:

Sandberg S, Christensen NB, Thue G, Lund PK. Performance of dry-chemistry instruments in primary health care. Scand J Clin Lab Invest 1989; 49: 483–8.

Innehållet i de insända artiklarna kommer inte att genomgå vanlig granskning med referee-system. Redaktionskommittén kommer emellertid att värdera alla manuskript innehållsmässigt och redaktionellt och eventuellt föreslå ändringar.

Almqvist & Wiksell Tryckeri, Uppsala 1990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