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Minimally processed fresh frozen human reference sera: preparation, testing, and application to international external quality assurance

G. M. HENRIKSEN,* M. M. PEDERSEN,* I. NØRGAARD,† M. BLOM,† L. BLOU,‡ O. BLAABJERG§ & A. ULDALL*

*Danish Institute for External Quality Assurance for Laboratories in Health Care, DEKS. Herlev University Hospital, Herlev, Denmark; †Department of Clinical Biochemistry and Blood Bank, Hjoerring Hospital, Hjoerring, Denmark; ‡Department of Plasma Fractionation, Statens Serum Institute, Copenhagen, Denmark; §Department of Clinical Biochemistry, Odense University Hospital, Odense, Denmark

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The preparation of unmodified or minimally processed fresh frozen human sera is described, as well as the previous use of such sera, e.g. in Nordic and international external quality assurance (EQA) activities. The unmodified serum is prepared from fresh donors' blood collected in dry bags and allowed to coagulate. The serum is collected "on the clot", pooled, filtered, mixed, dispensed in polypropylene vials and frozen at −80°C without further processing. Some batches were slightly modified by spiking or dilution. Critical steps of the production and use of the sera are described and improvements are discussed. A total of 34 different batches have been prepared since 1985. Results from homogeneity and stability studies are presented. The studies cover 18 routine components in serum stored at +4°C to 37°C for up to 34 days. Good stability was observed for storage of all components, with the exception of triglyceride. Amylase, creatininium, glucose, gamma-glutamyltransferase, urate (and perhaps carbamide) showed deterioration after 13 days of incubation at 37°C. The long-term stability at −80°C is reviewed and new data are presented, e.g. as consensus values from EQA schemes, where the same serum has been sent out three times over 5 years, and from reference measurement procedure values that have been assigned twice with an interval of 5 years. Furthermore, a 10-year stability study has been started.

Key words: Calibration; control; control material; homogeneity; preparation; reference; stability

Gitte M. Henriksen, DEKS, 54M1, Herlev University Hospital, DK-2730 Herlev, Denmark. Tel. +45 4488 3310, fax. +45 4453 5369, e-mail. gitte.m.henriksen@deks.dk

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INTRODUCTION

With the increasing demand for sample materials in clinical laboratories, the requirements for quality control materials have greatly increased in order to monitor performance objectively. Unmodified fresh frozen sera with reference measurement procedure values are preferred, but this is not always practically and economically possible to achieve, because of non-availability or the high cost of the reference measurement procedure values and the unmodified sera.

Synthetic, animal or recombinant reference materials used for calibration or control in the clinical laboratory should be preferred for ethical reasons. However, reference material of human origin is often the material of choice due to the analytical and practical requirements. Reference materials need to be stable and lyophilization has been the most popular technique for achieving suitable stability in spite of commutability problems. However, nowadays, manufactured liquid materials are also frequently preferred by the user, because no reconstitution is needed. Most liquid materials often require addition of stabilizers as part of the production, which may also give rise to commutability problems. Furthermore, the long-term stability of stabilized liquid serum is often shorter than that of lyophilized serum.

Use of donors' blood as starting material for reference sera

The processing of donors' blood for medical purposes always yields some surplus of plasma material, which could be used for the preparation of reference serum instead of being discharged. Therefore, it has been convenient to use surplus materials as control material for routine analysis in the hospital. Various procedures have been used in the hospital environment to manufacture frozen reference serum from either plasma from out-dated blood [1, 2] or surplus plasma from platelet concentrate [3].

However, it is questionable whether the described frozen sera are optimal as reference sera with the commutability needed for external quality assurance (EQA) schemes. This is due to the changed properties when the sera are converted from plasma to serum in an "artificial way". The starting material may also be altered because of long-term storage at $+4^{\circ}C$ or $+35-37^{\circ}C$ (outdated material). For this reason, fresh frozen unmodified serum is preferred. The necessity of using fresh frozen unmodified serum has also been documented by the Center for Disease Control (CDC) in the USA [4]. To accommodate this preference, a technique for collecting fresh blood and immediate preparation of serum ("on the clot") was developed [5] and has been used since 1985 to prepare reference sera for EQA or sera offered as certified reference materials. Examples of major uses of this type of reference sera are:

- as control material in national EQA schemes from DEKS (the Danish Institute for External Quality Assurance for Laboratories in Health Care) (previously DSKK/AKU) [5];
- as control materials in Nordic EQA schemes from Labquality (an international organizer of EQA schemes based in Finland) in the general clinical chemistry survey, "Short Term Survey" with more than 1000 participants;
- as calibrator in the Nordic Reference Interval Project, NORIP [6];
- as control materials with certified reference values in the surveys of the International Measurement Evaluation Programme (IMEP) of the EU Institute for Reference Materials and Measurement (IRMM) [7–9].

Our techniques for preparation and testing of unmodified or minimally processed fresh frozen human serum are described below. The acronym FHK is used for these materials. Details of the techniques have been improved slightly in the past 18 years since the first application. The procedure described below has been in use almost unchanged since 1994.

MATERIALS

Blood donors

Blood collected from voluntary Danish donors, consenting to the intended purpose of the donation, is used as starting material. The donors' blood was tested and found negative for HIV 1+2 antibodies, Hepatitis B virus(Ag) and Hepatitis C virus(Ag).

Blood collection bags

Baxter, Fenwal®, plain plastic bags were used.

Filter materials

Materials included a 10-inch cartridge filter Millipore Polysep™ (CP0671S03 (pore size 0.50 μm)), a 10-inch cartridge "dept" filter (Milligard® CWSS 71S03, final pore size 0.20 μm), a sterile filter, Sartorius Sartobran™ P-capsuli 5231307H9-SO-A, pore size 0.45 μm plus 0.20 μm, and an air exhaust filter, Milipore Aervent™-50, 0.20 μm.

Vials and stoppers

These comprised 10-mL polypropylene vials produced by PLM Langeskov A/S (Haunstrup Plast, now included in the Rexam Incorporation); Teflon® coated stoppers; outer metal seal (removable without tools); 5-mL vials, NUNC Cryo tubes with silicon seals and stoppers were used for the special reference sera, used as internal controls within the NORIP [6].

Plastic bottles

Disposable 2.5-L plastic bottles sterilized by irradiation were used.

Chemicals

Only chemicals of "pro-analysis" quality were used. Enzyme preparations of high purity were used. Water was distilled and sterile.

METHODS

Collection of blood and preparation of a serum pool

Blood was collected in the blood bank of Hjoerring County Hospital. About 450 mL blood is drawn into a plain plastic collection bag, without any anticoagulants or additives. After 3–4 h at room temperature the blood begins to clot spontaneously. The blood is then centrifuged and the harvested raw serum is moved to and pooled in a 2.5-L plastic bottle and stored for a few days at −40°C until the bottle is filled up with serum. The yield of serum is about 170–200 mL from each donation. The full plastic bottles are stored at −80°C. The entire pool of raw serum collected is sent to Statens Serum Institute (SSI) in Copenhagen, in frozen state (approx. −20°C), where the pool is stored at −20°C until the preparation takes place, usually within 1–2 weeks.

The following preparation took place at SSI—a typical protocol—is divided into four major steps: Thawing, filtering and modification if any, filling and freezing. The preparation takes place at room temperature.

1. The raw serum is thawed at ambient temperature for approximately 18 h after which the temperature in the plastic bottles is typically 0–10°C.
2. The entire pool typically consisting of 22–25 L raw serum is transferred to a sterile stainless steel tank (MT 34). Modification (if any) is done at this stage of the process as dilution or spiking. The raw serum is then filtered through a cartridge filter, followed by a further filtration through a cartridge "dept" filter into a sterile 21-L glass bottle mounted with a magnetic stirrer, a sterile filter, the necessary tubes, and an air exhaust filter. The entire filtration assembly is sterilized in steam at 120°C (autoclaved) for 1 h before use.
3. After slow stirring overnight (approx. 18 h) the serum is filled into autoclaved polypropylene vials in a sterile chamber, using a tube pump and closed with stoppers under strict sterile conditions. The first 50 mL serum is discharged. Usually 5 mL serum is filled into each vial. After the filling, the vials are sealed with a metal cap. To check for homogeneity, the first and last two filled vials are kept separate as well as one out of every 100 vials in the filling line.
4. Immediately after filling, the vials are moved to the Herlev University Hospital, where they are put into −80°C freezers. A number of vials corresponding to 2–3 L serum are put into each freezer, Freezing of all serum within 6 h is plotted in Figure 1. The serum remains stored at −80°C until use.

Minimally processed FHK: techniques for modification of the concentration of components

Usually only one concentration level of FHK was used, the natural concentration, arising

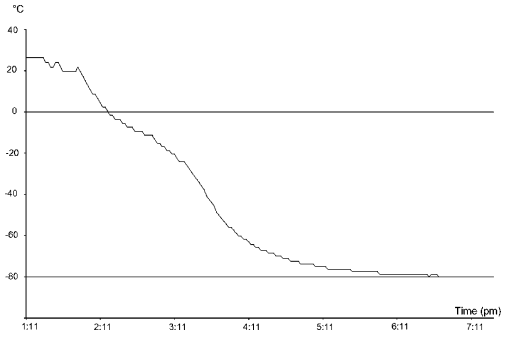

FIG. 1. Time taken to freeze approximately 2–3 L FHK serum in 5 mL vials to −80°C. The curve shows the temperature (abscissa) as a function of the time (ordinate). After 6 h, the serum is frozen.

from preparation of the serum pool from healthy donors. However, for practical purposes it is desirable to have sets of reference sera at different concentration levels of which the unmodified FHK is the most important material. Supplementary materials may be obtained through the processing of the unmodified FHK. In this situation commutability aspects needs to be taken into account.

A "high level control" was produced from unmodified FHK by removal of water with salts using either ultra filtration through an artificial kidney [5], or slow thawing of the frozen serum followed by discharge of the initially created aqueous phase [10]. In both cases the "concentrated" serum might be further enriched by spiking with important components, which may be desirable at a particularly high concentration.

A "low level control" is achieved by addition of water (one volume water plus one volume concentrated serum) and spiked with appropriate electrolytes [5].

An example of a modest "minimally processed reference serum" is shown in the section below as the IMEP-17 Material 2, involving minimal commutability problems.

Modification of FHK to achieve Material 2 of the IMEP-17 study

For the IMEP-17 Material 2 [7–9], the desired concentrations were in principle reached as described in Table I. However, for practical reasons, the procedure was performed in different steps: at Herlev University Hospital 1 L of a previously pooled serum was spiked with the entire amount of components needed for the final preparation of 21 L. The rest of the preparation took place at SSI, where the concentrated pooled serum was added to the main pool as well as the 1.6 L HCl 3.125 mmol/L. The serum was processed as described above and 9.5 mL was filled into each of the 10-mL polypropylene vials.

Preparation of reference sera for internal control within NORIP

Five reference sera, "CAL", "X", "P", "HIGH" and "LOW" were prepared as described above. The sera were filled into Cryo-tubes.

- "CAL": unmodified FHK with 17 components determined with reference methods by DGKC (the German Society for Clinical Chemistry).
- "X": unmodified FHK
- "P": unmodified FHK (the blood originated exclusively from women using hormonal contraceptives).
- "HIGH": the serum pool was concentrated using a freezing-thawing technique [10].
- "LOW": the serum was prepared by dilution of one volume "HIGH" with one volume of water containing 80 mmol/L sodium chloride.

Procedure for testing homogeneity

Homogeneity testing is done using the following procedure [11]: 20 representative vials were selected at random, from the beginning, middle and end of the filling line (a minimum of 10 vials were selected). The vials were thawed and the content mixed as described in the section "How to handle the FHK". Two test portions from each vial were prepared and analysed independently of each other. To evaluate the homogeneity of the vials, a number of representative components were selected, e.g.: albumin, alkaline phosphatase (ALP), amylase, calcium, creatine kinase (CK), creatininium, glucose, iron, lactate dehydrogenase (LD), magnesium, phosphate, sodium, triglyceride, urate, carbamide and zinc.

The serum was analysed on a Vitros 950 chemistry analyser in the laboratory at Herlev University Hospital. An independent control

TABLE I. Preparation of the IMEP-17 Material 2.

Component, unit	Molecular mass (g/mol)	Typical concentration of the primary native pool	Desired concentration of the final preparation[¤]	Amount to add to 21 L of the final preparation (mg)
Amylase, U/L		55	~85	15[*]
Creatininium, μmol/L	131.14	70	150	240
Gamma-glutamyltransferase (GT), U/L		36	~80	(~900 U)[#]
Glucose, mmol/L	180.16	4.5	8.5	16 647
Glycerol, mmol/L	92.10	1.2	2.1	1932
Lithium (chloride), mmol/L	42.40	0	1.0	890
Magnesium (chloride, 6 H_2O), mmol/L	203.3	0.83	1.25	2135
Potassium (chloride), mmol/L	74.55	3.7	5.5	3 070+(§)
Carbamide, mmol/L	60.06	5.0	10.0	6 940
Thyroxine (T4), nmol/L	776.93	100	~170	1.3 (partly dissolved, only)
Urate, μmol/L	168.11	286	500	850[§]
Zinc (chloride), μmol/L	136.29	17	25	29

IMEP = International Measurement Evaluation Programme.

[§]Urate (as uric acid) was dissolved in potassium hydroxide, 10 mL 1,0 mol/L (~10 mmol of additional K+) and diluted to 500 mL with water before addition to the serum.

[*]Amylase, EC number 3.2.1.1 from human saliva, 500 U (25°C) cat. no. 10092 ("BioChemica" purity; 100 U/mg) from Sigma-Aldrich

[#]Gamma-glutamyltransferase, EC number 2.3.2.2 from bovine kidney, 500 U (25°C) cat. no. G4756 (grade 2 purity; 26 U/mg) from Sigma-Aldrich

[¤]A dilution of the native serum (approx. 18.9 L serum + 2.1 L water) was intended through the present description, see § and †. In this way the initial concentration values of all components were, in principle, reduced by about one-tenth.

material with known values was included in every series of analyses, the first and after every 10 vials. To diminish evaporation, caps were used for the sample cups during the analytical run.

A one-way ANOVA (Microsoft® Excel version 9.0) was used for evaluation of the results; total, between and within standard deviations (s) were calculated. The samples were considered to be adequately homogeneous if: s between-samples $\leq 0.3 \times s$ proficiency testing [11].

Stability studies on thawed serum

The storage conditions of FHK in the stability studies were at 4°C, 22°C, 30°C and 37°C, for up to 34 days.

One vial at each storage temperature was removed after the planned storage time and analysed for a selection of components, mainly on Vitros 250/950. The experiment was planned so that it was possible to carry out the analysis of the vials from all storage temperatures and storage times under repeatability conditions (i.e. in one series of analyses). The following example demonstrates the principle: On day 0, all vials were stored at −80°C, but those vials that should be incubated for 30 days were moved to their appropriate temperatures; on day 7, those vials that should be incubated for 30−7 = 23 days were moved from the −80°C storage to their relevant temperatures; on day 14, the relevant vials that should be incubated in 30−14 = 16 days were moved from −80°C to the appropriate temperatures, and so on; on day 30, all vials were analysed and the results compared with the those obtained on the vials stored at −80°C, which was regarded as stable.

Several experiments of this type have been carried out in the past, essentially with the same outcome. In this publication the results in our studies cover the following components in serum: albumin, amylase, calcium, cholesterol, creatininium, gamma-glutamyltransferase(GT), glucose, IgG, iron, lithium, magnesium, potassium, sodium, thyroxin, triglyceride, urate, carbamide and zinc. To cover a larger number of components for the same amount of work, some components were only measured in one of

the two series. The results for a selection of components from the stability study of serum in thawed state are presented in Table III. One series was carried out on the slightly modified sera, FHK batch 2000-07 and one on FHK 2000-12 (see Annex A).

Stability studies: NFKK reference sera

The stability of the reference sera from the NORIP has been studied in different ways. One way is by following the consensus values on the reference serum "CAL" in the general clinical chemistry survey from Labquality, the "Short-Term Survey". CAL has been sent out three times: the first time in September 1997, the second time in April 2002 and the third time was in May 2003. Another way is by comparing the reference measurement procedure values assigned to "CAL" by DGKC [12] in May 1997 and transferred values assigned to CAL″ from "IMEP-17 Material 1" in 2002 with assigned values of the highest available metrological order in the IMEP-17 study [13]. A third way is by an in-house stability study [14]. The aim is to document stability over 10 years, counted from 2002, with the materials stored at −80°C. It is assumed that a stability study for the material X will be representative for all the materials in NORIP, since they are very similar with respect to matrix; typical unmodified FHK samples.

To document 10 years of stability at −80°C three different approaches are chosen. The components that are to have their stability documented are: alanine transaminase (ALT), albumin, ALP, amylase, aspartate transaminase (AST), bilirubin (total), calcium, cholesterol, CK, creatininium, glucose, iron, LD, magnesium, phosphate, potassium, protein, sodium, carbamide, urate and triglyceride:

1. the changes in absolute measurement values are observed, when material X is stored at −80°C;
2. measurement values for the material X stored at −150°C (fixed reference point) and the material X stored at −80°C are compared;
3. prediction of stability by accelerated experiments using the Arrhenius equation and a storage temperature of −20°C.

How to handle the FHK

Generally speaking, the laboratories should treat the FHK in the same way as normal patient sera. The usual way that serum is distributed to the participating laboratories is either as normal mail at ambient temperature (liquid form) or in frozen state using dry ice in a foam package. The mailing time is normally three days or less. When the serum is distributed in liquid form, it is stored at 4°C on arrival and analysed as soon as possible. If analysis cannot be performed immediately, the serum can be refrozen at −80°C (or −20°C).

If the serum is still frozen on the day foreseen for the analyses, vials should be placed on a table at ambient temperature, so that none of the vials touch each other, and left to thaw for 2 h. Before opening the vial, mixing should be performed by gently turning the vial upside-down for 10 min, e.g. on a slow-turning mixer [15].

RESULTS

Production lots

The 34 batches and subbatches of fresh frozen human sera manufactured since 1985 when preparation of serum "on the clot" from freshly collected donors' blood was initiated are reviewed in Annex A.

Results of a homogeneity check

In Table II we present an example of results from the most recent FHK batch. The samples in this example are considered to be sufficiently homogeneously based on the observation that the s between-samples for most of the components are low and because the criterion is met, with the exception of sodium (see Table II). The uncertainty regarding sodium was higher than expected, owing to the instability of the instrument at that time.

Stability of serum in thawed state

Generally speaking, all components studied were found to be stable under the present conditions: at 4°C, 22C, 30°C and 37°C, up to 31–34 days. However, all the results of potentially unstable components as observed

TABLE II. Example of a homogeneity check*. The samples are considered adequately homogeneous when the between-samples standard deviation *(s)* is ≤ 0.3 times the *s* for proficiency testing [11].

Component, unit	*s* between-samples	*s* proficiency testing** $\times 0.3$	*s* within-samples
Albumin, μmol/L	7.94	8.1	6.90
Alkaline phosphatase, U/L	1.44	3.9	2.05
Amylase, U/L	3.18	5.1	5.37
Creatininium μmol/L	0.45	2.37	1.32
Glucose, mmol/L	0.05	0.11	0.05
Iron, μmol/L	0.33	0.33	0.32
Lactat dehydrogenase, U/L	3.87	5.1	4.61
Magnesium, mmol/L	0.007	0.02	0.005
Phosphate, mmol/L	0.01	0.02	0.01
Sodium mmol/L***	1.31	0.51	0.70
Triglyceride, mmol/L	0.01	0.03	0.01
Urate, mmol/L	0	3.9	0
Carbamide, mmol/L	0.06	0.08	0.05
Zinc, μmol/L	0.41	0.42	0.36

*The name of the batch: FHK 0306. 10 vials were selected, CK (Creatine kinase) was not performed.
**Taken from the Labquality general clinical chemistry scheme: Short-Term Survey, May 2003.
***Unstable instrument.

in the present study are presented in Table III. The components shown in Table III were found stabile at 4°C for 31–34 days (with the exception of triglyceride). A single deviating value for glucose and one value for GT were ignored because of the size of the deviation and because it did not fit within the general pattern of the results. All the components show deviating results at 37°C after 15–19 days of incubation. However, it should be borne in mind that the results of this study are based on only a few observations and statistically should be viewed with some reservation. For example, the suggested instability of carbamide after 15–19 days may be erroneous, because there were only small deviations and they were only observed in one of the two series; The uncertainty of the initial value should also be taken into account.

Stability of FHK in frozen state

The results on material CAL for selected components and methods are shown in Table IV. No visible changes were detected from September 1997 to May 2003 in the Short-Term Survey and in the reference values.

In Figure 2 an example is shown from the 10 years' stability study of X. At this point in time, the study has been going on for one year. The graph indicates that ALT is stable when stored at −80°C.

DISCUSSION

Remarks and desirable further improvements in the preparation techniques of the pool of fresh frozen serum

The "fast" freezing of the serum dispensed in the vials helps to maintain an acceptable homogeneity of the total LD activity. This is because the magnitude of rearrangement of the LD subunits forming other LD-isoenzymes was diminished.

The present preparation technique has been developed for a product that can be mailed at ambient temperature for up to 3 days. Therefore, the importance of using a short time at room temperature for preparation will have a minor impact on the quality of the final product. However, for more refined use of the material, e.g. as reference serum for the analysis of lipids, further improvement of the preparation could include collection and processing at temperatures near 0°C, to maintain lipoprotein integrity; furthermore, the material could be shipped on dry ice [16]. According to the National Committee for Clinical Laboratory Standards (NCCLS) C37-A [4] and Gary Myers (pers. comm., 2002), it is possible to draw the blood into a collection bag that contains no additives or anticoagulants, but is immersed in an ice-water container during the collection. Within 15 min of blood collection, the bags are centrifuged to separate plasma from erythrocytes.

TABLE III. Results from the stability study of thawed serum for critical components.

	Serum batch FHK 2000-07							Serum batch FHK 2000-12					
Days of incubation and temperature	Creatininium µmol/L		Gamma-glutamyl-transferase (GT) U/L	Glucose mmol/L	Triglyceride Mmol/L	Urate mmol/L	Carbamide mmol/L	Amylase U/L	Creatininium µmol/L	Gamma-glutamyl-transferase (GT) U/L	Glucose mmol/L	Urate mmol/L	Carbamide mmol/L
Initial values at day 0, Mean (*m*) and *s* of 4 determinations	*m*	178	74.4	8.80	2.68	0.523	9.95	269	185	89.8	8.55	0.545	10.25
	s	1.4	0.5	0.13	0.008	0.005	0.10	7.5	2.6	0.5	0.13	0.006	0.10
3–4 days at 4°C	179		75	8.8	2.60*	0.53	10.0	277	187	88	8.7	0.54	10.1
6–8 days at 4°C	179		75	8.7	2.64*	0.52	10.2	276	184	88	8.6	0.54	10.2
12–13 days at 4°C	180		75	8.4*	2.63*	0.53	10.1	263	184	87*	8.6	0.55	10.2
15–19 days at 4°C	180		75	8.5	2.65	0.54*	10.2	282	184	88	8.7	0.55	10.4
31–34 days at 4°C	181		74	8.6	2.69*	0.53	10.0	269	186	90	8.8	0.54	10.3
3–4 days at 22°C	181		74	8.7	2.67	0.52	10.0	289	184	90	8.6	0.53	10.1
6–8 days at 22°C	179		74	8.6	2.81*	0.51	10.1	279	186	90	8.6	0.53	10.3
12–13 days at 22°C	189*		74	8.6	2.84*	0.50*	10.2	288	182	89	8.6	0.54	10.3
15–19 days at 22°C	191*		74	8.6	2.96*	0.49*	10.1	290	187	89	8.6	0.53	10.2
31–34 days at 22°C	197*		73	8.6	3.23*	0.43*	10.0	301*	184	89	8.6	0.52*	10.4
3–4 days at 30°C	177		74	8.8	2.72*	0.52	10.1	277	184	89	8.6	0.54	10.2
6–8 days at 30°C	181		74	8.7	2.96*	0.51	10.1	294	184	88*	8.5	0.54	10.4
12–13 days at 30°C	179		74	8.6	3.15*	0.50*	10.3*	285	179	90	8.6	0.53	10.4
15–19 days at 30°C	179		74	8.6	3.49*	0.49*	10.3*	294*	185	86*	8.5	0.53	10.3
31–34 days at 30°C	172*		73	8.5	3.82*	0.47*	10.1	312*	178	85*	8.5	0.52*	10.0
3–4 days at 37°C	177		73	8.7	2.90*	0.51	10.0	285	177	89	8.4	0.54	10.2
6–8 days at 37°C	175		73	8.7	3.19*	0.51	10.2	291	175	88*	8.3	0.52*	10.3
12–13 days at 37°C	174		73	8.5	3.43*	0.49*	10.2	315*	162*	87*	8.1*	0.52*	10.4
15–19 days at 37°C	164*		72*	8.6	3.75*	0.48*	10.4*	330*	159*	85*	8.0*	0.50*	10.2
31–34 days at 37°C	150*		69*	8.2*	3.71*	0.45*	10.3*	342*	143*	79*	7.8*	0.48*	10.5

*Deviation from the initial value by more than 3 *s* (for glucose, gamma-glutamyltransferase and carbamide the obtained *s* in the stability study for batch 2000-12 was also used for marking with an asterix on results from the stability study of batch 2000-7; this was because the initial *s* values were unrealistically low).

TABLE IV. Results on reference serum "CAL" for selected components and methods.

	Values assigned to CAL					Nordic External Quality Assurance Scheme								
	Reference measurement procedure value DGKC 1997		IMEP-17 Material 1 transferred values 2002 (14)			1997			2002			2003		
Component	Value	U, $k=2$	Value	U, $k=2$	Method	Mean	s	N	Mean	s	N	Mean	s	N
ALT U/L	17.8[8]				Vitros 250-950	24	4	59	21	3	11	22.7	3	52
Albumin g/L	40.8[1]		40.529	2.64	Bromcresol green	43.3	2.7	161	41.7	1.1	18	42.4	1.7	97
					Vitros 250-950	40.2	1.2	61	39.3	1	14	39.7	1.1	51
Bilirubin µmol/L	8.5[2]				Photometry	8.0	1.6	362	8.7	1.1	39	8.4	1.5	235
					Vitros 250-950	9.1	1.6	63	6.6	1.7	14	5.9	1.4	48
Calcium mmol/L	2.267[3]		2.2660	0.0080	AAS and fp	2.29	0.05	16	2.31		1	2.28	0.04	3
					Vitros 250-950	2.27	0.05	60	2.22	0.06	12	2.2	0.06	49
Chloride mmol/L	100.6[4]				ISE indirect	102.5	2.7	60	101.5	2.6	6	101	1.8	62
					ISE direct	100.7	2.3	94	98.8	0.8	6	100.3	1.9	46
Cholesterol mmol/L	4.94[5]		4.901	0.023	Vitros 250-950	5.04	0.15	59	5.06	0.22	11	4.94	0.12	41
CK U/L	118.8[8]				Vitros 250-950	100	11	58	114	6	10	92	9	46
Creatininium µmol/L	69.6[5]	1.05	70.57	0.58	Vitros 250-950	81.0	2.0	64	78.0	3.3	15	83.1	4.4	53
GT U/L	35.9[8]		35.83	0.96	Vitros 250-950	37	3	51	38	1	9	36.4	1	42
Glucose mmol/L	4.49[5]		4.464	0.035	Vitros 250-950	4.62	0.13	47	4.71	0.15	9	4.67	0.18	47
Magnesium mmol/L	0.807[3]		0.797	0.0064	AAS	0.81	0.04	16	0.79	0.03	4	0.81	0.05	4
					Vitros 250-950	0.77	0.03	47	0.78	0.03	10	0.8	0.03	40

TABLE IV. (Continued).

	Values assigned to CAL					Nordic External Quality Assurance Scheme								
	Reference measurement procedure value DGKC 1997		IMEP-17 Material 1 transferred values 2002 (14)			1997			2002			2003		
Component	Value	U, $k=2$	Value	U, $k=2$	Method	Mean	s	N	Mean	s	N	Mean	s	N
Phosphate mmol/L	1.03				Vitros 250-950	1.06	0.03	57	1.04	0.02	9	1.04	0.04	42
Potassium mmol/L	3.70[6]		3.738	0.022	ISE indirect	3.76	0.08	121	3.72	0.06	32	3.75	0.07	142
					ISE direct	3.77	0.10	320	3.73	0.06	6	3.77	0.09	237
					Vitros 250-950	3.75	0.06	66	3.79	0.07	15	3.78	0.08	53
Protein g/L	67.1[7]				Photometry	67.8	2.7	305	68.0	2.0	28	67.8	2.1	142
					Reflectometry	66.3	1.2	3	70.0		1	66		1
					Vitros 250-950	67.7	1.8	52	68.0	2	5	68.2	1.8	33
Sodium mmol/L	137.6[6]		137.36	0.73	ISE indirect	137.0	2.1	119	136.6	1.5	32	137	1.8	142
					ISE direct	136.8	1.7	324	137.1	1.0	6	136.9	1.7	237
					Vitros 250-950	138.2	1.7	66	137.7	2	15	137.3	1.4	53
Triglyceride mmol/L	1.31[5]				Vitros 250-950	1.44	0.04	56	1.38	0.04	10	1.41	0.05	37
Urate umo/L	285[5]	4.7	290.2	5.4	Vitros 250-950	290	8	60	277	7	14	280	6	46

Reference methods (DGKC): [1]RID, [2]Jendrassic (Basit *et al.*), [3]AAS. [4]Coulometry, [5]GC/IDMS, [6]Flame photometry, [7]Biuret (Dumas *et al.*), [8]IFCC 37°C.

Abbreviations: DGKC = German Society for Clinical Chemistry, fp = flame photometry, U = expanded uncertainty, k = coverage factor to obtain a 95% confidence interval, s = standard deviation, N = number of results, Mean = method mean; ALT = alanine aminotransferase; CK = creatine kinase; GT = gamma-glutamyltransferase.

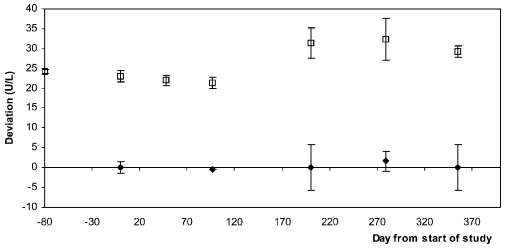

FIG. 2. Data on alanine aminotransferase (ALT) from the 10-year stability study on the Scandinavian Society of Clinical Chemistry (NFKK) reference serum X. (♦) Stability data obtained at −80°C with 95% confidence limits. (□) Difference between stability data obtained at −150°C and −80°C with 95% confidence limits.

After this step the plasma is transferred to a plastic centrifuge bottle for clotting. After 3–4 h the serum is separated from the clot. The typical yield is about 200 mL serum per donor. According to our experiences, this procedure requires fairly intense centrifugation (1000–1500 × *g* for approximately 30 min), because the erythrocytes trapped in the clot cannot support the separation. An attempt has be made to let the blood clot spontaneously at +4°C, but this took too long and potassium passed from the blood cells to the serum, leading to high potassium values in the serum. It is the intention to adapt the present procedure more closely to the NCCLS technique, as appropriate.

Techniques to achieve sets of "multilevel controls"

Usually only one concentration level of FHK was used. However, this covers only the natural concentration level of components from preparation of the serum pool from healthy donors. For practical use it is desirable to have more sets of reference sera at different concentration levels, of which the unmodified type is the most important because of its commutable quality. The most desirable alternative control material would be serum obtained from different groups of donors or patients, which would lead to different concentrations of analytes; FHK0306 (Annex A, serum from female donors) and the control serum P in NORIP (serum from oral anticontraceptive users) are examples of this type. Other reference sera in such a set will typically be modified. In this situation commutability aspects need to be taken into account.

More problematic were the sets of reference sera consisting of three levels (or more) based on more drastic concentrating and diluting of FHK. Such sets are found in both the early surveys with FHK materials [5] and the NORIP [6]. However, generally speaking, those problems were regarded as small compared to the commutability problems which may occur when using lyophilized sera.

Thawing and mixing

It is essential that the control serum in the original vial is fully thawed and thoroughly mixed before any subsample is removed from the vial. Otherwise, invalid results will arise both from the removed subsamples and from any later subsamples from the particular vial. Thawing by incubation of the vials at ambient temperature is slow. For vials with 9.5 mL serum (IMEP-17), visual inspection for ice crystals in the serum showed that it was fully thawed after 1.5 h of incubation. The Instructions for use presented in the certificate for X [16] specify more demanding procedures than presently known to be necessary; however, for practical reasons it seems wise to reduce any possible risks of handling errors; note that vials with 5 mL FHK are less prone to thawing and mixing errors than those with 9.5 mL. A recommendation for the future might be to thaw the samples in a water bath at 37°C.

Check of homogeneity

The present type of homogeneity check is performed according to ISO 13528 [11]. When an exact determination of the homogeneity of a given component is needed, more replicates should be performed on each vial [17]. Such an approach implies other problems, e.g. increased costs of reagents and samples.

The instability of a routine instrument can cause an incorrect increase of the standard deviation, as was the case with sodium in the example in Table II. This can lead to a false rejection of the material. When looking at the calculated standard deviations one should bear this in mind.

Stability of thawed serum

The stability data shown in Table III originate from two slightly modified sera. It was assumed that any instability of the investigated components in the unmodified sera also would be reflected by the present results from modified serum.

In native serum (unmodified) many other components are known to be unstable, e.g. phosphate and several enzymes. But these components have not been included in the present study, because it was performed in relation to the IMEP-17 study, where the number of components was restricted.

Stability of frozen serum

Judging from the results on CAL in the EQA scheme from Labquality and comparison of the reference measurement procedure values assigned to CAL, the FHK material is considered to be stable for at least 5 years for the measured components. Further distribution of CAL is planned in 2004 in the EQA scheme. In Figure 2, measurements on ALT illustrate the principle of the 10 years' stability study. The first measurement values at −80°C for ALT showed a drop in concentration, but then a marked increase in level was observed. These observed differences were caused by the variability of the analytical instrument over time. When looking at the difference between stability data obtained at −150°C and −80°C, we conclude that ALT is stable when stored at −80°C.

From documented stability studies lasting 2–7 years, and knowledge from similar materials [18–20] and practical experience (A. Uldall, pers. comm., 2003), the expected stability of materials stored at −80°C is at least 10 years (see Table IV [21–24]).

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ANNEX A

Production lots of fresh frozen human sera.

Time of production and a systematic designation of the batch	Origin of blood	Preparation, filtration and filling into vials	Examples of uses	Remarks
1985 FHK85 a, b, c	Aarhus Municipality Hospital, Blood Bank	Aarhus Municipality Hospital, Blood Bank [5]	EQA in Denmark with four rounds per year. Also used as reference serum until the stock expired in 2002	In addition to the unmodified batch serum, "concentrated & spiked" and "diluted" batches of serum were also prepared. Values assigned by the so-called "high performance methods" [22] or by reference measurement procedures for selected components
1988 FHK88 a, b, c	Ibid.	Ibid.	Ibid.	Ibid. A further two subbatches were prepared for interference studies: lipidaemia (Travemulsion addition); icterus (bilirubin addition)
1991 FHK91 a, b, c	Hjoerring County Hospital, Blood Bank	SERO A/S, Billingstad, Norway	Ibid. This preparation (unmodified batch) also became the IMEP-3 material [8]	Ibid. as the 1985 preparation. For six further subbatches allowing interference studies were also prepared: Bilirubin, salicylate, paracetamol, ibuprofen, high glucose, and turbidimetry(turbidity removed by ultracentrifugation at the Dept. of Clinical Biochemistry, Odense University Hospital)
1994 – Mar FHK9403	Hjørring County Hospital, Blood Bank	Statens Serum Institute (SSI) Department of plasma fractionation. The preparation technique described in this paper applies generally	Labquality: General clinical chemistry monthly survey ("Short-Term Survey") April 1994	1994 – 1997: The number of vials in each batch of production increased from about 1500 vials in 1994 to about 4000 – 4100 in 1997. Later batches consisted of about 4000 – 4100 vials (only for 5 mL vials)
1994 – Dec FHK9412	Ibid.	Ibid.	Ibid. survey, round: December 1994	
1995 – Mar FHK9503			Ibid. survey, round: May, October 1995	
1996 – Apr FHK9604	Ibid.	Ibid.	Ibid. survey, round: May, October 1996. This batch was used as reference serum for cholesterol, Ca and Mg until the stock was used up (2002) This batch was also used as the IMEP-7 material [23]	Reference measurement procedure values from DGKC on cholesterol, Ca, Mg were assigned

1997 – Apr FHK9704	Ibid.	Ibid.	Ibid. Survey, round: September 1997, April 2002 (only Danish and Finish laboratories participating in the Nordic Reference Interval Project, NORIP), May 2003. This batch was used as calibrator (CAL) in the NORIP	Reference measurement procedure values from DGKC were assigned for 17 components, see [13]
1998 – May FHK9805			This batch was reserved for the NORIP. It is now designated NFKK reference serum X and is to be used as reference serum in Nordic laboratories	The values were transferred from CAL and IMEP-17 material through the NORIP and the Nordic Trueness Study [18]
1999 – June FHK9906	Ibid.	Ibid.	Labquality survey as described above, round: September 1999, January and September 2000	
2000 – Apr FHK0004	Ibid.	Ibid., but prepared as the above unmodified serum, dispensed with 9.5 mL in each vial	IMEP-17 material 1 Used in 2000 – 2002 in the IMEP-17 study	[8]
2000 – July FHK0007	Ibid.	Ibid., but prepared similarly to IMEP-17 material 2. See Table I. 9.5 mL per vial	Used in various European EQA in 2003	
2000 – Dec FHK0012	Ibid.	Ibid., but prepared as described in Table I. 9.5 mL per vial	IMEP-17 material 2. Used in 2000 – 2002 in the IMEP-17 study	[8]
2001 – Aug FHK0108	Ibid.	Ibid., prepared as above for unmodified serum, 5 mL per vial; however pH of the serum was 7.9, [24]	Used in various European EQA in 2003. Key comparison for calcium of CCQM	A value for Ca was assigned by CCQM [25]
2001 – Sept FHK0109	Ibid.	Ibid, as above, pH about 7.5	Labquality survey, round September 2001	
2001 – Dec FHK0112	Ibid.	Ibid.	Stock; intended for Labquality surveys	
2002 – Apr FHK0204	Ibid.	Ibid.	December 2002, Labquality surveys	
2003 – Jan FHK0301			Stock; intended for Labquality surveys	
2003 – Apr FHK0304			Stock, intended for Labquality surveys	
2003 – June FHK0306			Labquality hormone surveys	Pool of sera from female blood donors. Reference measurement procedure values may be assigned