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Standard Reference Materials:

The Measurement of the Catalytic (Activity) Concentration of Seven Enzymes in NBS Human Serum SRM 909

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Standard Reference Materials:

The Measurement of the Catalytic (Activity) Concentration of Seven Enzymes in NBS Human Serum SRM 909

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PREFACE

Standard Reference Materials (SRM's) as defined by the National Bureau of Standards are well-characterized materials, produced in quantity and certified for one or more physical or chemical properties. They are used to ensure the accuracy and compatibility of measurements throughout the Nation. SRM's are widely used as primary measurement standards in many diverse fields in science, industry, and technology, both within the United States and throughout the world. They are also used extensively in the fields of environmental and clinical analysis. In many applications, traceability of quality control and measurement processes to the national measurement system is carried out through the mechanism and use of SRM's. For many of the Nation's scientists and technologists it is therefore of more than passing interest to know the details of the measurements made at NBS in arriving at the certified values or supplemental data for the SRM's produced. An NBS series of monographs, of which this publication is a member, called the NBS Special Publication - 260 Series, is reserved for this purpose.

This 260 Series is dedicated to the dissemination of information on different phases of the preparation, measurement, certification and use of NBS-SRM's. In general, much more detail will be found in these monographs than is generally allowed, or desirable, in scientific journal articles or on the SRM certificate. This enables the user to assess the validity and accuracy of the measurement processes employed, to judge the statistical analysis, and to learn details of techniques and methods utilized for work entailing the greatest care and accuracy. These monographs also should provide sufficient additional information not found on the SRM certificate so that new applications in diverse fields not foreseen at the time the SRM was originally issued will be sought and found.

The scope of the NBS Clinical SRM program has recently been extended to include the development of complex "matrix" type reference materials such as the anti-convulsant drugs in serum (SRM 900). More recently with SRM 909, a lyophilized human serum, the concentrations of a number of clinically-important analytes were certified by definitive methods. Analytes include total calcium, chloride, cholesterol, glucose, lithium, magnesium, potassium, and uric acid. (See Appendix 1, The Certificate of Analysis for SRM 909.) The catalytic (activity) concentrations of seven enzymes also have now been determined by the reference method type protocols included in this publication.

NBS policy generally precludes certifying a property of any SRM unless one of its laboratories and NBS staff have actively and fully participated in the analytical work. For determination of the enzyme activities in SRM 909, NBS only coordinated the efforts of a voluntary group of 28 cooperating scientists from 18 laboratories. The list of cooperating scientists and laboratories, the team leaders, and the names of other participating personnel and their institutional affiliation is given in the Introduction.

Prior to the start of the experimental work for this study, several general features of its organization and design were discussed and agreed upon. Among these were:

1) The scientific aspects of the work would be under the general direction of G. N. Bowers, Jr. and a team of enzyme measurement experts working with him.

2) Team leaders would be recognized for the specific enzyme methods assigned to them.

3) As much as possible, the reference method type protocols to be used would follow the recommendations of the Expert Panel on Enzymes of the International Federation of Clinical Chemistry (EPE/IFCC). Team leaders would write measurement protocols for each of the enzymes after discussion and agreement with G. N. Bowers, Jr.

4) Because the control of temperature is critical in enzyme analysis, it was agreed that a uniform reaction temperature would be used by all laboratories. The set-point would be defined by the melting point of gallium, 29.77 °C, and the temperature would be controlled within narrow limits. Accordingly, each team was supplied with an NBS Gallium Melting Point standard (SRM 1968).

5) The design of the testing schedules and statistical analysis of the resultant data would be performed by NBS under the direction of K. R. Eberhardt. Unless the preliminary data were to prove unsatisfactory, three rounds were to constitute the work program for evaluation of each enzyme method. Rounds 1 and 2 were to be considered preliminary - in the nature of a "training" exercise - and only Round 3 data would be used for reporting final results. The number of participating laboratories for the specific enzyme analysis was to range from a minimum of 3 to not more than 7.

6) Citation of the enzyme data as it would appear on the SRM 909 Certificate would be solely an NBS responsibility. However, a full report of the interlaboratory tests would be made, through an NBS publication - this publication being the result.

7) The organization of schedules, sample distribution, data collection, and similar duties were initially the responsibility of J. P. Cali. After July 1981, R. Alvarez assumed this responsibility.

The catalytic (activity) concentration values given in this publication are consensus values, obtained from data submitted by the cooperating laboratories. They are provided for information only; they are not NBS certified values.

Full recognition should be given to G. N. Bowers, Jr. for coordinating the scientific activities, to J. P. Cali for planning and organizational contributions, and to R. Schaffer for special editorial contributions. Special recognition is also

given to the 28 scientists who dedicated their time and talents to providing the analytical data that resulted in a successful project.

Inquiries concerning the technical content of this document should be directed to the authors. Other questions concerned with the availability, delivery, price, etc. of SRM 909, will receive prompt attention from:

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ABSTRACT

We determined the catalytic (activity) concentrations of seven enzymes (ACP, ALP, AlaAT, AspAT, CK, LDH and γ-GT) in the NBS lyophilized human serum Standard Reference Material (SRM 909). SRM 909 enzyme activity values should provide the basis for compatibility among methods for determining enzyme values in serum. Separate teams of clinical chemistry experts selected and carried out the analyses for the individual enzymes. The methods used are primarily those recommended by the Standards Committees of the AACC and/or the IFCC, as candidate reference methods; however, a reaction temperature set-point of 29.77 °C (the gallium melting point) was used. This monograph describes the serum reconstitution protocol, the specific enzyme methods, and the results of the third (and last) round of the interlaboratory test program, which we used to derive mean values for enzyme catalytic (activity) concentrations in SRM 909. Interlaboratory standard deviations of less than 10% were achieved. Measurements made one year after the conclusion of the interlaboratory study showed unchanged enzyme activities, within the uncertainties of the original assigned values.

Key words: enzyme; enzyme (catalytic) activity; human serum; interlaboratory reproducibility; reference method; Standard Reference Material.

DISCLAIMER

In order to describe materials and experimental procedures adequately, it was occasionally necessary to identify commercial products by the manufacturer's name or label. In no instances does such identification imply endorsement by the National Bureau of Standards nor does it imply that the particular products or equipment are necessarily the best available for that purpose.

I. INTRODUCTION

Measurement processes have been studied extensively from both an experimental and a statistical point of view. Statistical quality control of industrial goods originated with the work of Shewhart [1] who introduced the concept of control charts over 50 years ago. In 1946, Wernimont [2] applied the use of control charts to chemical analysis. The tremendous post-World War II expansion of industrial and space-related technology led to an unprecedented demand for better measurement technology. In the period 1945 to 1965 many staff members of the NBS devoted considerable effort to the systematic evaluation of the critical statistical parameters that describe measurement processes, including precision, systematic error, and accuracy. Also developed were a number of important statistical concepts used in experimental design, interlaboratory testing, the description of functional relationships, and the evaluation of experimental data. NBS Special Publication 300 [3] contains a collection of over 30 papers covering these topics along with an excellent set of general references on measurement science and statistics. Particularly relevant to the general field of chemical analysis are the following papers contained in that reference manual:

1. C. Eisenhart's two papers on precision and accuracy of calibration systems, and the expression of the uncertainties of final results (see in particular [4])

2. A series of papers by W. J. Youden on uncertainties in calibration, instrumental drift, experimental design, and interlaboratory testing

3. M. G. Natrella's paper on planning of experiments

4. The paper by J. Mandel and T. W. Lashof on interlaboratory evaluation of test methods

5. The paper on accuracy in chemical analysis using linear calibration curves by J. Mandel and F. J. Linnig.

Our purpose here is not to present a detailed discussion involving the statistical analysis of experimental data, but rather to point out the key measurement concepts necessary to establish the validity of analytical data and, in particular, to establish measurement compatibility on a national scale. An important goal of the work cited above is to develop measurement systems that will allow meaningful communication among different measurement stations over long periods of time and large geographical distances. This leads us to the concept of measurement compatibility. If measurements are to be useful in fostering meaningful economic or scientific communication between two or more parties, the different parties must agree on the results of the measurement and the meaning of the numbers associated with the measurement [5-9]. This agreement should take into consideration any imprecision and/or inaccuracies in the measurement process under consideration. Huntoon [10] in an extension of his earlier work [11] defining the National Measurement System concept, has also cited the importance of measurement compatibility and described the general role of reference materials in achieving compatibility.

If a measurement process is to be meaningful, then the data obtained should ideally be both precise and free of systematic error within agreed upon end-use requirements. An accurate measurement is both precise and free of systematic error [7]. Accurate measurement systems are necessarily compatible. Additional requirements for measurement processes, such as wide dynamic range, high sensitivity, or rapid operation, might also be desirable. However, such requirements are not necessary in achieving accuracy but rather represent practical considerations. If a measurement system is accurate, the numerical value obtained for the properties being measured are "true values" within the uncertainties of the measurement processes being used.

The true value determined by measurement processes is referred to as the "quaesitum" by Dorsey and Eisenhart and extensively discussed in their paper on absolute measurements [12]. A pragmatic operational definition of the "true value" of

a property is the value determined by precise measurement methods that are free of systematic error, i.e., an accurate method. Such values should ultimately be experimentally traceable to the base units of measurement such as mass, length, and time.

Unfortunately, the state-of-the-art in enzyme activity measurements is such that concepts such as accuracy and "true value" are not well-defined. Thus, an alternative must be sought to bring about measurement compatibility. One approach is to set up a measurement system based solely on interlaboratory precision whereby all laboratories achieve compatibility through the use of defined, reproducible measurement methods. We used that approach with mutually acceptable, well tested, stable methods of measurement, to provide "method-dependent" enzyme activity values for a homogeneous, stable reference material.

The argument for demonstrating measurement compatibility is straightforward. If two laboratories obtain the same analytical results for SRM 909, as stated herein (also given in the SRM 909 Informational Insert) for a specific enzyme when using the reference method recommended, then with a fairly high degree of assurance, both can apply the recommended method to the same unknown with confidence that they will obtain the same result. When this occurs, then measurement compatibility is achieved. The need to demonstrate interlaboratory compatibility with enzyme reference method measurement systems has been the rationale for this project.

A. Enzyme Project Background

When NBS decided to undertake to certify the analytes in a human serum SRM, several clinical chemists suggested that the utility of this SRM would be greatly enhanced if some of the most widely measured enzymes were also characterized with respect to activity. Primarily, with the advice and leadership of one of the authors (G. N. Bowers, Jr.), and the encouragement of many other leaders in the U.S. clinical chemistry community, a network of cooperating individuals from clinical laboratories was recruited to perform the enzyme activity measurements, according to a well-defined, interlaboratory testing protocol.

The team leaders for each enzyme and the scientists who contributed and their affiliations are given below:

Team #1 - Acid Phosphatase (ACP)

L. M. Ewen, Royal Columbian Hospital, New Westminster, B. C., Canada, (Team Leader);
R. J. L. Bondar, Worthington Diagnostics, Freehold, NJ;
W. Miller, Du Pont - aca Division, Wilmington, DE;
G. N. Bowers, Jr. and M. Onoroski, Hartford Hospital, Hartford, CT; and
L. M. Shaw, Hospital of the Univ. of Pennsylvania, Philadelphia, PA

Team #2 - Alkaline Phosphatase (ALP)

G. N. Bowers, Jr., Hartford Hospital, Hartford, CT, (Team Leader);
R. Miller, Technicon Instrument Company, Tarrytown, NY;
W. D. Fellows, Health Safety & Human Factors Laboratory, Kodak Park, Rochester, NY;
J. P. Bretaudiere, New York Department of Health, Albany, NY;
D. C. Hohnadel, Christ Hospital, Cincinnati, OH; and
W. Sowers, Hyland Division of Travenol, Round Lake, IL

Team #3 - Alanine Aminotransferase (AlaAT)

R. Rej, New York State Department of Health, Albany, NY, (Team Leader);
R. Miller, Technicon Instrument Company, Tarrytown, NY;
W. T. Ryan, Beckman Instruments, Inc., Brea, CA;
L. M. Shaw, Hospital of the Univ. of Pennsylvania, Philadelphia, PA; and
G. M. Sims, Hycel Inc., Houston, TX

Team #4 - Aspartate Aminotransferase (AspAT)

R. Rej, New York State Department of Health, Albany, NY, (Team Leader);
L. M. Shaw, Hospital of the Univ. of Pennsylvania, Philadelphia, PA;
W. D. Fellows, Health Safety & Human Factors Lab., Kodak Park, Rochester, NY;
N. W. Tietz, Univ. of Kentucky Medical Center, Lexington, KY; and
A. D. E. Bacharach, Strong Memorial Hospital, Rochester, NY

Team #5 - Creatine Kinase (CK)

R. Elser, York Hospital, York, PA (Team Leader);
R. Davis, Du Pont - aca Division, Wilmington, DE;
W. T. Ryan, Beckman Instruments, Inc., Brea, CA;
J. H. Stromme, Ulleval Hospital, Oslo, Norway; and
J. F. O'Brien, Mayo Clinic, Rochester, MN

Team #6 - Lactate Dehydrogenase (LDH)

R. B. McComb, Hartford Hospital, Hartford, CT (Team Leader);
K. Y. Jackson, New York State Department of Health, Albany, NY;
B. Howell, National Bureau of Standards, Washington, DC;
S. Buhl, Technicon Instrument Company, Tarrytown, NY; and
D. S. Young, and J. F. O'Brien, Mayo Clinic, Rochester, NY

Team #7 - γ-Glutamyltransferase (γ-GT)

L. M. Shaw, Hospital of the Univ. of Pennsylvania, Philadelphia, PA (Team Leader);
R. Miller, Technicon Instrument Company, Tarrytown, NY;
S. Osaki, Hycel Inc., Houston, TX;
J. H. Stromme, Ulleval Hospital, Oslo, Norway; and
A. W. Wahlefeld, Boehringer Mannheim, Tutzing, West Germany

B. General Criteria for Participants and Operations

The general requirements for individual team members and operation of the interlaboratory test program were:

(1) the team member must have experience in enzyme activity measurements;

(2) the laboratory technical staff under the team members' direction would follow the agreed-on testing protocol in all regards;

(3) sufficient priority would be given the work to allow reasonable scheduling;

(4) data would be collected and reported on an agreed-on format on data sheets supplied by NBS;

(5) work would be done voluntarily and thus would not add to the cost of SRM 909 to the ultimate user; and

(6) all results would be made available to the entire clinical chemistry community through publication.

II. GENERAL COMMENTS ON ENZYME ACTIVITY MEASUREMENTS

Measurements of enzyme activity in body fluids have been used in clinical medicine since the early 1900's when Wohlgemuth [13] first introduced amylase measurements of urine as a means of diagnosing acute pancreatitis. Today a general clinical laboratory may make measurements of about a dozen different enzymes found in serum. The proportion of enzyme measurements many laboratories report may be 15% of total test volume, as illustrated in Table 1 [14]. While analytical precision of day-to-day analyses within a laboratory may range between 5 and 15% (cv)*, the between-laboratory agreement is less satisfactory. The data in reference [15] shows that in large national surveys, interlaboratory cv's for commonly measured enzymes, e.g. AspAT can exceed 50%.

The Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology has shown that the use of a single, strictly defined, "standardized" enzyme method in a network of 100-200 Scandinavian hospital laboratories can lead to much improved interlaboratory precision. In Scandanavia, it should be noted, most laboratories utilize similar instrumentation, have well-trained personnel, and purchase enzyme reagents from a limited number of suppliers. In 1969, before the use of "standardized" methods had been initiated, the overall interlaboratory cv for values for five enzymes (AspAT, AlaAT, LDH, ALP and γ - GT) ranged from 30 to 49%. In 1978, after the widespread adoption of the "standardized" methods, cv's were in the range of 7 to 17% [16].

* cv denotes the coefficient of variation

TABLE 1

Number of Enzyme Activity Measurements in the Clinical Chemistry Laboratory at Hartford Hospital - October 1980 through September 1981.

Enzyme	
Alanine aminotransferase (AlaAT)	39 612
Aspartate aminotransferase (AspAT)	38 986
Lactate dehydrogenase* (LDH)	37 253
Alkaline phosphatase* (ALP)	29 972
Creatine kinase* (CK)	19 767
Amylase	15 647
Lipase	3 576
Acid phosphatase* (ACP)	1 573
Gamma-glutamyltransferase (γ-GT)	1 465
Cholinesterase	65
Ceruloplasmin	30
Enzyme Total	189 750

*Includes isoenzymes by electrophoresis or immunoinhibition

**Total clinical chemistry tests were 1,162,184 (excluding RIA, qualitative urine and coagulation tests).

The achievement of enzyme standardization in the United States would present a problem of greater magnitude from that in Scandinavia, since compatibility of results in 10 000 or more hospital and clinical testing laboratories would be necessary. Furthermore, a wide variety of methods are used in the U.S., ranging from simple kits to complex multichannel instrument systems, laboratory personnel vary markedly in training, and supplies of reagents for enzyme analyses are obtained from literally hundreds of sources.

It has been suggested by the U.S. clinical chemistry community that the "best" approach to achieve compatible results in such an enormous number of laboratories is to develop and promulgate for use, an interrelated hierarchy of reference methods and reference materials [17]. The National Reference System for Clinical Chemistry (NRSCC), which functions under the administrative guidance of the National Committee for Clinical Laboratory Standards (NCCLS), has been created to carry out this task. For clinical enzymology, reference-type enzyme methods would be needed. The methods would be used for assigning enzyme activity values to the enzyme(s) in appropriate reference materials, and these reference materials would be made readily available to all.

The development of reference-type enzyme methods has reached an advanced state within the Expert Panel on Enzymes of IFCC and standards groups in the U.S. As a consequence, it is now possible to write protocols reasonably like those that will ultimately be recommended by these panels of experts.

The availability of the NBS Human Serum, SRM 909, which contains several stable enzymes, provided a unique opportunity to examine the problems associated with interlaboratory use of these reference type enzyme methods [18].

At the time when the selection of an appropriate human serum preparation for use as the SRM was begun, several manufacturers were visited to assess their capabilities for the production of a suitable freeze-dried material that would meet target specifica-

tions. The final choice was based on the low inhomogeneity of pilot runs of the materials that were screened. The SRM would be a freeze-dried serum in the form of multiple, small pellets, and would be dispensed into vials by weight.

Enzymes levels in the final manufactured lot of serum were specified in accord with the broad guidelines suggested by the manufacturer, and in consultation with several clinical chemists. The choice of tissue sources for the enzymes to be added was left to the manufacturer.

The enzymes, their associated activities, and source of added enzymes are shown in Table 2.

TABLE 2

Catalytic (Activity) Concentrations (U/L)

Enzyme	Total Planned	Base Level*	Amount Added*	Found Here**	Tissue Source of Supplement
ACP	2.2 ± 0.7	ND	ND	0.23	None
ALP	100 ± 25	49	86	75	Calf Intestine
AlaAT	28 ± 8	5	31	24	Pig Heart
AspAT	28 ± 8	15	33	31	Pig Heart
CK	150 ± 50	112	179	123	Pig Heart
LDH	150 ± 50	97	144	229	Pig Heart
γ-GT	30 ± 15	ND	ND	16	None

* at 37 °C, Dupont aca (automatic clinical analyzer)

**at 29.77 °C

III. PROTOCOLS FOR ENZYME MEASUREMENTS

This chapter gives general information concerning the specific enzyme methods chosen. It also presents details of the protocol for reconstituting the lyophilized SRM 909 because this part of the procedure was critical to the overall success of the study. Chapter IV gives specific information on the protocols used for each enzyme.

A. General Comments and Observations on the Enzyme Reference Methods Chosen

The Expert Panel on Enzymes of the Committee on Standards of the IFCC has now published Parts 2-7 of recommendations [19-23] on reference methods for the measurement of the catalytic concentrations (activity per unit of volume) of the following enzymes: aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, alkaline phosphatase and creatine kinase. It has also published recommendations with regard to general considerations on the determinations of enzymes [24] and methods for preparation of enzyme reference materials [25]. In general, the specific protocols used in this study closely follow these IFCC recommendations if they were available in draft form in mid-1979. The major exception is our use of the melting point temperature of gallium (29.77 °C), as recommended by Mangum [26] and Bowers and Inman [27] as the reaction temperature set-point instead of 30.00 °C.

Each team leader was responsible for the choice of reference method after conferring with Dr. Bowers. The team leader wrote the detailed protocol and circulated it among his team for comment and concurrence. Thereafter, team members would follow the final protocol without modification. Enzyme team members were recruited mainly from among individuals who were previous participants in other enzyme method development activities or transfer projects conducted, for example, by AACC, EPE/IFCC, CDC or CAP. Team members were from governmental, academic, hospital, and industrial laboratories.

The protocols for AlaAT and AspAT were prepared by Dr. Rej, for CK by Dr. Elser, and for γ-GT by Dr. Shaw. These four protocols were almost identical to EPE/IFCC methods already published or in early draft stages in 1979. The modifications introduced are discussed under the specific protocols in Chapter IV.

The state of current prostatic acid phosphatase measurement technology, which includes recently introduced radioimmunoassay methods, does not permit the selection solely of an activity concentration method as the reference method at this time. Nevertheless, the method of Ewen and Spitzer [28] which employs thymolphthalein monophosphate as substrate and a 1-hour incubation at 29.77 °C was used in our study for measuring the activity of this enzyme. Dr. Shaw arranged for SRM 909 samples to be analyzed concurrently by an immunological (RIA) prostatic acid phosphoresis method. Unfortunately and inadvertently, seminal fluid acid phosphatase was not added to the serum base pool from which SRM 909 was made, and as a consequence, it contains only very low levels (background) of acid phosphatase. In order to enhance our study of acid phosphatase, a special small batch of lyophilized material similar in composition to SRM 909, but which contained added human seminal fluid acid phosphatase, was analyzed in parallel with SRM 909. In addition to the development of our ACP protocol, the purity of thymolphthalein monophosphate preparations and specifications for use of this material as a high quality substrate were studied [29,30].

Alkaline phosphatase was based on the manual reference method of Bowers and McComb [31]. However, the method was updated by the inclusion of specifications for p-nitrophenol [32] and p-nitrophenylphosphate [33]. Although originally planned, no study was made to compare the 2-amino-2-methyl-1-propanol preparations used as buffer in various laboratories, with respect to potential inhibitor content [34]. The EPE/IFCC draft recommendation for alkaline phosphatase [22] that has now been published, was unavailable at the start of this study.

The LDH reference method was developed further by Dr. McComb. It utilizes the highly purified sodium pyruvate supplied by NBS as SRM 939. The NADH supplied to each laboratory was from a single lot that was tested by Dr. McComb and found free of LDH-inhibitor [35]. Special algorithms were employed to calculate the "initial" activity from the non-linear progress curves.

Amylase, although present in SRM 909, was not included in our study. The development of a consensus on a reference method for amylase is not likely in the near future. There is neither agreement on the methodologic principle for an amylase reference method, nor especially an optimum substrate.

B. Reconstitution Protocol

When determining the definitive values for the non-enzymatic constituents of SRM 909, NBS reconstituted the material at room temperature. However, reactivation of the enzymes in a reproducible manner at lower reconstitution temperatures was needed.

Our study of activity versus the procedure for reconstituting (rehydrating) SRM 909 lyophilized serum led to adoption of a uniform reconstitution protocol. Some of the SRM's catalytic activities were found to be dependent on reconstitution variables and to change during storage at different temperatures. Because we needed to reconstitute the lyophilized serum at below room temperature to obtain reproducible enzymatic activity, ice-water baths were used during the reconstitution process. That provided an easily achievable uniform temperature for the 1-hour allowed for reconstitution. The separate enzyme protocols provide individual details where needed for handling the reconstituted material after 1 hour. The reconstitution procedure is as follows:

1. Remove vials of SRM 909 lyophilized serum and diluent from refrigerator. Keep the vial of diluent in an ice

water bath for 5 min prior to use. Ice and water (not air and ice) should contact the vial.

2. Tap vial bottom of lyophilized serum on a solid surface to dislodge freeze-dried particles adhering to the stopper. Remove the metal cap and stopper; be careful not to lose any of the dried particles.

3. Use a Class A volumetric pipette at room temperature to add exactly 10.0 mL of the ice-cold diluent to the vial of lyophilized serum as follows: Dispense the diluent slowly against the inside neck of the vial while continually turning it, to wet all the serum particles.

4. Replace the stopper; swirl gently and invert the vial 10 times, then reimmerse it in the ice-bath. Repeat the gentle swirling and inverting operations at 10 min intervals for 1 hour, always returning the vial to the ice-bath.

5. Before use, inspect to ensure the absence of undissolved particles.

6. Proceed to the directions given with individual enzyme protocols.

IV. SPECIFIC ENZYME PROTOCOLS

This chapter contains the detailed descriptions of the specific measurement protocols for the enzymes. Each section is for one enzyme, and contains descriptions of (1) the methodological principle, (2) instrumentation and equipment, (3) reagents, (4) the analytical procedure, and (5) calculations. A summary of the data obtained in the interlaboratory testing for each enzyme is given in Chapter V.

A. Prostatic acid phosphatase

(Orthophosphoric monoester phosphohydrolase, acid optimum, EC 3.1.3.2)

Principle: This measurement of the catalytic (activity) concentration of prostatic acid phosphatase (ACP) in human serum utilizes the method of Roy et al. [36] as modified by Ewen and Spitzer [28]. The reaction sequence is as follows:

$$\underset{\text{monophosphate}}{\text{Thymolphthalein}} + H_2O \xrightarrow{\text{ACP}} \text{thymolphthalein} + H_3PO_4.$$

The spectrophotometric absorbance of thymolphthalein, the product liberated from the substrate thymolphthalein monophosphate (TMP) in proportion to the activity of the acid phosphatase during its reaction, is measured after conversion of the thymolphthalein to its colored form at an alkaline pH.

Final Reaction Conditions:

Reaction temperature	29.77	°C
pH	5.4	
Acetate buffer	0.15	mol/L
Thymolphthalein monophosphate (TMP)	1.0	mmol/L
Brij-35	1.5	g/L
Volume fraction (sample/total)	1:12	(0.083)

Instrumentation and Equipment

Glassware: Volumetric glassware should meet the National Bureau of Standards (NBS) Class A specification or be individually calibrated prior to use. For pipetting serum specimens, "to-contain," self-adjusting micropipettes, accurate to within ±0.2% of volume are preferred. Alternatively, repetitive dispensing devices with disposable tips may be used; however, their precision and accuracy should be checked and be within 0.5% (95% confidence limit). Tubes used in the assay must be chemically clean and preferably made of borosilicate glass.

Instrumentation: Specifications for the instrumentation, pH meter, waterbath, and spectrophotometer are given in Notes 1, 2 and 3, respectively.

Reagents: Specifications for TMP and thymolphthalein are given in Notes 4 & 5, respectively [29]. All other chemical reagents including water should meet American Chemical Society (ACS) reagent grade specifications.

1. Acetic acid (5 mol/L). Use a buret to transfer 28.87 mL of glacial acetic acid into a 100 mL volumetric flask. Dilute to volume with water. Mix well.

2. Sodium acetate (5 mol/L). Dissolve 68.0 g of sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$) in water in a 100 mL volumetric flask. Dilute to volume. Mix well.

3. Acetate buffer (5 mol/L, pH 5.4 at 25 °C). Add sufficient 5 mol/L sodium acetate (reagent 2) to 5 mol/L acetic acid (reagent 1) to adjust to pH 5.4 at 25 °C.

4. Acetate buffer (0.25 mol/L, pH 5.4 at 25 °C). Pipette 5 mL of acetate buffer (reagent 3) into a 100 mL volumetric flask. Dilute to volume with water. Mix well.

5. Buffered substrate. Dilute a 300 g/L Brij-35 solution to a concentration of 3.24 g/L. Use 50 mL of this diluted solution to dissolve the amount of $Na_2TMP \cdot xH_2O$ required to give a TMP concentration of 1.1 mmol/L when the volume of this reagent is brought to 100 mL; (eg., if using $Na_2TMP \cdot 11H_2O$, molecular weight 752.65, the appropriate quantity is 82.8 mg). Add 1.92 g of sodium acetate trihydrate to the solution, mix to dissolve, adjust the pH of the solution to 5.4 at 25 °C with 0.1 mol/L HCL, and finally bring volume to 100 mL with reagent grade water. Store in refrigerator.

6. Alkaline reagent (0.1 mol/L Na_2CO_3, in 0.1 mol/L NaOH). Dissolve 10.6 g of anhydrous Na_2CO_3 and 4.0 g of NaOH in distilled water. Dilute to 1000 mL. Mix well.

7. Thymolphthalein stock standard (3 mmol/L). Dissolve 129.2 mg of thymolphthalein in 70:30 (V/V) n-propanol-water in a 100-mL volumetric flask. Fill to the mark. Mix well.

Analytical Procedure:

1. Preparation of standard curve (standardization to a catalytic concentration of 60 U/L): Into 10 mL volumetric flasks pipette 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mL of thymolphthalein stock standard (reagent 7). Fill each flask to the mark with 70:30 (V/V) n-propanol-water mixture. Mix well. Into a set of seven tubes, dispense 550 µL of buffered substrate (reagent 5) and 1.0 mL of alkaline reagent (reagent 6). Pipette 50 µL of the 70:30 n-propanol-water mixture into the first tube of the set and

50 µL of one of each of the diluted standards into the remaining tubes. Mix well. Read the absorbance of these solutions at 595 nm, using the first tube as blank. Plot absorbance versus U/L on linear graph paper. These absorbances are equivalent to absorbance changes obtained by the action of 10, 20, 30, 40, 50, and 60 U/L of acid phosphatase when a 30-minute assay incubation time is used, or to one-half of these activities if a 60-minute assay is used.

2. Measurement of the catalytic concentration of acid phosphatase: Use six tubes for each specimen to be assayed, five "test" tubes, labeled "15", "30", "45", "60" (for minutes) and "total", and also one "blank" tube. Into tubes labeled 15, 30, 45, 60 and blank, pipette 1.0 mL of the alkaline reagent (reagent 6). Into the remaining empty tubes labeled total, pipette 5.5 mL of the buffered substrate (reagent 5). Bring only the tubes labeled total to the 29.77 °C assay temperature (see Note 2) in a water bath, and to each at convenient intervals (e.g., 30 s) transfer 500 µL of a serum specimen, mix gently and allow to incubate at the assay temperature. At exactly 15, 30, 45 and 60 minutes after addition of specimen, withdraw aliquots from each incubating total tube and transfer exactly 0.6 mL to the tubes correspondingly labeled 15, 30, 45, and 60.

While the total tubes are incubating, or following incubation--whichever is more convenient--add 550 µL of buffered substrate (reagent 5) to each blank tube followed by 50 µL of each serum specimen, and mix.

Read the absorbances of test and blank solutions at 595 nm against reagent grade water; subtract blank from test absorbances; check that the reaction is linear with time for each specimen; and obtain the catalytic (activity) concentration of acid phosphatase either by reference to the standard curve or calculation as described below.

Calculations:

The catalytic (activity) concentration of acid phosphatase

in SRM 909 measured by the above protocol at 29.77 °C, is calculated in terms of International Units per liter, (1.0 U/L = 1.0 $\mu mol \cdot min^{-1} \cdot L^{-1}$) as follows:

$$\text{Catalytic concentration (in U/L)} = (dA/dt)V \cdot \varepsilon^{-1} \cdot b^{-1} \cdot v^{-1}$$

where dA is the change of absorbance at 595 nm over the specified rate measurement interval, dt is the measurement interval in minutes, V is the volume of the final reaction mixture in liters, ε is the micromolar absorptivity of thymolphthalein ($39\ 200 \times 10^{-6}$ $L \cdot \mu mol^{-1} \cdot cm^{-1}$) under the final reaction conditions, b is the cuvette pathlength in cm, and v is the sample volume in liters.

By substituting the numerical values for V, ε, b, and v:

$$\text{Catalytic concentration (in U/L)} = \frac{(dA/dt) \cdot (1.6 \times 10^{-3})}{(39\ 200 \times 10^{-6})\ (1)\ (0.05 \times 10^{-3})}$$

$$= 816.3 \cdot dA \text{ per min.}$$

Catalytic (activity) concentration in katals per unit volume (kat/L) can be calculated from the relationship 1.0 U/L = 16.67 nkat/L [37].

Notes:

1. The pH measuring device should be calibrated at 25 °C against reference buffers from an authoritative source (e.g., NBS or IUPAC).

2. Temperature of assay. The melting point of gallium (29.77 °C) is the reaction temperature set-point for this reference method. A water bath with a continuous agitation capability is used to maintain ±0.05 °C control throughout the water mass.

3. The method is relatively insensitive to variation in wavelength setting or spectral bandwidth if measurements are made

on spectrophotometers in which stray light is low. A wavelength setting error of ±2 nm with a spectral bandwidth of 8 nm results in an absorbance error of less than 1%. Cuvettes should have parallel faces of optical glass that transmit light without significantly reducing the light intensity due to absorption, reflections or other scattering effects. The internal pathlength should be 10.00±0.01 mm.

4. Specifications for TMP as substrate. The $Na_2TMP \cdot xH_2O$ from different manufacturers may vary in content of thymolphthalein, in content of water of crystallization and in color. To be acceptable as substrate, the supplier's analysis for the $Na_2TMP \cdot xH_2O$ should show that the material meets the following specifications [29]:

a. Formula: $C_{28}H_{29}O_7PNa \cdot xH_2O$

b. Molecular weight, anhydrous: 554.5.

c. Water content (by Karl Fischer method): range 0.15-0.26 as mass fraction or 5.5:1 to 11:1 as mole fraction.

d. Appearance: white to pale yellow crystals; clear, colorless to pale yellow, 25 g/L aqueous solution.

e. pH of aqueous solution: range 8.0-8.5.

f. Apparent molar absorptivity: range 2150 - 2500 at 445 nm.

To check molar absorptivity, dissolve 100.0 mg of $Na_2TMP \cdot x\ H_2O$ in 0.1 mol/L Na_2CO_3 in a 100 mL volumetric flask. Dilute to volume with 0.1 mol/L Na_2CO_3. Prepare a 1 in 10 dilution with additional 0.1 mol/L $NaCO_3$. Measure absorbance ($\underline{A}$) at 445 nm with 0.1 mol/L Na_2CO_3 as reference. Calculate the apparent molar absorptivity using label value for mole fraction of H_2O in $Na_2TMP \cdot xH_2O$ as follows:

$$\text{Apparent molar absorptivity} = \frac{(A)\ (554.5)}{(1\text{-mass fraction } H_2O)\ (0.100)}$$

g. Thymolphthalein content: less than 5 mmol/mol

To check mole fraction of thymolphthalein in TMP, add 1.0 mL of the alkaline reagent (reagent 6) to 0.5 mL of buffered substrate (reagent 5). The molar absorptivity at 595 nm (25 °C) should be less than 0.075 relative to the blank.

h. Storage: 4 °C.

5. High purity thymolphthalein in a solution of 0.06 mol/L Na_2CO_3 solution (as under assay conditions) has been found to have a molar absorptivity of 40 500±200 $L \cdot mol^{-1} \cdot cm^{-1}$ at 600 nm (at 25 °C and 10 mm pathlength). Test for this by transferring 25.00 mL of the thymolphthalein stock standard (reagent 7) to a 1-L volumetric flask. Dilute to volume at 25±1 °C with 70:30 (V/V) n-propanol-water. A mixture of 3 ml of this solution and 5 mL of the alkaline color reagent (reagent 6) should have an absorbance at 600 nm of 1.134±0.005 at 25 °C after blank correction.

6. Prepare tubes needed for serum sample preservation by pipetting 25 µL of acetate buffer (reagent 3) into chemically clean borosilicate glass tubes. Place tubes in an oven at 60 °C, and allow buffer solution to evaporate but do not over-dry. Stopper and store tubes at room temperature until required. These tubes contain sufficient buffer for acidifying 0.5 mL of patient sample. Check tubes on initial preparation and periodically throughout use by adding 0.5 mL of serum and measuring the pH after dissolving the buffer.

7. Collect blood with minimal venous stasis. Plasma obtained by collection into sodium heparin at a concentration of 0.2 g/L is preferred. Centrifuge whole blood for 10 minutes at 3000xG at 25 °C. Immediately thereafter transfer 0.5 mL of the plasma to a tube containing dried acetate buffer. Agitate gently to dissolve the buffer. Store specimens at -20 °C if they are not to be assayed on the same day.

8. Alternative reconstitution of SRM 909: (for use if only acid phosphatase is to be assayed). Follow reconstitution protocol given above, except that 0.25 mol/L acetate buffer (reagent 4) is used in place of the diluent supplied with SRM 909. Store aliquots at -20 °C if required for use on subsequent days.

Comments by Team Leader (L. M. Ewen)

Final results were calculated from data submitted by R. J. L. Bondar, M. Onoroski/G. N. Bowers, Jr. and L. M. Ewen. The prostatic acid phosphatase activity found in SRM 909 was extremely low. We would have preferred to have an NBS material with a prostatic acid phosphatase level at or above the upper limit of the reference range for the method, e.g., to a level of 2 U/L.

Because of the low activity of this enzyme in SRM 909, no data were gathered on its reactivation. SRM 909 was not supplemented with prostatic acid phosphatase and the activity found by our method was extremely low (the ΔA per 60 min was 0.02). This was confirmed by radioimmunoassay (RIA) performed by L. M. Shaw which detected no prostatic acid phosphatase in the SRM. As a check on the RIA methodology, samples similar to SRM 909, but supplemented with prostatic acid phosphatase to a level of 5 U/L, were assayed simultaneously with SRM 909 by RIA, and the level of prostatic acid phosphatase in the enriched material was found to be 11 µg/L.

The method used in this work should not be viewed by standardizing agencies as an official recommendation of the procedure. Several subcommittees such as the Enzyme Study Group of the Standards Committee of the American Association for Clinical Chemistry, are actively engaged in projects with the objective of the development of a specific recommended method for prostatic acid phosphatase. Their recommendations, while needed, are not yet available.

B. Alkaline Phosphatase
(Orthophosphoric monester phosphohydrolase, alkaline optimum, EC 3.1.3.1)

Principle: This protocol for measuring the total catalytic (activity) concentration of alkaline phosphatase in SRM 909 is a slightly modified version of the manual reference method described by Bowers and McComb [31]. The method differs from earlier published methods [38] only slightly in final reagent conditions. The buffer pH is raised to 10.5, the substrate concentration is increased to 16 mmol/L and the magnesium concentration is increased to 1 mmol/L. However, the procedural steps have been markedly altered. In order to avoid prolonged exposure of the enzyme to 2A2M1P [39], the serum or other sample is preincubated with 3 mmol/L magnesium for at least 5 min before the activity is measured. Furthermore, the reaction rate is followed only within the first two minutes to avoid subsequent slower rates thought to be due to the removal of zinc from the enzyme by chelation to 2A2M1P and/or to inhibitory compounds in the buffer [34].

The appearance of the yellow color of p-nitrophenol (NP) is monitored continuously in a spectrophotometer at 402 nm as the alkaline phosphatase(s) catalyzes the hydrolysis of the colorless substrate, p-nitrophenyl phosphate (NPP). This enzymatic reaction takes place in a transphosphorylating buffer, 2-amino-2-methyl-1-propanol (2A2M1P), with all reaction parameters carefully controlled.

$$NPP + 2A2M1P \xrightarrow{ALP} NP + 2A2M1P{\cdot}P.$$

Final reaction conditions:

Reaction temperature	29.77	°C
pH	10.5	
2-Amino-2-methyl-1-propanol (2A2M1P)	1.0	mol/L
p-Nitrophenyl phosphate (NPP)	16.0	mmol/L
Magnesium acetate	1.0	mmol/L
Volume fraction (sample/total)	0.0164	(1:61)

Reagents:

Specifications for the 2A2M1P [34], NP [33] and the p-nitrophenyl phosphate [32] are given in the Notes and should be met. All other chemical reagents and water should meet American Chemical Society (ACS) reagent-grade specifications. Glassware should meet the National Bureau of Standards (NBS) Class A specification or be individually calibrated prior to use.

1. Magnesium solution (3 mmol/L): Dissolve 650 mg of magnesium acetate·$4H_2O$ in water in a 1-L volumetric flask. Dilute to volume. Mix well. This solution is stable indefinitely at 4 °C.

2. 2A2M1P buffer (1.5 mol/L, pH 10.50 at 29.77 °C): Warm the 2A2M1P (see Note 1) at 30-35 °C until completely liquefied. Weigh 135 g of this liquid directly in a 1-L volumetric flask. Add about 500 mL of reagent-grade water, mix, and then add 190 mL of 1.000 mol/L HCl. Allow solution to cool to 25±1 °C, and adjust to volume with reagent-grade water. Mix well. At 29.77 °C, this buffer has a pH of 10.50±0.05 when measured after calibration, using special Tris-type electrodes (see Note 2) and NBS buffers, and sloping (i.e., adjusting the slope controls) of the pH meter. When stored at room temperature with protection from atmospheric CO_2, this buffer is stable for 1 month.

3. p-Nitrophenyl phosphate buffered substrate (24.5 mmol/L in 2A2M1P buffer): For each 1 mL of reagent 3 required, dissolve 9.1 mg of $Na_2NPP·6H_2O$ (see Note 3) in 2A2M1P buffer (reagent 2). Make fresh daily.

4. Stock standard NP (1 mmol/L): Dissolve 139.1 mg of high-purity NP (see Note 4) in water. Dilute to volume in 1-L volumetric flask at 25±1 °C with water. Mix well. This solution is stable for many months if protected from light.

5. Working standard NP (25 μmol/L in 2A2M1P buffer): Place 900 mL of 2A2M1P buffer (reagent 2) in a 1-L volumetric flask. Add exactly 25.00 mL of stock standard NP (reagent 4). With contents at 25±1 °C, fill flask to the mark with water and mix. The absorbance of this reagent at 402 nm (at 29.77 °C in a 10.00 mm cuvette) is 0.476 when a blank made from 9 parts of reagent 2 and 1 part of water is subtracted. This is equivalent to a molar absorptivity of 19 050 $L \cdot mol^{-1} \cdot cm^{-1}$. If the value obtained differs by more than ±1% from this value, the observed value should be used to calculate the activity. The user should be able to explain the reasons for the difference. Reagent 5 has been shown to be stable for at least 8 weeks.

Analytical Procedure:

Into a test tube containing 1.0 mL of magnesium solution (reagent 1), add 50 μL of sample. A calibrated mechanical diluter/pipette or a "to-contain" pipette may be used to deliver sample; however, adequate wash-out of the pipette must be ensured. Mix thoroughly and place tube in a water bath at 29.77 °C for exactly 5 min so that magnesium activation and temperature equilibration occur before the rate measurement is made. Immediately on completion of the 5-minute preincubation with magnesium, add 2.0 mL of buffered substrate (reagent 3) which has been prewarmed to 29.77 °C. Mix well. Immediately transfer the reaction mixture into a 1.000-cm cuvette in a spectrophotometer set to 402±1 nm (see Note 6) and equipped to allow the cuvette and contents to be rapidly adjusted to a reaction temperature of 29.77±0.05 °C (see Note 5). Immediately record absorbance at 402 nm vs. time continuously or at regular intervals (e.g., 10 s) for a total of 120 s against an air blank. Obtain the rate of absorbance change per minute from the spectrophotometric data by graphic or mathematical means.

Calculations:

The catalytic (activity) concentration of alkaline phosphatase in SRM 909 measured by this protocol at 29.77 °C is calculated in terms of International Units per liter (1.0 U/L = 1.0 $\mu mol \cdot min^{-1} \cdot L^{-1}$) as follows:

$$\text{Catalytic concentration (in U/L)} = dA/dt \cdot V^{-1} \cdot \varepsilon^{-1} \cdot b^{-1} \cdot v^{-1}$$

where dA is the change of absorbance at 402 nm over the specified rate measurement interval, dt is the measurement interval in minutes, V is the total volume of the final reaction mixture in liters, v is the sample volume in liters, b is the cuvette pathlength in cm, and ε is the micromolar absorption coefficient of NP, which is 19 050 x 10^{-6} $L \cdot mol^{-1} \cdot cm^{-1}$ under the final reaction conditions. By substituting numerical values,

$$\text{Catalytic concentration (in U/L)} = \frac{(dA/dt)\ (3.05 \times 10^{-3})}{(19\ 050 \times 10^{-6})\ (1)\ (0.05 \times 10^{-3})}$$

$$= 3203 \cdot dA \text{ per min.}$$

Catalytic (activity) concentration in katals per unit volume (kat/L) can be calculated from the relationship 1.0 U/L = 16.67 nkat/L [37].

Notes:

1. 2A2M1P has been shown by many workers to contain inhibitors. The 2A2M1P used should be inhibitor-free by both enzymatic and special testing, as described by Rej et al. [34]. They suggest that 5-amino-3-azo-2,2,5-trimethyl-hexanol is one of these inhibitors.

2. Measurements of pH at 10 and above in amino propanol solutions require special "Tris-type" electrodes, which can be obtained either from Sigma Chemical Co., St. Louis, MO 63178, as "Trisma" electrode No. E4878, or from the Beckman Instrument Co., Fullerton, CA 92634, as described in Applications Research Technical Report No. 542.

3. The quality of $Na_2NPP \cdot 6H_2O$ may vary from lot to lot and manufacturer to manufacturer [33]. Variations in water content may cause differences of ±5%. The specifications for this substrate are as follows:

a. Molar absorptivity: At 311 nm and 25 °C in 10 mmol/L NaOH, the molar absorptivity has been found to be 9866 $L \cdot mol^{-1} \cdot cm^{-1}$. If all other specifications are met, $Na_2NPP \cdot xH_2O$, when x is in the range of four to six, may be acceptable. The molar concentration of NPP in solution is established from its absorbance in NaOH at 311 nm.

b. Test for NP: The mole fraction of NP in NPP should be less than 0.1%. The absorbance of a 100 mmol/L solution of NPP in a 1.000-cm cuvette at 432 nm should be less than 0.300 [32].

c. Inorganic phosphate in $Na_2NPP \cdot xH_2O$. Amount allowable is less than 1%. Test for inorganic phosphate in a 100 mmol/L Na_2NPP solution by methods used normally in a clinical chemistry laboratory to determine phosphorous in human serum.

4. High-purity NP after being recrystallized from water, dichloromethane, and again water [32] has been found to have a molar absorptivity of 18 380±90 $L \cdot mol^{-1} \cdot cm^{-1}$, at 401 nm and 25 °C, in 10 mmol/L NaOH (1.000-cm pathlength). Check by transferring 25.00 mL reagent 4 into a 1-L flask and diluting to volume at 25±1 °C with 10 mmol/L NaOH. The absorbance of this 25 μmol/L solution after blank correction should be 0.460±0.002.

5. Temperature of assay: The melting point of gallium, 29.77 °C, is the reaction-temperature set point for this reference method. A water bath with a continuous agitation capability is used to maintain ±0.05 °C-control in the reaction mixture [27].

6. Temperature factors for this reference method for alkaline phosphatase on pooled human serum at 25, 30, and 37 °C relative to 29.77 °C are 0.79, 1.01 and 1.33, respectively. Correction is not made for thermal expansion or temperature effects on absorbance. Similar temperature-conversion factors have been found with a crude preparation of alkaline phosphatase prepared from human liver and added to human serum pool.

7. The method is relatively insensitive to variation in wavelength setting or spectral bandwidth when measurements are made on spectrometers in which stray light is low. A wavelength setting error of ±3 nm with a spectral bandwidth of 5 nm results in an absorbance error of less than 1% [40].

8. The reference intervals for healthy individuals for this method at 29.77 °C (see Note 5) are as follows:

Population	Catalytic concentration, (U/L)
Newborns, 1 week	Up to 250
Girls	
1-12 years	Up to 350
>15 years	See adults
Boys	
1-12 years	Up to 350
12-15 years	Up to 500
>20 years	See adults
Adults	25-100

Comments by Team Leader (G. N. Bowers, Jr.)

Final results (RRII) were calculated from data supplied by all members of the team (see list in the Introduction). The human alkaline phosphatase activity level in the base pool after the initial processing, but before supplementation, was 49 U/L at 37 °C as measured by the manufacturer on a Du Pont aca. Partly purified, calf-intestinal alkaline phosphatase was added to the base pool by the manufacturer prior to lyophilization, to give a total activity of about 100 U/L (by aca at 37 °C). Since the activity factor for altering the reaction temperature from 29.77 °C to 37 °C with most alkaline phosphatase methods and materials is approximately 1.33, we expected the activity for SRM 909 to be about 75 U/L. Many other factors, such as pH and buffer concentration, preincubation with magnesium, and length of exposure to buffer before and during assay would also affect the activity [36]. Fortuitously, the activity found by use of this protocol (at 29.77 °C) was near 75 U/L.

One laboratory's initial difficulty with the protocol is worth noting. Its results for the two preliminary test rounds

were markedly different from all of the others. The laboratory discovered two causes for its discrepant results: (1) the temperature at the cuvette was too low because of insufficient water flow, and (2) instead of air or water, a substrate/magnesium/2A2M1P had been used as the blank. $Mg(OH)_2$ had precipitated, which caused high blank readings. With these problems avoided, its results for RRIII, then corresponded with the other laboratories.

RRI and RRII were carried out in the spring and summer of 1980. The interlaboratory mean values were 73.2±4.3 U/L (S.D. for individual laboratories) and 74.4±3.5 U/L respectively. For RRIII data, which were obtained during the winter of 1980-81, the mean value was 75.4±2.0 U/L. Using the above protocol, a European government R&D clinical laboratory found a value of 76.5±1.0 U/L in the spring of 1981.

The participants and instruments used in RRIII are given below.

Participant	Instrument Used
G. N. Bowers, Jr.	Varian Cary 219
W. N. Fellows	Beckman ASTAR
D. C. Hohnadel	Gilford 300N
J. P. Bretaudiere	Varian Cary 219
R. Miller	Varian Cary 219
W. Sowers	Varian Cary 219

The greatest variable one faces in measuring alkaline phosphatase activity in lyophilized material is the increase in activity seen with time and incubation temperature after reconstitution. Figure 1 shows the SRM 909 alkaline phosphatase activity increases observed as a function of time and temperature, the samples being held at various temperatures after undergoing a uniform 1-h reconstitution in an ice-water bath. Although higher activities can be reached more rapidly by incubating the reconstituted material at higher temperatures, the maximum activities thus obtained are not stable. In fact a

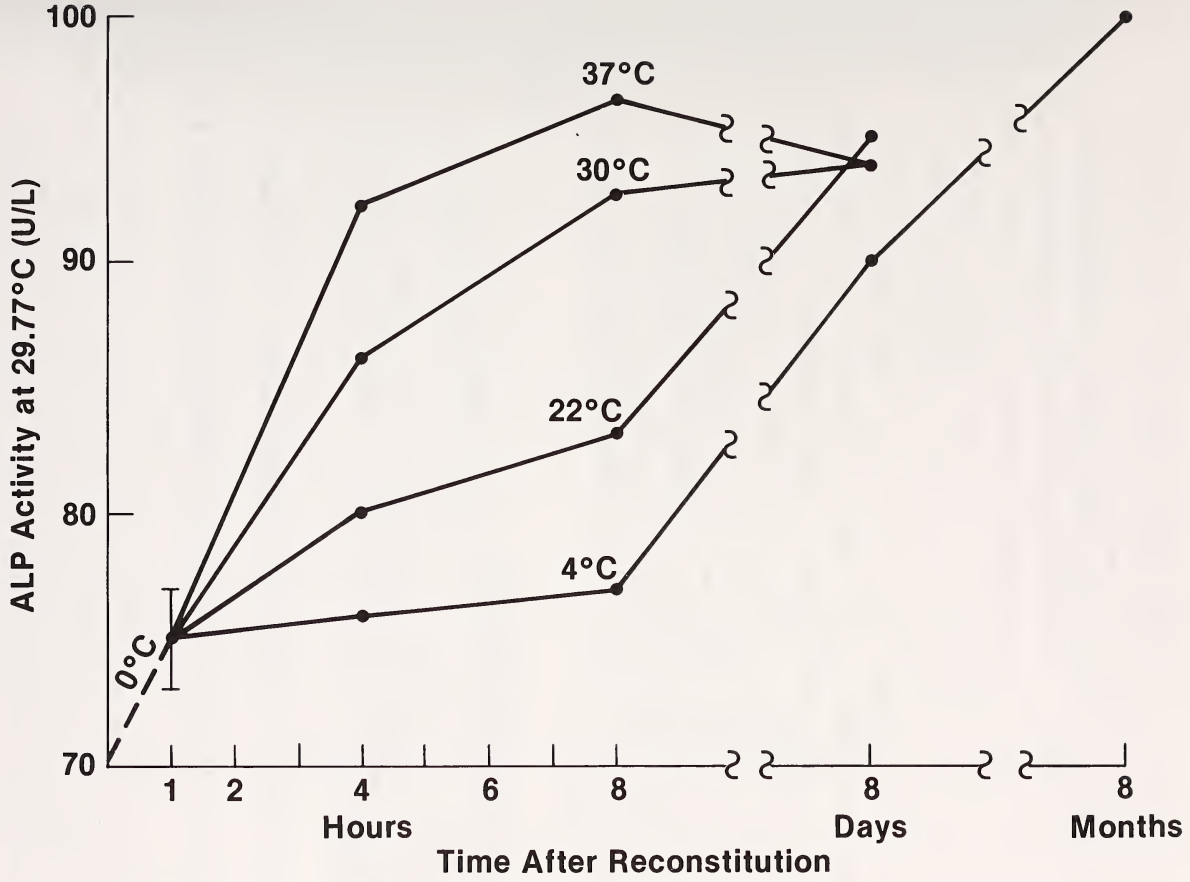


Figure 1: ALP activity changes measured at 29.77 °C after reconstitution at temperatures in the range 4-37 °C.

completely reproducible procedure was not found. By warming samples for 8 h at 37 °C, a peak activity of 95 U/L was reached; but this activity level subsequently decreased slightly.

Observations were made over an 18 month period on 19 reconstituted samples that were held at 4 °C. Initially, these activities were 75±2 U/L, but after 8 months, the range was from 26 to 104 U/L. In the absence of a satisfactory means for stabilizing activity, we decided to reconstitute and hold samples at 0 °C in an ice-water bath. This would reduce variation in the temperature and consequently, the rate of reactivation among laboratories. The decision to use 0 °C for reconstituting was applied to all enzymes, and was in effect for Round Robin III. For the non-enzymatic constituents of SRM 909, see Certificate of Analysis for SRM 909, Appendix 1; NBS independently released its final recommendations for reconstitution at room temperature (23±2 °C). A systematic comparison of the differences in enzyme reactivation resulting from these two procedures has not been made, but one can expect to add 2 U/L of phosphatase activity from reconstituting at room temperature for 1-h. Steady increases in activity with time would occur thereafter, as shown in Figure 1.

C. Alanine Aminotransferase
(L-Alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2)

Principle: Alanine aminotransferase (AlaAT), formerly called glutamate pyruvate transaminase (GPT), catalyses the following reaction:

$$\text{L-alanine} + \text{2-oxoglutarate} \xrightarrow{\text{AlaAT}} \text{pyruvate} + \text{L-glutamate}$$

The activity of AlaAT is measured by converting the pyruvate thus

formed into lactate with reduced nicotinamide adenine dinucleotide (NADH) and lactate dehydrogenase (LDH) as indicator enzyme [20, 41]:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+$$

The quantity of NADH oxidized, which is equivalent to the amount of pyruvate formed, is measured by the decrease in absorbance at 339 nm.

Final Reaction Conditions:

Reaction temperature	29.77	°C
L-Alanine	500	mmol/L
$pH_{30\ °C}$	7.30	
Tris buffer	100	mmol/L
2-Oxoglutarate	15	mmol/L
Pyridoxal 5'-phosphate	0.10	mmol/L
NADH (assuming $NADH \cdot Na_2 \cdot 2H_2O$)	0.165	mmol/L
LDH	1200	U/L (25 °C)
Volume fraction (sample/total)	0.083	(1:12)

Reagents:

1. L-Alanine (615 mmol/L) in Tris (110 mmol/L, pH 7.30): To about 800 mL of deionized water in a 1-L or larger beaker, add 13.32 g of Tris and 54.80 g of L-alanine. Stir contents with beaker immersed in a water bath to achieve a temperature of 30±2 °C. While monitoring the temperature, adjust pH to 7.30±0.02 with 1 mol/L HCl. Transfer quantitatively to a 1.00-L class A volumetric flask. Equilibrate at 20±2 °C and bring volume to mark. Mix well. Refrigerate working solution; freeze the remainder.

2. 2-Oxoglutarate (180 mmol/L) in Tris (110 mmol/L, pH 7.30): To about 600 mL deionized water in a 1-L or larger beaker, add 26.30 g of 2-oxoglutaric acid and 13.32 g of Tris. Stir contents with flask immersed in a water bath to achieve a temperature of 30±2 °C. While monitoring the temperature,

adjust pH to 7.30±0.02 with 10 mol/L and 1 mol/L NaOH. Transfer quantitatively to a 1.00-L class A volumetric flask. Equilibrate at 20±2 °C and bring volume to mark. Mix well. Freeze in aliquots of about 10 mL.

3. Tris (110 mmol/L, pH 7.30): To about 800 mL of deionized water in a 1-L or larger beaker, add 13.32 g of Tris. Stir contents with flask immersed in a water bath to achieve a temperature of 30±2 °C. While monitoring temperature, adjust pH to 7.30±0.02 with 1 mol/L add HCl. Transfer quantitatively to a 1.00-L class A volumetric flask. Equilibrate at 20±2 °C and bring volume to mark. Mix well. Store at 4 °C.

4. Pyridoxal phosphate (5.0 mmol/L) in Tris (110 mmol/L, pH 7.30): For each 100 mL of reagent 4 required, dissolve 133 mg of pyridoxal 5'-phosphate in 100.0 mL of reagent 3. Portion out aliquots of about 10 mL. Store at $\leq$-20 °C. This reagent can be used for 5 days when kept at 4 °C.

5. Lactate dehydrogenase (3.06 kU/mL): Purchase hog muscle LDH containing 550 U/mg at 25 °C as a 10 mg/mL solution in glycerol (See Note 3). Add 0.8 mL of a 50:50 (V/V) glycerol: water mixture to each 1.0 ml of enzyme solution. LDH activity should be verified by the method given in reference [41].

6. Combined assay mixture: To prepare sufficient mixture for the number of assays to be performed, combine the following in the quantities shown:

No. of assays:	10	20	30	50	100
Reagent 1, (in mL) (Alanine)	20	40	60	100	200
Reagent 5, (in mL) (LDH)	0.01	0.02	0.03	0.05	0.1
Reagent 4, (in mL) (Pyridoxal-P)	0.5	1.0	1.3	2.5	5.0
$Na_2NADH \cdot 2H_2O$, (in mg) (dry powder)	3.0	6.0	9.0	15.0	30.0

Analytical Procedure:

Add 0.2 mL of serum to a cuvette. Pipette 2.00 mL of the combined assay mixture (reagent 6) into the same cuvette and incubate at 29.77 °C for 15 min. When the absorbance of the mixture at 339 nm is stable, add 0.2 mL of 2-oxoglutarate (reagent 2), mix, and record absorbance changes. The reaction must be followed continuously. The reaction rate is obtained over the linear portion of dA/dt, usually after 2 or 3 minutes.

One blank reaction must be assessed as follows: Combine 2.0 mL of reagent 6 as above and 0.2 mL of distilled water as a sample. Assay in triplicate each day. The value for this blank measurement should be less than 5 U/L.

Calculations:

The catalytic (activity) concentration of alanine aminotransferase in SRM 909 measured by this protocol at 29.77 °C is calculated in International Units per liter, 1.0 U/L = 1.0 ($\mu mol \cdot min^{-1} \cdot L^{-1}$) as follows:

$$\text{Catalytic concentration (in U/L)} = (dA/dt) \cdot V \cdot \varepsilon^{-1} \cdot b^{-1} \cdot v^{-1}$$

where dA is the change of absorbance at 339 nm over the specified rate measuring interval, dt is the measuring interval in minutes, V is volume of the final reaction mixture in liters, v is the sample volume in liters, b is the cuvette pathlength in cm, and ε is the micromolar absorptivity of NADH which is 6 300 x 10^{-6} $L \cdot \mu mol^{-1} \cdot cm^{-1}$ under the final reaction conditions. By substituting the known values:

$$\text{Catalytic concentration (in U/L)} = \frac{(dA/dt) \cdot (2.4 \times 10^{-3})}{(6.30 \times 10^{-3})\ (1)\ (0.20 \times 10^{-3})}$$

$$= 1905 \cdot dA \text{ per min.}$$

Calibration of reagent blank activity is identical; the final result is calculated by deducting the value of the reagent blank (U/L) from that found with SRM 909 (U/L).

Catalytic (activity) concentration in katals per unit volume (kat/L) can be calculated from the relationship 1.0 U/L = 16.67 nkat/L [37].

Notes:

1. Reagents 1 and 4 should be filtered through microporous filter (0.22 μm).

2. Assay volume may be reduced to 1.00 mL of reagent 6 and 0.100 mL each for sample and reagent 2.

3. Boehringer-Mannheim hog muscle LDH (catalog 127.868 or 127.221) is suitable for preparation of reagent 5.

Comments by Team Leader (R. Rej)

The method selected for use in these round robins is based on that recommended by the AlaAT Study Group of the Standards Committee of the American Association for Clinical Chemistry [41] and the Expert Panel on Enzymes of the International Federation of Clinical Chemistry [20]. This procedure was developed with considerable cooperation and collaboration between these two groups and therefore represents both a national and international reference method for measurement of AlaAT activity. There are slight mechanical and methodological differences between these two methods. However, in terms of substrate concentrations and measurement pH, they are identical. The major difference lies in the measurement of four different catalytic rates for each specimen in the IFCC procedure. The AACC method -- and that used in these round robins -- recommends the measurement of a single blank measurement: a reagent blank using distilled water as a specimen. The major sources of catalytic activity in this blank measurement are contaminant AlaAT activity within the LDH (reagent 5) and the reduction of 2-oxoglutarate by LDH itself:

$$\text{2-oxoglutarate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{2-hydroxyglutarate} + \text{NAD}^+$$

The muscle type isoenzyme of LDH (LDH-5, M4) catalyzes this reaction at a rate several-fold slower than that catalyzed by the heart type isoenzyme (LDH-1, H4); therefore this isoenzyme was recommended for these measurements. The K_m of this isoenzyme for

pyruvate, however, is larger than that of LDH-1 and the lag phase (the time required after addition of 2-oxoglutarate before a linear change in absorbance with time is observed) is measurable. Reaction rates for NBS SRM 909 were determined 3 min after addition of 2-oxoglutarate. The magnitude of the reagent blank was always measurable and the mean of triplicate measurements within each laboratory was deducted from the catalytic rate measured with SRM 909 (see Figures 5 and 5b). The rates given in Table 4 are those determined with such blank correction.

The additional two blanks proposed by the IFCC [20] may compensate for interferences in some patient sera. No such interferences were observed with NBS SRM 909 after the required 15 min preincubation.

The preincubation before addition of 2-oxoglutarate may be performed at 30±2 °C rather than the rigorously controlled 29.77 needed for measurement of catalytic activity -- this is particularly important if a thermostatted flow-cell is used for measurements. All laboratories used the NBS Gallium Cell (SRM 1968) for calibrating laboratory thermistors. NBS SRM 922 (Tris base) was used for preparation of all buffers and substrates. The temperature of reconstituting liquid did not appear to affect results. Individual spheres of the dry serum were found to be attracted to the stopper and neck of vials. A piezo-electric anti-static pistol (Zerostat by Discwasher) was used to reduce static charges.

The participants and instruments used in the all round robins are:

R. Rej	Varian Cary 219
L. Shaw	Gilford Stasar III
G. Sims & S. Osaki	Beckman 24 and 34
W. Ryan	Beckman DU-8
R. Miller	Varian Cary 219

D. Aspartate Aminotransferase
(L-Aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1)

Principle: Aspartate aminotransferase (AspAT), formerly glutamate oxalacetate transaminase-GOT, catalyzes the following reaction:

$$\text{L-aspartate} + \text{2-oxoglutarate} \xrightarrow{\text{AspAT}} \text{oxalacetate} + \text{L-glutarate.}$$

The activity of AspAT is measured by converting the oxalacetate into L-malate with reduced nicotinamide adenine dinucleotide (NADH) and malate dehydrogenase (MDH) as indicator enzyme:

$$\text{oxalacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+.$$

The quantity of NADH oxidized, which is equivalent to the amount of oxalacetate formed, is measured by the decrease in absorbance at 339 nm.

The assay employs the final conditions recommended by the AACC Standards Committee working group [42]; this assay correlates well with the proposed IFCC methods [19]. Three modifications are incorporated: 1) the melting point of gallium, at 29.77 °C, is the measurement temperature; 2) the volume fraction of specimen was changed from 0.0667 (1:15) to 0.083 (1:12) to allow the use of convenient volumetric glassware for all additions, i.e., 2.00 and 0.200 mL or 1.00 and 0.100 mL pipettes can be used for dispensing reagents and specimen; and 3) LDH is not used. The catalytic rate of the reaction mixture prior to the addition of 2-oxoglutarate is monitored in the spectrophotometer prior to adding the 2-oxoglutarate.

Final reaction conditions:

Reaction Temperature	29.77	°C
pH	7.80	
Tris buffer	89	mmol/L
L-Aspartate	175	mmol/L

2-Oxoglutarate	15	mmol/L
Pyridoxal 5'-phosphate	0.11	mmol/L
NADH (assuming $NA_2NADH \cdot 2H_2O$)	0.165	mmol/L
MDH (EC 1.1.1.37)	0.95	U/mL(25 °C)
Volume fraction (sample/total)	0.083	(1:12)

Reagents:

1. L-aspartate (216 mmol/L) in Tris (100 mmol/L, pH 7.8 at 30 °C): Add 28.8 g of L-aspartic acid and 12.1 g of Tris to 700 mL of deionized water in a 1-L beaker. Stir continuously and monitor the pH. While temperature is controlled to 30±2 °C, add NaOH pellets slowly (no more than 5 g). Then adjust pH to 7.8±0.02 at 30 °C with 10 mol/L NaOH. Cool solution to ambient temperature and quantitatively transfer to a 1.0-L class-A volumetric flask. Bring to the mark. Mix well. Stable for at least 2 weeks.

2. Malate dehydrogenase (235 U/mL): To 10 mL of 50:50 (V/V) glycerol-water, add 0.2 mL of porcine MDH 10 mg/mL in glycerol (about 1200 U/mg at 25 °C). Mix gently by inversion. Refrigerate.

3. Pyridoxal phosphate (5.44 mmol/L) in water: Dissolve 288 mg of pyridoxal phosphate monohydrate in 200 mL of distilled water. Portion out in about 10 mL aliquots and freeze at $\leq$ 20 °C. Can be used for 4 days when refrigerated (at $\sim$4 °C).

4. 2-Oxoglutarate (180 mmol/liter) in Tris (100 mmol/liter, pH 7.8): Add 2.63 g of 2-oxoglutaric acid and 1.21 g of Tris to about 70 mL of deionized water. While controlling the temperature at 30±2 °C, adjust pH to 7.80±0.02 with 1.0 mol/L and 10 mmol/L NaOH. Quantitatively transfer into a 100.0 mL class A-volumetric flask and bring to mark at ambient temperature. Stable at 4 °C for at least 2 days. Freeze in portions of about 10 mL ($\leq$-20 °C).

5. Combined assay mixture: To prepare sufficient assay mixture for the tests to be performed, combine the following in quantities shown below:

Reagent 1 (Aspartate)	60	mL
Reagent 2 (MDH)	0.3	mL
Reagent 3 (Pyridoxal phosphate)	1.5	mL
$Na_2NADH \cdot 2H_2O$ (dry powder)	9.0	mg

Analytical Procedure:

Add 0.2 mL of serum to a cuvette. Pipette 2.00 mL of the combined assay mixture (reagent 5) into the same cuvette; incubate at 29.77 °C at least 15 min. When absorbance at 339 nm is stable, add 0.2 mL of 2-oxoglutarate (reagent 4), mix, measure rate at 339 nm. One blank reaction must be assessed as follows. Combine 2.0 mL of reagent 5 and 0.2 mL of distilled water as sample. Assay in triplicate each day. The value for the blank should be less than 0.8 U/L.

Calculations:

The catalytic (activity) concentration of aspartate aminotransferase in SRM 909 measured by this protocol at 29.77 °C is calculated in International Units per liter ($1.0\ U/L = 1.0(\mu mol \cdot min^{-1} \cdot L^{-1})$) as follows:

$$\text{Catalytic concentration (in U/L)} = dA/dt \cdot V \cdot \varepsilon^{-1} \cdot b^{-1} \cdot v^{-1}$$

where dA is the change of absorbance at 339 nm over the specified rate measurement interval, dt is the measuring interval in minutes, V is the total volume of the final reaction mixture in liters, v is the sample volume in liters, b is the cuvette pathlength in cm, and ε is the micromolar absorption coefficient of NADH which is $6300 \times 10^{-6}\ L \cdot \mu mol^{-1} \cdot cm^{-1}$ under the final reaction conditions.

By substituting values,

$$\text{Catalytic concentration (in U/L)} = \frac{dA/dt\ (2.4 \times 10^{-3})}{(6.3 \times 10^{-3})\ (1)\ (0.2 \times 10^{-3})}$$

$$= 1905 \cdot dA \text{ per min.}$$

Reagent blank is calculated in an identical manner. The reagent blank is typically 0.4±0.4 U/L and need not be deducted from the overall rate measurement.

Catalytic (activity) concentration in katals per unit volume (kat/L) can be calculated from the relationship 1.0 U/L = 16.67 nkat/L [37].

Notes:

1. Reagents 1 and 4 should be filtered through microporous filter (0.22 μm).

2. Boehringer-Mannheim porcine heart MDH (product 127892 and 127906) meets these specifications. The glycerol-based material must also show low AspAT contamination.

3. Assay volume may be reduced to 1.00 mL of assay mixture (reagent 5) and 0.100 mL each of serum and 2-oxoglutarate (reagent 4).

Comments by Team Leader (R. Rej)

The method selected for use in these round robins is based on those recommended by the AspAT Study Group of the Standards Committee of the American Association for Clinical Chemistry [42] and the Expert Panel on Enzymes of the International Federation of Clinical Chemistry [19]. There are several methodological differences between these two methods. These include differing substrate concentrations and different procedures for measurement

of blank activity. As with the AlaAT procedure described previously, four separate measurements are determined for each specimen in the IFCC method. Because of the interference due to addition of LDH (also see AlaAT procedure) LDH was not added to the procedure used for the NBS SRM 909 study. LDH is often added to AspAT assays to more quickly remove endogenous pyruvate. The pyruvate concentrations of SRM 909 do not appear excessive so that there was no requirement to correct for this potential interference with SRM 909. This is not necessarily the case with patient specimens. Reagent blanks (measurement of catalytic) rate with distilled water as a specimen) were performed by all participants but the magnitude of these catalytic rates was less than the overall imprecision for these determinations. In the majority of laboratories this blank rate was the equivalent of <0.8 U/L. It was therefore decided to use the measurement of the catalytic rate found with SRM 909 without deduction of this reagent blank (Table 4). The reagent blank was, however, determined in each round robin to insure that its magnitude did not exceed 0.8 U/L. The omission of LDH from the procedure used likely contributed to the insignificance of the reagent blank.

Two additional blanks proposed by the IFCC [19] may compensate for interferences in some patient sera. No such interferences were observed with NBS SRM 909 after the the required 15 min preincubation.

The preincubation before addition of 2-oxoglutarate may be performed at 30± 2 °C rather than the rigorously controlled 29.77 °C needed for measurement of catalytic activity -- this is particularly important if a thermostatted flow-cell is used for measurements. All laboratories used the NBS Gallium Cell (SRM 1968) for calibrating laboratory thermistors. NBS SRM 922 (Tris base) was used for preparation of all buffers and substrates. The temperature of reconstituting liquid did not appear to affect results. Individual spheres of the dry serum were found to be attracted to the stopper and neck of vials. A piezo-electric anti-static pistol (Zerostat by Discwasher) was used to reduce static charges.

The participants and instruments used in all three round robins are:

R. Rej	Varian Cary 219
N. Tietz	Beckman ACTA CIII
A. Bacharach	Varian Cary 118
W. Fellows	Beckman ACTA CIII
L. Shaw	Gilford Stasar III

E. Creatine Kinase

(ATP:Creatine N-phosphotransferase, EC 2.7.3.2)

Principle: This protocol was developed for measuring the total creatine kinase (CK) catalytic (activity) concentration in SRM 909 lyophilized human serum. It was adapted with two modifications from the Scandinavian Committee on Enzymes (SCE) method [43]. The modifications include: 1) changing the reaction temperature from 37 °C to the melting-point of gallium, 29.77 °C, and 2) omitting adenylate kinase (AK) inhibitors by using instead a discrete measurement of a blank to correct for the presence of AK in the reference material.

The appearance of NADH is continuously monitored in a spectrophotometer at 340 nm as the enzyme creatine kinase catalyzes the transfer of a phosphate group from creatine phosphate to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP), which is coupled to the indicator reaction, according to the following scheme.

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{D-glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{D-glucose-6-phosphate}$$

$$\text{D-glucose-6-phosphate} + \text{NAD}^+ \xrightarrow{\text{G6PD}} \text{D-gluconate-6-phosphate} + \text{NADH} + \text{H}^+$$

Final reaction conditions:

Reaction temperature	29.77	°C
pH (at 29.77 °C)	6.60	
Imidazole acetate	100	mmol/L
Creatine phosphate	30	mmol/L
Adenosine-5'-diphosphate (ADP)	2	mmol/L
D-Glucose	20	mmol/L
Nicotinamide adenine dinucleotide (NAD^+)	2	mmol/L
Hexokinase (HK) (EC 2.7.1.1)	2500	U/L
D-Glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49) (L. mesenteroides)	1500	U/L
Magnesium acetate	10	mmol/L
N-Acetylcysteine (NAC)	20	mmol/L
Ethylenediaminetetracetic acid, sodium salt, Na_2(EDTA)	2.0	mmol/L*
Volume fraction (sample total)	0.043	(1:23)

*This concentration is not critical and may range from 1.5 - 3.0 mmol/liter.

This method differs from other CK methods by eliminating the use of adenylate kinase inhibitors [43, 44]. A blank measurement must be made in the absence of creatine phosphate to account for adenylate kinase activity [45]. EDTA has been included [44]. Sample and incomplete reagent (the latter lacks creatine phosphate) are preincubated for 5 minutes before activity measurements are begun.

Reagents:

All reagents should meet American Chemical Society reagent-grade (A.R.) specifications. Water should be at least Type II grade (2.0×10^6 ohm cm at 25 °C).

1. Imidazole acetate stock solution [128 mmol/L in pH 8.0 buffer (25 °C) with magnesium acetate (12.8 mmol/L) and EDTA (2.55 mmol/L)]: Dissolve imidazole (8.72 g), disodium ethylenediamine tetracetate·$2H_2O$ (0.95 g) and magnesium acetate·$4H_2O$ (2.75 g) in about 950 mL of Type II H_2O. Add acetic acid (1 mol/L) to adjust to pH 8.0 (at 25 °C). Then add more H_2O to a total volume of 1000 mL. Reagent is stable for 2 months at +4 °C and for a minimum of six months at -20 °C.

2. Working reagent A. Imidazole acetate (115 mmol/L) pH 8.0 (25 °C), magnesium acetate (11.5 mmol/L), EDTA (2.3 mmol/L). N-acetyl cysteine (23.0 mmol/L), ADP (2.3 mmol/L), D-glucose (23.0 mmol/L), NAD^+ (2.3 mmol/L), hexokinase (2900 U/L), D-glucose-6-phosphate dehydrogenase (1750 U/L): To prepare 100 mL, combine 90 mL of reagent 1 with N-acetylcysteine (375 mg), adenosine-5'-diphosphoric acid (98 mg), D-glucose (414 mg), and nicotinamide adenine dinucleotide (153 mg). Mix well. Adjust temperature to 29.77 °C before adjusting pH to 6.60 with 1 mol/L acetic acid. Then add between 260-290 U (25 °C) of hexokinase and approximately 175 U (20 °C) of glucose-6-phosphate dehydrogenase (L. mesenteroides). Type II water is then added to the 100 mL volume (see notes 1 and 2). Store at +4 °C or -20 °C. The reagent is stable for 6-h at room temperature; for 24-h at +4 °C; and for a minimum of 1 week at -20 °C.

3. Working reagent B. Creatine phosphate (345 mmol/L): Dissolve 1.125 g of creatine phosphate tetrahydrate disodium salt in 7 mL of type II water. Dilute to 10.0 mL. Mix well. See note 3. Store at +4 °C or -20 °C. Stable for 3 months at +4 °C, and for a minimum of 1 year at -20 °C.

Analytical Procedure:

Measurement of total CK activity plus adenylate kinase (AK) activity.

Pipette 100 µL of serum into a test tube containing 2.000 mL of reagent 2 that has been prewarmed to approximately 30 °C. Mix and incubate for 5 min at approximately 30 °C. Add 200 µL of reagent 3 that has been prewarmed to approximately 30 °C. Mix and pour into a 1.000-cm cuvette. Place the cuvette in a controlled cell compartment maintained at 29.77 °C of a spectrometer set to read at 340 nm. The rate of the steady state reaction which follows the lag phase of up to 120 s, is determined by continuous recording of the 340 nm absorbance. Record the absorbance vs. time for a total of 300 s against a blank made from 2.000-mL of reagent 2, 100 µL of Type II water and 200 µL of reagent 3.

Procedure for blank (AK activity only): Into a test tube containing 2.000-mL of reagent 2 that has been prewarmed to approximately 30 °C, pipette 100 µL of serum. Mix and incubate for 5 min at approximately 30 °C. Add 200 µL of Type II water that has been prewarmed to approximately 30 °C. Mix and pour into a 1.000-cm cuvette. Place the cuvette into the spectrophotometer's controlled cell compartment. The rate of the steady state reaction which follows the lag phase of up to 120 s, is determined by continuous recording of absorbance at 340 nm. Record the absorbance vs. time for a total of 300 s against a blank made of 2.000 mL of reagent 2, and 300 µL of Type II water.

Calculations:

The catalytic (activity) concentration of creatine kinase in SRM 909 measured by this protocol at 29.77 °C, is calculated in International Units per liter, (1.0 U/L = 1.0 $\mu mol \cdot min^{-1} \cdot L^{-1}$) as follows [46].

$$\text{Catalytic concentration (in U/L)} = dA/dt \cdot V \cdot \varepsilon^{-1} \cdot b^{-1} \cdot v^{-1}$$

where d$\underline{A}$ is the change of absorbance at 340 nm over the specified rate measuring interval, d$\underline{t}$ is the measuring interval in minutes, $\underline{V}$ is the total volume of the final reaction mixture in liters, $\underline{\varepsilon}$ is the micromolar absorption coefficient of NADH, which is 6317 x 10^{-6} $L \cdot \mu mol^{-1} \cdot cm^{-1}$ under the final reaction conditions [47], $\underline{b}$ is the cuvette pathlength in centimeters, and $\underline{v}$ is the sample volume in liters. Therefore:

$$\text{Catalytic concentration (in U/L)} = \frac{(dA/dt)\ (2.3 \times 10^{-3})}{(6317 \times 10^{-6})\ (1)\ (0.1 \times 10^{-3})}$$

$$= 3641 \cdot dA \text{ per min.}$$

Catalytic (activity) concentration in katals per unit volume (kat/L) can be calculated from the relationship 1.0 U/L = 16.67 nkat/L [37].

Notes:

1. ATP concentration in reagent 2 should be less than 10 µmol/liter; as a consequence the increase in absorbance on addition of the auxiliary enzymes should be less than 0.060.

2. The absorbance of reagent 2 at 340 nm should be less than 0.200. Certain lots of imidazole have been found to contain an unknown substance which contributes significantly to absorbance at 340 nm. These lots should be identified and not used in the preparation of stock imidazole buffer. Although at present, it is not clear whether the recovery of activity is affected, the range of linearity of measurement is compromised by starting with a high initial absorbance.

3. The absorbance of reagent 3 at 340 nm should be less than 0.150.

COMMENTS BY TEAM LEADER (R. C. Elser)

The amount of residual CK activity present in the base pool after processing but before supplementation with exogenous enzyme was estimated to be 112 U/L. Partly purified porcine heart CK was added to the base pool prior to lyophilization to increase the final activity to the 150-200 U/L range when assayed on a DuPont *aca* at 37 °C.

The participants and instruments used in the final round robin (III) are given below for the participants who submitted results for Round Robin 3.

Participant	Instrument Used
R. C. Elser	Gilford Stasar III
J. H. Stromme	GEMSAEC
R. H. Davis	Gilford 250
J. F. O'Brien	GEMSAEC

The reconstitution protocol followed for CK was identical to that recommended for reconstitution of this material in the assay of other enzyme constituents of SRM 909. A systematic comparison of the differences in reactivation from reconstituting SRM 909 at 0 °C and at room temperature has not been made, but it is to be expected that approximately 20% less activity might be recovered with reconstitution at room temperature.

Davis, *et al*. [44] have shown that the temperature at which lyophilized sera are reconstituted has an effect on the recovery of CK activity. SRM 909 was reconstituted and incubated at 2, 6, 10, 20, 25 and 30 °C and assayed at 30, 60, and 90 minutes following reconstitution. The assay method, which was performed at 30 °C, used the DuPont *aca* optimized CK formulation. Figures 2 and 3 show the combined effects of time and temperature on recovered values. It is clear that both time and temperature, especially in the 2-10 °C range, affect recovery. It appears that no particular time and temperature combination is superior.

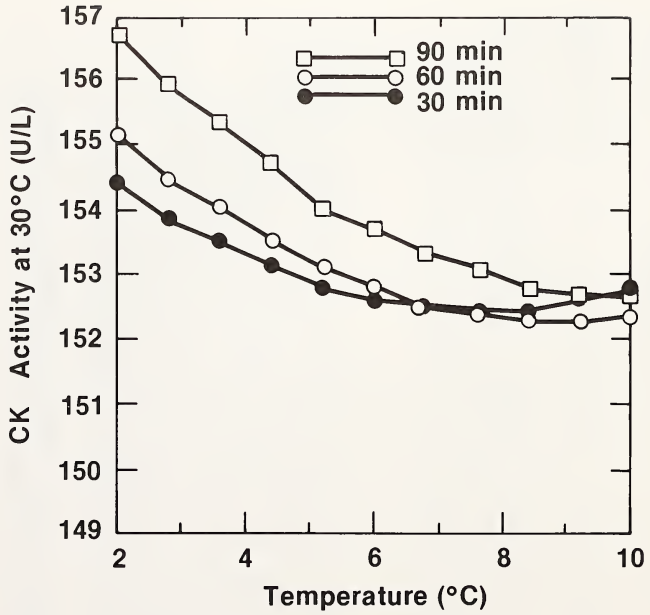


Figure 2

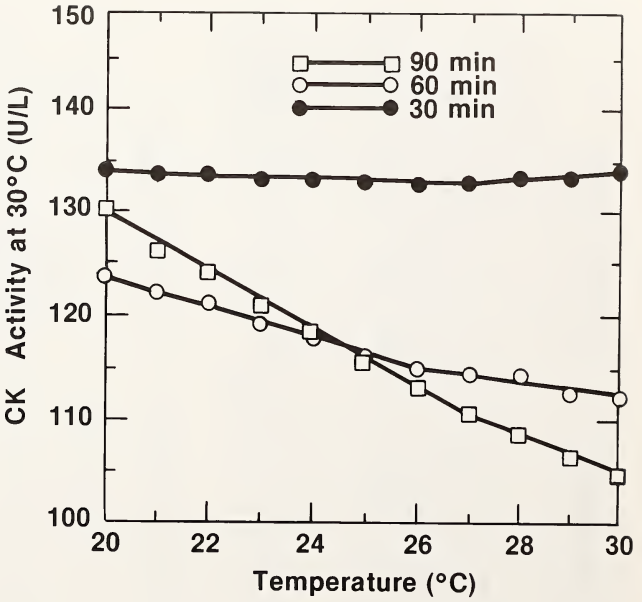


Figure 3

Figures 2 and 3: Effect of time and incubation temperature on CK activity measured with an aca method at 30 °C. Reconstitution temperature ranges are 2-10 °C and 20-30 °C for figures 2 and 3, respectively.

However, the reproducibility of the temperature of an ice bath meets the needs of temperature constancy from laboratory to laboratory and from time to time. Time of rehydration at this temperature may vary from 30 to 90 minutes with less than 2% variation in recovery of activity.

The stability of CK-3 (CK-MM) has been examined by Whitner, et al. [47a]. The presence of ADP and N-acetylcysteine increased the half-life of CK activity by approximately 30-fold when lyophilized material was stored at -20 °C.

F. Lactate Dehydrogenase
(L-Lactate:NAD^+ oxidoreductase, EC 1.1.1.27)

Principle: Lactate dehydrogenase (LDH) catalyzes the following reaction:

$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-lactate} + NAD^+.$$

Lactate dehydrogenase activity is determined by measuring the decrease in absorbance at 339 nm due to the oxidation of NADH as pyruvate is reduced to L-lactate [48-51].

Final reaction conditions:

Reaction temperature	29.77	°C
pH (at 29.77 °C)	7.35	
Tris buffer	0.0968	mol/L
Sodium pyruvate	1.2	mmol/L
NADH	0.15	mmol/L
Volume fraction (sample/total)	0.016	(1:61)

Reagents:

1. Tris buffer (0.15 mol/L): In a 1-L volumetric flask, dissolve 18.22 g of tris(hydroxymethyl)aminomethane (NBS SRM 922) in 800 mL of deionized water. To this solution add exactly 124.5 mL of 1.000 mol/L HCl and mix thoroughly. Dilute to mark with deionized water. Verify that the pH at 29.77 °C is 7.35±0.02. This reagent should be stable for 2 weeks at room temperature.

2. NADH (0.229 mmol/L): Weigh the appropriate amount of dry $Na_2NADH \cdot xH_2O$ (Note 1) into a 200 mL volumetric flask and dissolve in Tris buffer (reagent 1). Adjust volume to the mark and mix. The absorbance of this solution at 339 nm should be 1.447±0.05 and the pH should be 7.35±0.02 at 29.77 °C. The solution is stable at room temperature for 4 hours and at 4 °C for 24 hours.

3. Sodium pyruvate (3.66 mmol/L): In a 100 mL volumetric flask, dissolve 40.2 mg of sodium pyruvate (NBS SRM 910) in 50 mL of reagent grade water. Dilute to volume with additional water and mix. Stable for 8 hours at room temperature or for 24 hours at 4 °C.

4. Potassium dichromate (0.205 mmol/L): Dissolve 60.1 mg of $K_2Cr_2O_7$ (NBS SRM 136) in a 1-L volumetric flask in approximately 500 mL of deionized water. Add 0.10 mL of concentrated $HClO_4$ and dilute to volume with water.

Analytical Procedure:

To a test tube containing 2.0 mL of reagent 2, add 50 µL of sample (Note 2). Incubate this mixture for 10 min at 29.77 °C. Initiate the LDH reaction by adding 1.00 mL of reagent 3 that has been preincubated at 29.77 °C for at least 5 min. Mix and quickly transfer a portion of this mixture to a 1.00 cm cuvette (Note 3) that is at 29.77±0.1 °C (Note 4). Record the 339-nm absorbance of the reaction mixture at 30 s intervals for a total of 180 s starting 30 s after addition of the pyruvate.

Calculations:

The time course of the LDH reaction must be corrected for non-linearity [52]. The initial rate is calculated from the corrected curve. The value obtained is used to calculate the activity as follows:

1. The natural logarithm (ln) corresponding to each 30 s data point (lnA) is plotted vs. time (in min). From the resulting straight line, calculate the slope and y intercept (ln A_0). A_0 is obtained from the antilog of lnA_0 (see Note 5).

2. Calculate from these constants, the initial rate of absorbance change.

$$(dA/dt)_o = \text{slope} \times A_o = \text{initial rate}$$

3. From this initial rate of absorbance change, the catalytic (activity) concentration of lactate dehydrogenase in SRM 909 measured by the above protocol at 29.77 °C is calculated in International Units per liter (1.0 U/L = 1.0 $\mu mol \cdot min^{-1} \cdot L^{-1}$) as follows:

$$\text{Catalytic concentration (in U/L)} = (dA/dt)_o \cdot V \cdot \varepsilon^{-1} \cdot b^{-1} \cdot v^{-1},$$

where dA is the change of absorbance at 339 nm over the specified rate measuring interval, dt is the measuring interval in minutes, V is the total volume of the final reaction mixtures in liters, v is the sample volume in liters, b is the cuvette pathlength in cm, and ε is the micromolar absorption coefficient (molar absorptivity) of NADH which is 6300 x 10^{-6} $L \cdot \mu mol^{-1} \cdot cm^{-1}$ under the final reaction conditions. Therefore:

$$\text{Catalytic concentration (in U/L)} = \frac{(dA/dt)o}{(6300 \times 10^{-6})\ (1)\ (0.05 \times 10^{-3})}$$

$$= 9682 \cdot dA_o \text{ per min.}$$

Catalytic (activity) concentration in katals per unit volume (kat/L) can be calculated from the relationship 1.0 U/L = 16.67 nkat/L [37].

Notes:

1. Some suppliers of NADH (and catalogue numbers) are Boehringer-Mannheim (107727), Sigma (7179), and P-L Biochemicals (6500). The quality and formula weight of commercially available NADH vary considerably from lot to lot. The material to use should appear as dry white crystals and, according

to the labeling, should contain a minimum of 98% NADH as disodium salt hydrate. Water content is different even among the best lots of NADH so that amounts used to prepare reagent 2 will vary. The amounts of NADH given below are needed to prepare 100 mL of reagent 2, based on formula weights (F.W.) stated on the labeling provided with every lot of NADH. The amounts needed compensate for water of hydration and ethanol content.

F.W.	Amount Needed (mg)
750	17.3
755	17.4
760	17.5
765	17.6
770	17.7
775	17.8
780	17.9
785	18.0
790	18.2
795	18.3
800	18.4

2. The delivery of each sample from the TC pipette should be followed by 2 washes with the diluted sample mixture. A mechanical pipetor-dilutor may be used as an alternative, if the accuracy and precision of this apparatus can be shown to be within 0.5% (95% confidence) for both sample and diluent.

3. The pathlength of this cuvette should be 1.00±0.01 cm. An indirect check of this dimension can be made by measuring the absorbance of acid dichromate at 350 nm [53]. The absorbance of a 0.205 mmol/L solution at 25 °C against distilled water should be 0.645±0.006 (±2SD).

4. The temperature of the reaction mixture should be checked just before the 30 s reading and just after the 300 s reading by use of a small-mass temperature probe that is connected to a measuring system which is calibrated against a gallium cell.

   If the set temperature is different from 29.77 °C (e.g., 30.0 °C), activities should be corrected to 29.77 °C. Reaction temperatures that differ by more than 0.3 °C from the 29.77 °C reference point should not be used.

5. As an alternative, the time course data can be curve fitted to the exponential equation $y = a^{bx}$. In this case $a = A_0$ and b = slope; thus axb = slope x A_0 = initial rate or $(dA/dt)_0$.

COMMENTS BY TEAM LEADER (R. B. McComb)

The average LDH catalytic concentration increased substantially between RRI, RRII, and RRIII. This probably reflects changes in the protocol that were introduced to provide more accurate estimates of the initial reaction rates. We decreased the measurement intervals from five 1-min readings to five 30 s readings. In RRIII, all participants used the same high quality NADH preparation. The NADH was supplied by Dr. J. Siegal (P. L. Biochemicals, Cat. No. 6500). Based on high performance liquid chromatography, 95% of the 260 nm absorbing material in this preparation was present in the NADH peak. (NBS has developed specifications for SRM NADH, but material meeting these specifications is not available as an SRM.)

The lab-to-lab variation in results was least in RRIII. The coefficient of variations were 4.9%, 8.2% and 6.4% in RRIII, RRII, and RRI, respectively. Results obtained by performing the assay at temperatures other than 29.77±0.3 °C are corrected to this reference temperature by using the correction factor 7.2% per degree.

The effects of reconstitution and storage temperature on LDH activity were tested during RRIII. One vial of SRM 909 was reconstituted according to the SRM 909 protocol and stored at 0 °C. A second vial was reconstituted in an identical fashion but both reconstitution and storage was at 22 °C. LDH activity was measured in triplicate on samples taken from each vial at 1, 6, and 24 hours. Average activity increased 4% over the 24 hour period in both vials. Activity in the vial that was reconstituted at 0 °C was consistently less by an average of 1.6% than that in the vial reconstituted at 22°C. These are small differences but suggest the need for strict adherence to the reconstitution protocol when using SRM 909.

A report from France [60] suggests that the inclusion of 200 mmol/L sodium chloride in the reaction mixture eliminates non-linearity in the time-course of the P to L reaction. If this effect can be confirmed, this modification would constitute a major improvement in methodology for the measurement of LDH activity.

G. γ-Glutamyltransferase

[(γ-Glutamyl)-peptide: amino acid γ-glutamyltransferase, EC 2.3.2.2]

Principle: The biochemical and clinical chemistry literature shows that a number of donor substrates have been used for the assay of γ-GT activity. We use L-γ-glutamyl-3-carboxy-4-nitroanilide (GluNA-CO_2) and glycylglycine (glygly), and this conforms with the recommendation of the Expert Panel on Enzymes of IFCC [21]. GluNA-CO_2 is the preferred substrate since it has the practical advantage of being readily soluble in aqueous solution. The rate of absorbance change at 410 nm found with human sera is 21% higher with GluNA-CO_2 than with an equivalent concentration of L-γ-glutamyl-3-sulfonic-4-nitroanilide, another possibly utilizable water-soluble substrate. The absorbance contribution of 4-nitroanilide substrates is significant at 405 nm, but decreases substantially as wavelength is increased. Glycylglycine is the preferred acceptor substrate since a significantly higher catalytic rate (and, therefore, more readily observable change in absorbance) is obtained using it as compared with other acceptor substrates.

γ-GT in human serum catalyzes two reactions simultaneously, namely, transpeptidation and autotransfer. In the former reaction glygly is the γ-glutamyl acceptor substrate; in the latter reaction GluNA-CO_2 is the acceptor substrate:

(1) TRANSPEPTIDATION

$$\text{GluNA-CO}_2 + \text{glygly} \xrightarrow{\gamma\text{-GT}} \gamma\text{-gluglygly} + \text{NA-CO}_2$$

(2) AUTOTRANSFER

$$\text{GluNA-CO}_2 + \text{GluNA-CO}_2 \xrightarrow{\gamma\text{-GT}} \text{GlugluNA-CO}_2 + \text{NA-CO}_2$$

In the case of human serum γ-GT, the reaction rate obtained at a concentration of 10 mmol/L of GluNA-CO_2 in the absence of glygly is 13% of the rate obtained at the same GluNA-CO_2

concentration but with 150 mmol/L of glygly present. The relative rate of transpeptidation to autotransfer at substrate concentrations used in our method, 6 mmol/L GluNA-CO_2 and 150 mmol/L glygly, is less than 1%. The substrate glycylglycine also serves as buffer.

Final Reaction Conditions:

Reaction temperature	29.77	°C
pH (29.77 °C)	7.90	
L-γ-glutamyl-3-carboxy-4-nitroanilide	6	mmol/L
Glycylglycine	150	mmol/L
Volume fraction (sample/total)	0.091	(1:11)

The assay is initiated by the addition of serum to the combined substrate/buffer mixture, equilibrated to 29.77 °C. The assay is followed at 410 nm for at least 300 s. Venous blood is collected under conditions that minimize stasis. Serum is the preferred specimen. Plasma obtained from whole blood anticoagulated with any anticoagulant tends to give lower values than the corresponding serum specimen. Investigations with this method have shown that γ-glutamyltransferase in serum remains stable for a minimum of 5 days at +4 °C and for a minimum of 7 months at -80 °C.

Instrumentation and Equipment:

Glassware: Volumetric glassware should meet the National Bureau of Standards (NBS) Class A specification or be individually calibrated prior to use. For pipetting control material or serum specimens, mechanical pipettes with disposable tips may be used, but the accuracy and precision of the device used should be checked and be within 0.5% (95% confidence interval).

Instrumentation:

1) pH meter. Calibrate meter at 25 °C against reference buffers from a recognized authoritative source (NBS or IUPAC), but use at 29.77±0.3 °C.

2) Temperature of the assay. The melting point of gallium, 29.77 °C, is the set point temperature for this reference method. A water bath is required, having continuous agitation capability to maintain uniform conditions throughout the water mass. Temperature should be controlled at the set-point ±0.05 °C.

3) Spectrophotometer. A wavelength setting error maximum of ±2 nm with a spectral bandwidth of 8 nm is required. Cuvettes should have parallel faces of optical glass which transmits light without significantly reducing the light intensity due to absorption, reflections or other scattering effects. The internal pathlength should be 1.00±0.01 cm.

Reagents:

To prevent the growth of micro-organisms in the solutions, sterilized containers should be used. All solutions should be prepared in calibrated flasks with freshly distilled and deionized water.

1. N-Glycylglycine (183.3 mmol/L, pH 7.90 at 29.77 °C): Dissolve 24.222 g of glycylglycine (free base, MW 132.12) in approximately 800 mL water in a 1-L volumetric flask, adjust pH to 7.90 at 29.77 °C with 2 mol/L NaOH against standardized reference buffers (NBS or IUPAC). Adjust volume to the mark with water. Mix well. (See Note 1.)

2. L-γ-Glutamyl-3-carboxy-4-nitroanilide (66.00 mmol/L): Dissolve 2.286 g of L-γ-glutamyl-3-carboxy-4-nitroanilide monoammonium salt monohydrate (MW 364.30) in water in a 100 mL volumetric flask. Fill to the mark. Mix well. (See Note 2.)

3. Reagent mixture L-γ-glutamyl-3-carboxy-4-nitroanilide (6.6 mmol/L) and glycylglycine (165 mmol/L, pH 7.90 at 29.77 °C): Combine 90.00 mL of reagent 1 and 10.0 mL of reagent 2 in a 100 mL volumetric flask. Mix well. The absorbance at 410 nm of this solution measured against water should not exceed 0.500. (See Note 3.)

4. Reagent solution glycylglycine (165 mmol/L, pH 7.90 at 29.77 °C: Combine 90.0 mL of reagent 1 and 10.0 mL of water in a 100 mL volumetric flask. Mix well.

5. Sodium chloride solution (154 mmol/L): Dissolve 9.00 g sodium chloride in 1000 mL water.

Analytical Procedure:

Subprocedures that constitute one measurement:

Measurements of the rates of two reaction mixtures are required for determining the γ-GT activity of the serum sample:

A, the rate of the overall reaction; and
B, the rate due to the reagents (as a reagent blank).

The two measurements are made on mixtures having a 2.20 mL final volume; the cuvette pathlength is 1.00±0.01 cm; the cuvette compartment of the spectrophotometer is thermostated at 29.77±0.05 °C; and absorbances are measured at 410±1 nm.

The two mixtures are prepared as follows:

for rate A, add 0.200 mL of serum to 2.000 mL of reagent 3;
for rate B, add 0.200 mL of water to 2.000 mL of reagent 3.

The temperatures of the serum, water, and reagents 3 and 4 are at 29.77 °C prior to being combined. After pipetting the separate components into the cuvette and mixing them, the absorbances are recorded for 300s.

For sera with catalytic concentrations of γ-GT up to about 300 U/L, the values of dA/dt are constant over an interval of at least 300 s. If the values of dA/dt are found to be greater than 0.215 per minute, dilute the serum 5- or 10-fold with reagent 5 and repeat the measurements.

Corrections for blank reactions

The overall γ-GT reaction rate (A) is corrected for the reagent blank rates (B) as follows:

$$(dA/dt)\ \text{corrected} = (dA/dt)_A - (dA/dt)_B$$

The corrected value of (dA/dt) is used in the following calculations. It corresponds to the initial rate of the reaction catalyzed by γ-glutamyltransferase. The serum blank is usually zero. The reagent blank (due to autohydrolysis of L-γ-glutamyl-3-carboxy-4-nitroanilide) is very small, but

measurable. It should be within the range of 0.0002 - 0.0005A per min under the conditions of the assay.

Calculations:

The catalytic (activity) concentration of γ-glutamyltransferase in SRM 909 measured by the above protocol at 29.77 °C, is calculated in International Units per liter (1.0 U/L = 1.0 μmol·min^{-1}·L^{-1}) as follows:

$$\text{Catalytic concentration (in U/L)} = (dA/dt)\cdot V\cdot\varepsilon^{-1}\cdot b^{-1}\cdot v^{-1}$$

where dA is the change of absorbance at 410 nm over the specified rate measuring interval, dt is the measuring interval in minutes, V is the total volume of the final reaction mixture in liter, v is the sample volume in liters, b is the cuvette pathlength in cm, and ε is the micromolar absorption coefficient of 5-amino-2-nitrobenzoate which is 790 x 10^{-6} L·mol^{-1}·cm^{-1} under the final reaction conditions (see Note 4). Therefore,

$$\text{Catalytic concentration (in U/L)} = \frac{(dA/dt)\ (2.2 \times 10^{-3})}{(7.9 \times 10^{-4})\ (1)\ (0.2 \times 10^{-3})}$$

$$= 1391\cdot dA \text{ per min.}$$

Catalytic (activity) concentration in katals per unit volume (kat/L) can be calculated from the relationship 1.0 U/L = 16.67 nkat/L [37].

Notes:

1. Glycine can be a contaminant of glycylglycine preparations. Glycine is an inhibitor of γ-GT (competitive with the donor substrate) and its level must be kept to the minimum achievable (less than 0.1% on a molar basis). We have found that currently available lots of this substrate from Sigma or BMC satisfy this requirement.

2. The pure L-steroisomer is required since the D-form is only 35% as reactive. Other minor contaminants found in preparations of this substrate, L-α-glutamyl-3-carboxy-, 4-nitroanilide and 5-amino-2-nitrobenzoate, inhibit the γ-GT reaction minimally or not at all. However, their proportions in GluNA-CO_2 preparations must, nevertheless, be as low as practically achievable (i.e. less than 0.5% and 0.1% by weight, respectively. L-substrate that meets the above criteria has been available from BMC. Other vendors of the highly pure substrate may be used. When ordering, specify pure L substrate.

   An analysis report must be obtained from the manufacturer of this substrate. A pale yellow color is obtained when dissolved in aqueous solution. The absorbance at 410 nm of a 1 mmol/L solution of this substrate in 150 mmol/L glycylglycine (pH 7.9) has been found to be 0.045-0.050.

3. The reagent solutions should be stoppered and stored either in a refrigerator at 4 °C (short term) or in a freezer at -20 or -80 °C (long term). No measurable decrease in catalytic activity of γ-glutamyltransferase in serum pools was obtained after 1 month of storage of reagents 1 and 2 at 4 °C or after 7 months at either -20 or -80 °C. At room temperature, bacterial contamination is a limiting factor. Moreover, at room temperature spontaneous hydrolysis of L-γ-glutamyl-3-carboxy-4-nitroanilide produces a gradual increase in absorbance at 410 nm. (After 5 days at room temperature an absorbance increase of about 0.3 is produced.) The rate of spontaneous hydrolysis is even greater in reagent 3 at room temperature.

4. High purity 5-amino-2-nitrobenzoic acid is available from BMC. This material, which has ∿0.2% water by weight, was found to be chromatographically pure (TLC, GLC), but is greater than 98% pure on the basis of nitrogen analysis. The value for the molar absorption coefficient of 2-amino-2-nitrobenzoate under the final reaction conditions has been reported to be 790 $L \cdot mol^{-1} \cdot cm^{-1}$ [59].

Comments by Team Leader (L. Shaw)

The stability of γ-GT catalytic activity in SRM 909 was tested in each of the three round robins by Drs. Rauscher and Wahlefeld. After reconstitution according to the SRM 909 protocol, γ-GT activity was determined 5 times. SRM 909 was then stored in the refrigerator at 4 °C and 5 replicate assays were performed at the following times; 30-min, 60-min, 2-h and 7-h. The results of round robin 3 are included in Table 3. The results show good short-time stability of γ-GT in SRM 909. This result is, of course, consistent with the well-known stability (in human serum) of γ-GT.

Table 3. Protocol for determination of γ-glutamyltransferase activity by continuous monitoring - From RR III

DATE	REPLICATE NUMBER	γ-GT-ACTIVITY (U/L) FOUND AFTER RECONSTITUTION TIME				
		30-min	60-min	2 h	7 h	
week 1	1	15.1	16.8	18.8	15.5	
day 1	2	16.8	16.8	17.4	16.8	
	3	16.4	17.4	17.4	16.8	
A2 82	4	16.4	17.4	18.3	17.6	
	5	16.0	16.8	16.4	16.0	
	$\bar{x}$	16.1	17.0	17.6	16.5	16.8
	cv %	4.0	1.9	4.5	4.9	3.6
week 1	1	15.5	16.0	15.1	15.1	
day 2	2	15.5	15.1	15.1	15.1	
	3	15.5	16.8	16.8	16.0	
B2 5	4	16.4	16.8	15.5	15.5	
	5	15.5	16.0	15.5	16.4	
	$\bar{x}$	15.7	16.1	15.6	15.6	15.8
	cv %	2.6	4.4	4.5	3.7	3.8
week 2	1	15.1	14.7	16.0	16.0	
day 1	2	16.0	15.5	16.0	16.0	
	3	15.1	15.5	16.0	16.8	
A2 8	4	15.5	15.5	17.2	16.8	
	5	15.5	15.5	16.4	17.2	
	$\bar{x}$	1.4	15.3	16.3	16.6	15.9
	cv %	1.2	1.2	1.6	1.6	3.7
week 2	1	16.0	16.0	15.1	15.5	
day 2	2	15.5	16.0	14.7	16.0	
	3	15.5	16.0	14.7	16.0	
A2 54	4	16.8	16.0	15.1	15.5	
	5	16.8	17.6	16.0	16.4	
	$\bar{x}$	16.1	16.3	15.1	15.9	15.9
	cv %	2.0	2.2	1.8	1.2	3.9

V. STATISTICAL ANALYSIS OF CATALYTIC CONCENTRATION DATA ON SRM (909) ROUND ROBIN III

The data summarized in this chapter are only from RRIII of the interlaboratory enzyme study. The raw data were taken directly from the data reporting forms, each form showing five replicate values the lab obtained for each of four vials of reconstituted SRM 909. One lab in the ACP team had not participated in RRII, but submitted results for ACP (LO and HI) in RRIII. Since these results were highly discordant with the RRIII results from the other labs in the team, they are not included in this evaluation.

The raw data were reduced by taking the arithmetic mean of the replicate values for the catalytic concentration of individual vials. This produced a reduced data set in which, within a given enzyme-team, each lab is represented by four values-one for each vial.

Figures 4-8 provide graphical summaries of the reduced data set for each of the seven enzyme-teams. The data for ACP were summarized separately for the enriched (non-SRM) and unenriched (SRM 909) material (Figures 4a and 4b). The data for the AlaAT team were summarized both with and without use of the blank correction (Figures 5a and 5b). The data corresponding to the 19 individual participating laboratories are coded by letters A through U (I and O are not used). In Figs. 4-8, the four values from each lab are plotted about a horizontal solid line segment which indicates the mean concentration (i.e. mean of four mean values) for that lab. Distances along these lines have no meaning except to show, from left to right, that the four values were derived from four individual vials of serum analyzed by that lab on (week 1, day 1), (week 1, day 2), (week 2, day 1), (week 2, day 2), respectively. A dashed line is plotted at the overall mean value for the team, and dotted lines are plotted above and below this mean at a distance of 1 "reproducibility" standard deviation (see Table 4). Table 4 gives a numerical summary of the results in a form which follows ANSI/ASTM E 691-79, "Standard

TABLE 4

HUMAN SERUM ENZYME STUDY, PRECISION SUMMARY FOR ROUND ROBIN III

ANALYTE (number of labs)	(1) OVERALL MEAN ($\mu mol \cdot min^{-1} \cdot L^{-1}$)	(2) REPEATABILITY STD. DEV. W/IN LABS S_r	(cv) (%)	(3) BETWEEN LAB STD. DEV. S_L	(cv) (%)	(4) REPRODUCIBILITY STD. DEV. $S_R = \sqrt{(S_L)^2+(S_r)^2}$	(cv) (%)
ACP (LO) (3 labs)	0.2281	0.0164	(7.2)	0.0326	(14.3)	0.0365	(16.0)
ACP (HI)* (3 labs)	5.051	0.1430	(2.8)	0.2116	(4.2)	0.2554	(5.1)
ALP (6 labs)	75.35	1.909	(2.5)	1.148	(1.5)	2.227	(3.0)
AlaAT - with blank corr. (5 labs)	24.23	0.8367	(3.5)	1.577	(6.5)	1.785	(7.4)
AlaAT - w/out blank corr. (5 labs)	25.49	0.5611	(2.2)	2.166	(8.5)	2.238	(8.8)
AspAT (5 labs)	30.68	0.3735	(1.2)	0.7731	(2.5)	0.8586	(2.8)
CK (4 labs)	122.97	9.685	(7.9)	3.293	(2.7)	10.23	(8.3)
LDH (4 labs)	229.20	5.011	(2.2)	8.155	(3.6)	9.571	(4.2)
γ-GT	16.43	0.2413	(1.5)	0.3617	(2.2)	0.4348	(2.6)

* Enriched material, not SRM 909

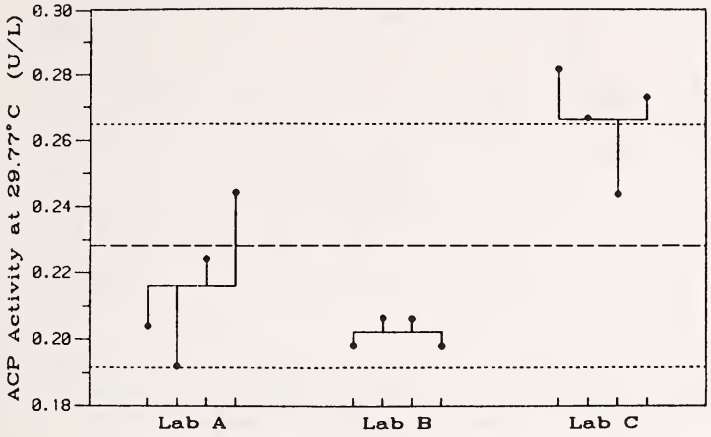


Figure 4a.

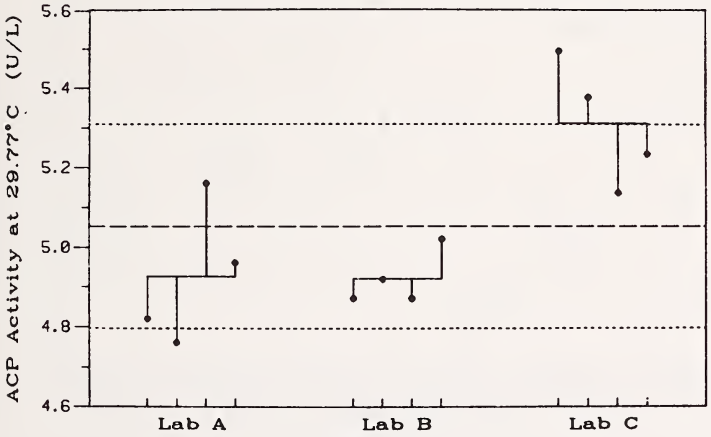


Figure 4b.

Figures 4a and 4b: Summary of ACP results for low activity (SRM 909) material (4a), and for enriched material (4b), showing the mean value per vial (closed circle, •), the mean values per laboratory (horizontal solid lines, ——), the mean value for all laboratories (dashed line, ----), and the 1-sigma "reproducibility" standard deviation limits (dotted lines, ·····).

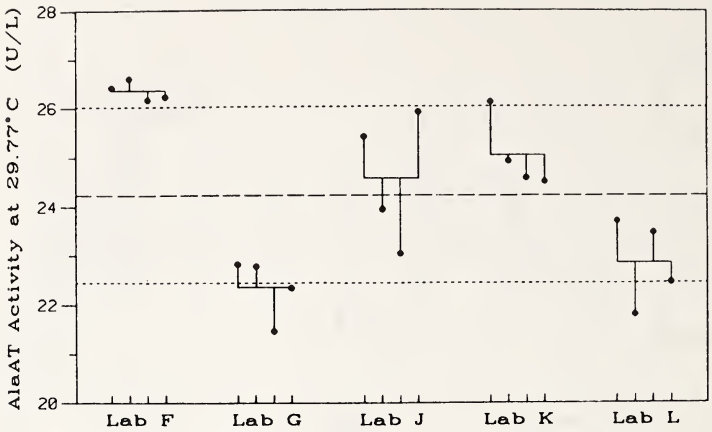


Figure 5a.

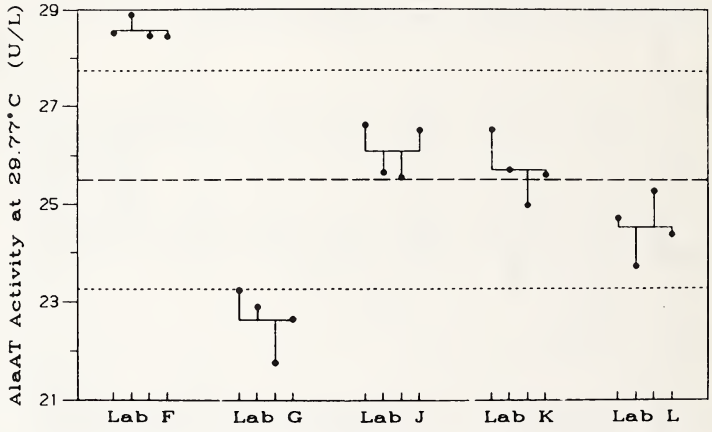


Figure 5b.

Figures 5a and 5b: Summary of results for AlaAT, with blank correction (5a) and without blank correction (5b), showing the mean value per vial (closed circle, •), the mean values per laboratory (horizontal solid lines, ——), the mean value for all laboratories (dashed line, ----), and the 1-sigma "reproducibility" standard deviation limits (dotted lines, ·····).

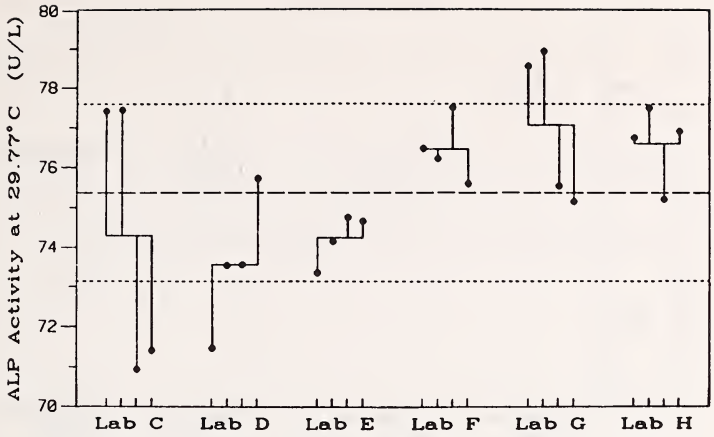


Figure 6a.

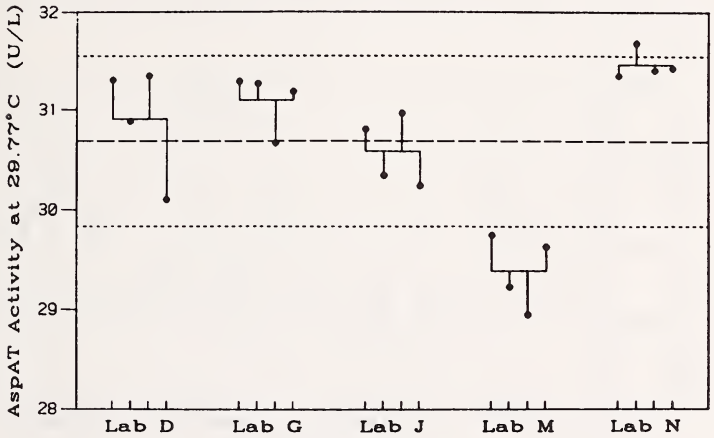


Figure 6b.

Figures 6a and 6b: Summary of results for ALP (6a), and for AspAT (6b), showing the mean value per vial (closed circle, •), the mean values per laboratory (horizontal solid lines, ——), the mean value for all laboratories (dashed line, ----), and the 1-sigma "reproducibility" standard deviation limits (dotted lines, ·····).

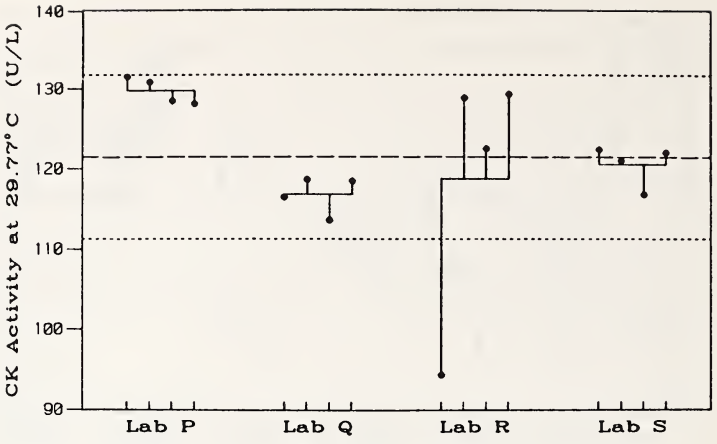


Figure 7a.

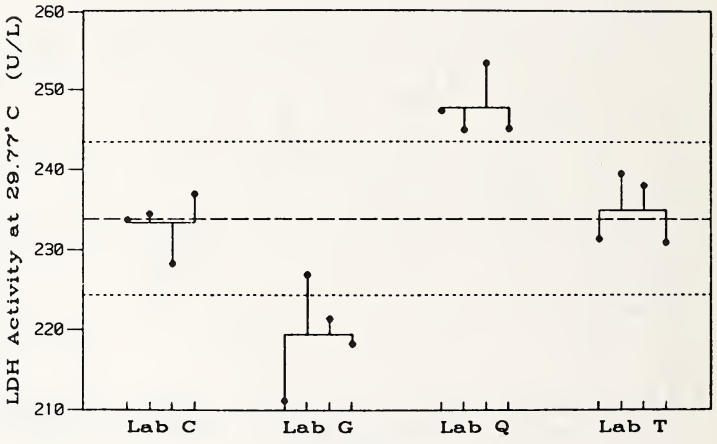


Figure 7b.

Figures 7a and 7b: Summary of results for CK (7a), and for LDH (7b), showing the mean value per vial (closed circle, •), the mean values per laboratory (horizontal solid lines, ——), the mean value for all laboratories (dashed line, ----), and the 1-sigma "reproducibility" standard deviation limits (dotted lines, ·····).

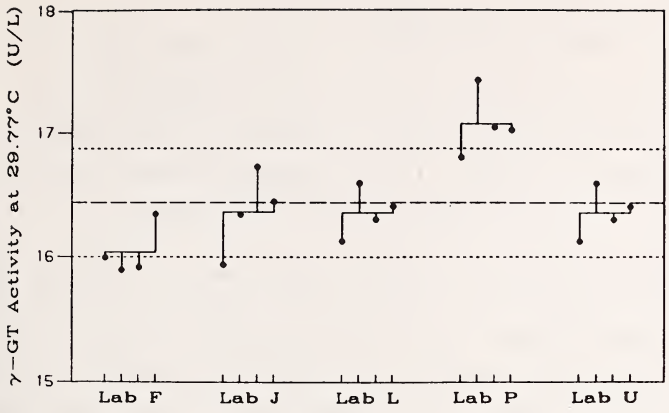


Figure 8: Summary of results for γ-GT, showing the mean value per vial (closed circle, •), the mean values per laboratory (horizontal solid lines, ——), the mean value for all laboratories (dashed line, ----), and the 1-sigma "reproducibility" standard deviation limits (dotted lines, ·····).

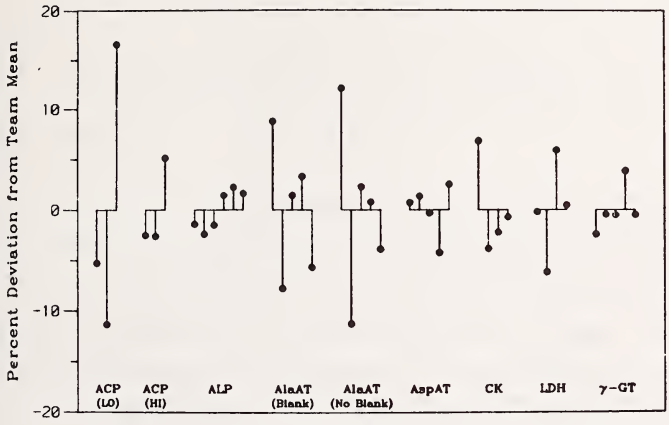


Figure 9: Summary of all results in terms of percent deviations from enzyme team means. Each laboratory is represented by its mean value (closed circle, •).

Practice for Conducting an Interlaboratory Test Program to Determine the Precision of Test Methods."

Table 4, column (1), contains the overall mean value of concentration in U/L ($\mu mol \cdot min^{-1} \cdot L^{-1}$). The "repeatability" standard deviation (S_r) and coefficient of variation (cv) are given in column (2). The S_r value is obtained by calculating the standard deviation of the four values representing each lab and pooling the results across labs for each enzyme-team.

The standard deviation S_L and corresponding cv are given in column (3). S_L estimates the between-lab component of variation from a random-effects model, one-way analysis of variance. This quantity represents the variability due strictly to between-lab differences, after the within-lab variability is taken out. The formula is:

$$S_L = \sqrt{\frac{\Sigma(\bar{X}_i - \bar{X})^2}{p-1} - \frac{(S_r)^2}{4}}$$

where

p = number of labs in a given enzyme-team
X_i = mean value for lab i ($i = 1,\ldots,p$)
$\bar{X}$ = mean value among all labs in an enzyme-team.

The "reproducibility" standard deviation in column (4) combines the within-lab and between-lab standard deviations. It represents the typical variability between results obtained by two different laboratories analyzing one vial each under the present protocol (which involves averaging five replicate values). The corresponding coefficient of variation for reproducibility (as a percent of the mean value) is given in parentheses.

Figure 9 presents a graphical summary of some of the information in Table 4. The values plotted against the vertical axis are percent deviations from the enzyme-team means. All enzyme-teams are represented on this graph. The values plotted represent individual lab means, although the labs are not individually identified. The formula for the "percent deviations" plotted is:

$$\% \text{ deviation} = \frac{(\text{lab. mean}) - (\text{enzyme-team mean})}{(\text{enzyme-team mean})} \times 100$$

These statistical results show that very good interlaboratory reproducibility of results may be achieved using the analytical methods described here. Except for measurements of the unenriched and therefore exceptionally low acid phosphatase level, the teams achieved reproducibility standard deviations of less than 9% for the enzyme catalytic (activity) concentrations.

We emphasize that the mean values (Table 4) for the catalytic activity concentrations of the seven enzymes in SRM 909 are consensus values, obtained by the cooperating laboratories through the use of the protocols described in this monograph. The assigned values for the catalytic (activity) concentrations in SRM 909 are "method dependent values" given for information purposes only. They are not certified by NBS.

In order for SRM users to achieve agreement with the assigned values, they must follow exactly 1) the serum reconstitution protocol at 0 °C for 1-h and 2) exactly adhere to every detail of the analytical methods protocols described in this document.

The long-term stability of the assigned enzyme catalytic (activity) concentration values also is not characterized. Several of the enzyme team leaders are monitoring the stability of the activity of these enzymes. Results obtained by the team leaders in the winter of 1982-1983 for all enzymes (except ACP-low) confirmed the values found in column (1) of Table 4 within the reproducibility standard deviations shown. SRM 909 users will be kept informed of any stability problems that are identified.

VI. SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

This study of the catalytic concentration of seven enzymes in SRM 909 was made possible by the voluntary cooperation of 28 scientists and their staffs in 17 U.S. laboratories and 3 elsewhere (in Canada, Norway and West Germany).

Two round robins like the final, third round robin, which was the one used to assemble data for assigning the catalytic (activity) concentration values, gave the participants pragmatic preliminary experience with the protocols and statistical design for these analyses. The reference type method protocol was chosen by each Team Leader who was very alert to the current status of IFCC and AACC recommendations for the enzyme reference method for which he (or she) was responsible. Although not entirely identical to IFCC methods, our methods are generally little different from them in final assay conditions. For the phosphatase and lactate dehydrogenase, no IFCC recommendations existed at the initiation of this study in 1979 to guide our selections; hence, these are exceptions.

The reaction temperature set-point was 29.77 °C, the gallium melting-point. NBS/SRM 1968 gallium cells were sent to each Team to assure standardization at 29.77 °C reaction temperature. The fact that 29.77 °C was used as the reaction temperature set point in this study should not be taken to signify that either NBS or all of the cooperating team leaders and members endorse this temperature as the optimum for determining enzyme catalytic activities. We used this means for standardizing reaction temperature to achieve interlaboratory reproducibility of enzyme catalytic concentration values. The temperature set point was thus rigorously established and controlled for our study.

The mean values for the catalytic (activity) concentration of the seven enzymes in SRM 909 represent method dependent values obtained through the use of a cooperating laboratory network. The assigned values are given for informational purposes only, they are not certified by NBS.

Information on changing the reaction temperature from 29.77 to 30 or 37 °C was not obtained as a multilaboratory cooperative effort. Such information was added subsequently to make the SRM 909 more useful. The information on "temperature factors" for SRM 909 enzymes was gathered only in the laboratories of the team leaders so identified and have not been checked or endorsed by the other scientists in the cooperating laboratories or by NBS personnel.

The main conclusion of this study is that excellent interlaboratory reproducibility of results (interlaboratory standard deviations of less than 10%) may be achieved for measurement of enzyme catalytic (activity) concentrations of stable, reference type materials, if participating laboratories adhere exactly to a rigorously defined reconstitution protocol and reference method protocol including the assurance of high quality and uniform reagent control.

Information obtained in this study should also be useful in the development of future NBS human serum SRM's. The study participants made the following Recommendations:

1. When securing serum material for certification, NBS procurement contracts should specify the enzyme additives in terms of species, tissue, purity, stability and activity per mL or mg protein.

2. The supplier should give details on all enzyme additives (as above).

3. Acceptable enzyme sources are as follows: ACP-human seminal fluid, ALP-beef kidney or liver (not placenta or intestine of any species), porcine heart for ASAT, ALAT, LDH, and CK and beef or porcine kidney for γ-GT.

The authors also recommend that all reference enzyme activity measurements should be made using the reference methods described in this monograph until they may be replaced by

reference methods adopted by the International Federation for Clinical Chemistry and/or the National Reference System for Clinical Chemistry. The authors further recommend that temperature factors relating enzyme activity at 29.77 °C--to activities at 25, 30 and 37 °C be established as part of any future study.

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U. S. Department of Commerce
Malcolm Baldrige
Secretary

National Bureau of Standards
Ernest Ambler, Director

APPENDIX

SEE ADDENDUM

(A REVISED SRM 909 CERTIFICATE WILL BE AVAILABLE SEVERAL MONTHS AFTER THIS MONOGRAPH IS PRINTED. COPIES OF THE REVISED CERTIFICATE CAN BE OBTAINED FROM NBS.)

National Bureau of Standards

Certificate

Standard Reference Material 909

Human Serum

This Standard Reference Material (SRM) is intended for use in evaluating the accuracy of clinical procedures for the determination of specified constituents in serum, in calibrating instruments and equipment used in these procedures, and in validating working or secondary reference materials.

CERTIFIED VALUES OF ANALYTES: The concentrations of the analytes were determined by methods having the highest accuracy, i.e., definitive methods. The certified values are given in two ways, depending on whether reconstitution is done with or without weighing the freeze-dried serum contents of a vial. Concentration values having smaller uncertainties may be obtained by weighing the freeze-dried serum and multiplying this mass by the certified concentration values of the analytes per unit mass of dried serum. These certified values appear in Table 1. When the contents of a vial are not weighed, the mean values for the certified concentrations and their uncertainties should be used. These values, which apply to all vials of reconstituted SRM 909, appear in Table 2.

NOTICE AND WARNINGS TO USERS

USE: HANDLE AS IF CAPABLE OF TRANSMITTING HEPATITIS! Although this product was tested with licensed third generation reagents and found nonreactive for the presence of hepatitis B surface antigen (HB_SAG), no known test method can offer assurances that products derived from human blood will not transmit hepatitis.

SRM 909 IS INTENDED FOR "IN VITRO" DIAGNOSTIC USE ONLY.

STORAGE: The freeze-dried serum should be stored in a refrigerator at a temperature between 2 and 8 °C. It should not be frozen nor exposed to sunlight or ultraviolet radiation. Under the recommended storage conditions, this SRM is expected to be stable for at least one year; should evidence indicate a more rapid degradation of the certified properties, purchasers will be notified by NBS. The material is not certified for use after one year from date of purchase.

The statistical analysis of the data was performed by K.R. Eberhardt of the Statistical Engineering Division.

The overall direction and technical measurements leading to the certification were under the chairmenship of E. Garner, H.S. Hertz, T.J. Murphy, D.J. Reeder, R. Schaffer and E. White V.

The technical and support aspects concerning the preparation, certification, and issuance of this Standard Reference Material were coordinated through the Office of Standard Reference Materials by R. Alvarez.

Washington, D.C. 20234
September 15, 1980
January 13, 1981 (revision)
October 8, 1982 (revision)

George A. Uriano, Chief
Office of Standard Reference Materials

(over)

Instructions for Use

HANDLE AS IF CAPABLE OF TRANSMITTING HEPATITIS! SRM 909 is supplied as a set of six vials of freeze-dried human serum and six vials of high-purity, diluent water for use in reconstituting the serum.

Two procedures for reconstituting SRM 909 are described. Selection of a procedure depends on the uncertainties required for the concentrations of the analytes. If lower uncertainties than those shown in Table 2 are required, the freeze-dried serum contents of a vial must be weighed as described in Procedure A.

Procedure A. Reconstitution with weighing of the freeze-dried serum: Completely remove label and adhesive by scraping the vial and then wiping it with a tissue moistened with a solvent, such as acetone or ethanol. Scratch an identification on vial. Remove metal closure and lightly tap bottom of vial to dislodge any serum particles adhering to the stopper. Dislodge stopper to equalize air pressure, then replace, wipe surface of vial, and weigh to the nearest 0.1 mg. (Use a clean empty vial of the same size as a tare.) Carefully remove stopper to avoid possible loss of serum particles. Use a Type 1 Class A volumetric transfer pipet or other dispenser of known accuracy to slowly add 10.00 ± 0.02 mL of the diluent water at 20 - 25 °C to the sides of the vial while continually turning the vial. Replace stopper, swirl vial two or three times, and let stand for 10 minutes. Mix contents by gently swirling, let stand for approximately 30 minutes, swirl again, let stand 10 minutes, and finally invert the vial several times. *Do NOT shake vigorously* because this will cause frothing. Total time for reconstitution is approximately 1 hour. After reconstitution, use contents as soon as possible. If not used immediately, store between 2 and 8 °C until ready for use, preferably within 8 hours. After the reconstituted serum has been used, clean and dry the vial and its stopper. Reweigh after replacing stopper. The tare is reweighed at the same time to compensate for changes in temperature and humidity. The mass of dry serum is given by the difference between the original and final weighings.

The concentration of an analyte, after the contents of a vial is weighed and reconstituted with 10.00 mL of diluent water, is calculated by multiplying the mass of freeze-dried serum, in grams, by the certified concentration of the analyte per gram of freeze-dried serum given in Table 1. For example, if the mass of freeze-dried serum in a vial is 0.8703 g, the concentration of uric acid in this vial would be:

$$0.5681 \text{ mmol}\cdot\text{L}^{-1}\cdot\text{g}^{-1} \times 0.8703 \text{ g} = 0.4944 \text{ mmol/L}.$$

The uncertainty is also calculated similarly and for this example would be:

$$0.0050 \text{ mmol}\cdot\text{L}^{-1}\cdot\text{g}^{-1} \times 0.8703 \text{ g} = 0.0044 \text{ mmol/L}.$$

Table 1. Certified Analyte Concentrations and Uncertainties per Gram of Freeze-Dried Serum after Reconstitution of SRM 909 according to Procedure A.

Analyte[1]	Concentration,[2] per gram, $\text{mmol}\cdot\text{L}^{-1}\cdot\text{g}^{-1}$	Uncertainty,[3] per gram, $\text{mmol}\cdot\text{L}^{-1}\cdot\text{g}^{-1}$
Calcium[a]	3.560	± 0.013
Chloride[a]	128.0	± 1.5
Glucose[a]	7.74	± 0.11
Lithium[a]	1.945	± 0.033
Potassium[a]	4.1546	± 0.0098
Uric Acid[a]	0.5681	± 0.0050

1. Analytical Methods
   a. Isotope dilution mass spectrometry.
2. The certified concentrations apply to reconstituted serum at room temperature (20-25 °C).
3. The uncertainties represent 95%/99% statistical tolerance limits for the concentrations per gram of dry serum reconstituted with 10.00 mL of high-purity water. The tolerance limits are constructed so that, at a confidence level of 95%, they will contain the concentration/mass ratios for at least 99% of the vials.

Procedure B. Reconstitution of SRM 909 without weighing freeze-dried serum: Remove metal closure and lightly tap bottom of vial to dislodge any serum particles on stopper. Carefully remove stopper to avoid possible loss of serum particles. As described in Procedure A, reconstitute with 10.00 ± 0.02 mL of the diluent water, and use immediately or store between 2 and 8 °C until ready for use, preferably within 8 hours. Table 2 gives the certified concentrations of the constitutents and the tolerance limits for use with this procedure.

Table 2. Certified Concentrations and Uncertainties for Analytes in Reconstituted SRM 909 for Use with Procedure B.

Analyte[1]	Concentration[2], $mmol \cdot L^{-1}$	Uncertainty[3], $mmol \cdot L^{-1}$
Calcium[a]	3.02	+0.17 −0.06
Chloride[a]	108	+7 −3
Glucose[a]	6.55	+0.46 −0.21
Lithium[a]	1.65	+0.12 −0.06
Potassium[a]	3.52	+0.19 −0.06
Uric Acid[a]	0.481	+0.031 −0.012

1. Analytical Methods:
   a. Isotope dilution mass spectrometry.
2. The certified concentrations apply to reconstituted serum at room temperature (20-25 °C).
3. The uncertainties represent 95%/95% statistical tolerance limits for the concentrations, and reflect the combined effects of measurement imprecision and the variability of the mass of dry serum among vials. They are constructed so that, at a confidence level of 95%, they will include the concentrations of at least 95% of all vials of SRM 909, when reconstituted according to Procedure B.

The major source of uncertainty in the certified concentrations shown in Table 2 is caused by vial-to-vial variability in the mass of freeze-dried serum. Information on the relation between mass variability and concentration was obtained directly during the course of experimentation by weighing the contents of each vial as an intermediate step in determining the concentration of an analyte. Supplementary information on the variability in mass was obtained from a differential refractometry experiment in which over 170 vials were analyzed. The data obtained from these two sources indicate that the distribution of masses is non-Gaussian and skewed in the direction of high mass. The largest two masses encountered were 5.2% and 2.7% above the mean mass, 0.8469 g. All other masses were contained in an interval from 1.5% below to 1.8% above the mean.

Source of Material: The human serum for SRM 909 was processed, vialed, and packaged by Hyland Division, Travenol Laboratories Inc., Round Lake, Illinois.

Analyses were performed in the NBS Center for Analytical Chemistry by I.L. Barnes, K.A. Brletic, R.G. Christensen, A. Cohen, J.W. Gramlich, W.R. Kelly, L.R. Machlan, J.R. Moody, L.J. Powell, L.T. Sniegoski, and M.J. Welch (Research Associate, College of American Pathologists).

This Standard Reference Material has been measured and certified at the Laboratories of the National Bureau of Standards, Gaithersburg, Maryland. All inquiries should be addressed to:

Office of Standards Reference Materials
Room B311, Chemistry Building
National Bureau of Standards
Washington, D.C. 20234

The date of issuance and certification of this Standard Reference Material is September 15, 1980.

U. S. Department of Commerce
Philip M. Klutznick
Secretary

National Bureau of Standards
Ernest Ambler, Director

Addendum to

National Bureau of Standards Certificate of Analysis

Standard Reference Material 909

Human Serum

ADDITIONAL CERTIFICATION

The following certified values are to be added to Tables 1 and 2.

Table 1. Certified Analyte Concentrations and Uncertainties per Gram of Freeze-Dried Serum after Reconstitution of SRM 909 according to Procedure A.

Analyte[1]	Concentration, [2]per gram, $mmol \cdot L^{-1} \cdot g^{-1}$	Uncertainty, [3]per gram, $mmol \cdot L^{-1} \cdot g^{-1}$
Cholesterol[a]	4.346	±0.030
Magnesium[a]	1.425	±0.072

Table 2. Certified Concentrations and Uncertainties for Analytes in Reconstituted SRM 909 for Use with Procedure B.

Analyte[1]	Concentration[2], $mmol \cdot L^{-1}$	Uncertainty[3], $mmol \cdot L^{-1}$
Cholesterol[a]	3.68	+0.22 −0.08
Magnesium[a]	1.21	+0.14 −0.10

Washington, D.C. 20234
November 14, 1980

George A. Uriano, Chief
Office of Standard Reference Materials

Enzymes - The catalytic concentrations in terms of U/L* of seven enzymes were determined in an interlaboratory study, and are given in Table 3. The results are in a form that follows ANSI/ASTM E691-79 "Standard Practice for Conducting an Interlaboratory Test Program to Determine the Precision of Test Methods".

Because of the state-of-the-art for the determination of enzymes, their concentrations cannot be stated on an accuracy basis. The enzyme values shown below are material and method-dependent. Thus, any laboratory using this SRM and the catalytic concentrations of these enzymes must follow the methods as described. The values given are consensus values, *not certified by NBS,* and were obtained through the interlaboratory study. They are provided for information only.

*Catalytic concentration is expressed in terms of International enzyme units per liter (U/L) where U is given as micromoles of substrate converted per minute. (1.0 U equals 16.67 nkat.)

Table 3

Catalytic Concentrations of Enzymes in Reconstituted SRM 909

Enzyme	Number of Laboratories	Overall Mean, U/L	Repeatability[1] Std. Dev.	Reproducibility[2] Std. Dev.
Acid Phosphatase[3]	3	0.23	0.02	0.04
Alkaline Phosphatase	6	75.4	1.9	2.2
Alanine Aminotransferase	5	24.2	0.8	1.8
Aspartate Aminotransferase	5	30.7	0.4	0.9
Creatine Kinase	4	123.0	9.7	10.2
Lactate Dehydrogenase	4	229.2	5.0	9.6
γ-Glutamyltransferase	5	16.4	0.3	0.4

1. Repeatability (within-laboratory standard deviation) for each enzyme was obtained by calculating the standard deviation of the four daily mean values (5 replicates) obtained in each laboratory and pooling the results across laboratories.

2. Reproducibility standard deviation combines the within-laboratory and between-laboratory standard deviations. It represents the typical variability between results obtained by two different laboratories analyzing one vial each under the present protocol (which involves averaging five replicate values).

3. Native catalytic concentrations. Prostatic acid phosphatase was not added to the serum base.

Reconsitution Procedure for Enzyme Determinations: The enzyme concentrations listed inTable 3 were measured after the lyophilized serum had been reconstituted with ice-cold water for about one hour as described below. This procedure is different from those given on pages 2 and 3 of the Certificate.

1. Remove the vials of SRM 909 lyophilized serum and diluent water from the refrigerator.
2. Immerse the vial of water in an ice-water slush for 5 minutes. Water-ice should contact the vial, not air-ice.
3. Tap the SRM 909 vial lightly on a solid surface to dislodge any serum particles adhering to the stopper. Remove metal cap and stopper, being careful to avoid loss of serum particles.
4. Transfer 10.0 mL of the cold diluent water* to the vial of serum as follows: Using a class A, 10-mL pipette at room temperature, dispense the water slowly to the side of the vial while continually turning the vial to wet all serum particles and enhance dissolution.
5. Replace the stopper, swirl to wet beads of serum, gently invert vial 10 times, and immerse the vial in ice water. Repeat the gentle swirling and inverting operations at 10 minute intervals for one hour, always returning the vial to the ice-water bath.
6. Inspect the reconstituted SRM 909 visually. Undissolved serum particles should not be evident. Invert the reconstituted SRM gently several times before use for specific enzyme procedures.

*A slight excess over 10.0 mL is actually delivered in this procedure. For enzyme concentration measurements, the error is not considered significant.

Enzyme Methods - The methods used in this study are outlined below. Details of the methods and the experience with their use during the interlaboratory study are to be written for publication in the NBS 260 Series. (This series is used to disseminate information on the preparation, measurement, certification, and use of NBS SRM's.) All methods use a reaction temperature of 29.77 °C, which can be verified by using a Gallium Melting Point Cell, SRM 1968 (1).

1. Acid Phosphatase [Orthophosphoric monoester phosphohydrolase (acid optimum), EC 3.1.3.2].

The catalytic concentration of acid phosphatase in U/L is determined using thymolphthalein monophosphate as substrate. Enzymatic hydrolysis at pH 5.4 yields a colorless product, thymolphthalein, which becomes a self-indicating chromogen (λ= 595 nm) when the enzymatic reaction is terminated by the addition of alkali. The method of Ewen and Spitzer (2) is employed with the following modifications: a) the reaction temperature is 29.77 °C and b) multiple tests are performed at 15, 30, 45, and 60 min. The final reaction conditions are:

Reaction temperature	29.77 °C
pH	5.4
Acetate buffer	0.15 mol/L
Thymolphthalein monophosphate	1.0 mmol/L
Brij-35	1.5 g/L
Volume fraction (sample/total)	0.083 (1:12)

2. Alkaline Phosphatase [Orthophosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1].

The catalytic concentration of alkaline phosphatase in U/L is measured by following the liberation of 4-nitrophenol from the substrate 4-nitrophenyl phosphate in the transphosphorylating buffer, 2-amino-2-methyl-1-propanol. It utilizes the method of Bowers and McComb (3) with the single modification of changing the reaction temperature from 30 °C to 29.77 °C. The final reaction conditions are:

Reaction temperature	29.77 °C
pH	10.5
4-Nitrophenylphosphate	16.0 mmol/L
2-Amino-2-methyl-1-propanol	1.0 mol/L
Magnesium acetate[1]	1.0 mmol/L
Volume fraction (sample/total)	0.0164 (1:61)

3. Alanine Aminotransferase (L-Alanine: 2 oxoglutarate aminotransferase, EC 2.6.1.2).

The catalytic concentration of alanine aminotransferase (AlaAt) in U/L is measured by coupling pyruvate production with reduced nicotinamide adenine dinucleotide (NADH) and lactate dehydrogenase (LDH). It utilizes the IFCC/EPE and AACC/AlaAt Study Group reference method conditions (4,5) at a reaction temperature of 29.77 °C instead of 30 °C. The volume fraction of serum allows convenient use of standard volumetric glassware. The starting reagent is 2-oxoglutarate, added after 15 minutes preincubation of specimen with an otherwise complete reaction mixture at 29.77 °C and after a stable absorbance is reached at 339 nm. The coupled catalytic reaction is followed for 300 seconds. A reagent blank reaction rate (water as specimen) is deducted from the overall rate. The final reaction conditions are:

Reaction temperature	29.77 °C
pH	7.3
L-Alanine	500 mmol/L
2-Oxoglutarate	15 mmol/L
Pyridoxal-5'-phosphate	0.11 mmol/L
Tris buffer	89 mmol/L
NADH (assuming NADH $Na_2 \cdot 2H_2O$)	0.16 mmol/L
LDH (EC 1.1.1.27)	2.2 U/mL (25 °C)
Volume fraction (sample/total)	0.083 (1:12)

4. Aspartate Aminotransferase (L-Aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1).

The catalytic concentration of aspartate aminotransferase in U/L is measured by coupling oxalacetate production with reduced nicotinamide adenine dinucleotide (NADH) and malate dehydrogenase (MDH). It utilizes the IFCC/EPE reference method conditions (6) at a reaction temperature of 29.77 °C instead of 30 °C and with minor modifications suggested by the AACC Study Group (7). In addition, lactate dehydrogenase is omitted from the reagent system. The starting reagent is 2-oxoglutarate, added after 15 minutes preincubation at 29.77 °C and after a stable absorbance is reached at 339 nm. The coupled catalytic reaction is followed for 300 seconds. The final reaction conditions are:

Reaction temperature	29.77 °C	
pH	7.8	
L-Aspartate	175	mmol/L
2-Oxoglutarate	15	mmol/L
Pyridoxal-5′-phosphate	0.11	mmol/L
Tris buffer	89	mmol/L
NADH (assuming NADH-Na_2·$2H_2O$)	0.16	mmol/L
MDH (EC 1.1.1.37)	0.95	U/mL (25 °C)
Volume fraction (sample/total)	0.083 (1:12)	

5. Creatine Kinase (ATP: creatine N-phosphotransferase, EC 2.7.3.2).

The catalytic concentration of creatine kinase (CK) is measured by coupling adenosine triphosphate production with nicotinamide adenine dinucleotide (NAD^+) through the use of intermediate reactions catalyzed by hexokinase and glucose-6-phosphate dehydrogenase. It follows the method recommended by the IFCC/EPE (8) and the AACC/CK Study Group (9) with the following modifications: a) adenylate kinase inhibitors are removed; b) a blank reaction is run; c) disodium ethylenediamine tetraacetate (Na_2EDTA) is added to the system; and d) the reaction temperature is 29.77 °C. The final reaction conditions are:

Reaction temperature	29.77 °C	
pH	6.6	
Imidazole acetate	100	mmol/L
Creatine phosphate	30	mmol/L
Adenosine-5′-diphosphate	2	mmol/L
D-Glucose	20	mmol/L
NAD^+	2	mmol/L
Hexokinase (EC 2.7.1.1)	2500	U/L
D-Glucose-6-phosphate dehydrogenase (EC 1.1.49) (from Leuconostoc mesenteroides)	1500	U/L
Magnesium acetate	10	mmol/L
N-Acetyl cysteine	20	mmol/L
Na_2EDTA	2	mmol/L
Volume fraction (sample/total)	0.043 (1:23)	

6. Lactate Dehydrogenase (L-Lactate: NAD^+ oxidoreductase, EC 1.1.1.27).

The catalytic concentration of lactate dehydrogenase in U/L is measured by following the oxidation of reduced nicotinamide adenine dinucleotide (NADH) at 339 nm. It utilizes the method of Bowers (10) with minor modifications at a reaction temperature of 29.77 °C. The final reaction conditions are:

Reaction temperature	29.77 °C	
pH	7.2	
Sodium pyruvate	1.2	mmol/L
NADH	0.15	mmol/L
Tris buffer	96.8	mmol/L
Volume fraction (sample/total)	0.016 (1:61)	

7. γ-Glutamyltransferase [(γ-Glutamyl)-peptidase: amino acid γ-glutamyltransferase, EC 2.3.2.2.].

The catalytic concentration of γ-glutamyltransferase in U/L is measured by following the liberation of the 4-nitroaniline at 410 nm. The acceptor substrate glycylglycine also serves as the buffer. This assay utilizes the IFCC/EPE proposed reference method (11) with a modification in reaction temperature from 30 °C to 29.77 °C. This assay is initiated by the addition of serum to the combined substrate/buffer mixture equilibrated to 29.77 °C. It is followed at 410 nm for at least 300 seconds. The final assay reaction conditions are:

Reaction temperature	29.77 °C	
pH	7.90	
L-γ-Glutamyl-3-carboxy-4-nitroanilide	6	mmol/L
Glycylglycine	150	mmol/L
Volume fraction (sample/total)	0.091 (1:11)	

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5. AUTHOR(S) G.N. Bowers, Jr., R. Alvarez, J.P. Cali, K.R. Eberhardt, D.J. Reeder, R. Schaffer, G.A. Uriano, R. Elser, L.M. Ewen, R.B. McComb, R. Rej, L.M. Shaw

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11. ABSTRACT (A 200-word or less factual summary of most significant information. If document includes a significant bibliography or literature survey, mention it here)

We determined the catalytic (activity) concentrations of seven enzymes (ACP, ALP, AlaAT, AspAT, CK, LDH and γ-GT) in the NBS lyophilized human serum Standard Reference Material (SRM 909). SRM 909 enzyme activity values should provide the basis for compatibility among methods for determining enzyme values in serum. Separate teams of clinical chemistry experts selected and carried out the analyses for the individual enzymes. The methods used are primarily those recommended by the Standards Committees of the AACC and/or the IFCC, as candidate reference methods; however, a reaction temperature set-point of 29.77 °C (the gallium melting point) was used. This monograph describes the serum reconstitution protocol, the specific enzyme methods, and the results of the third (and last) round of the interlaboratory test program, which we used to derive mean values for enzyme catalytic (activity) concentrations in SRM 909. Interlaboratory standard deviations of less than 10% were achieved. Measurements made one year after the conclusion of the interlaboratory study showed unchanged enzyme activities, within the uncertainties of the original assigned values.

12. KEY WORDS (Six to twelve entries; alphabetical order; capitalize only proper names; and separate key words by semicolons)

enzyme; enzyme (catalytic) activity; human serum; interlaboratory reproducibility; reference method; Standard Reference Material.

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