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An updated protocol based on CLSI document C37 for preparation of off-the-clot serum from individual units for use alone or to prepare commutable pooled serum reference materials

https://doi.org/10.1515/cclm-2019-0732
Received July 17, 2019; accepted September 20, 2019

Abstract: Manufacturers of *in vitro* diagnostic medical devices, clinical laboratories, research laboratories and calibration laboratories require commutable reference materials that can be used in the calibration hierarchies of medical laboratory measurement procedures used for human specimens to establish metrological traceability to higher order reference systems. Commutable materials are also useful in external quality assessment surveys. In order to achieve these goals, matrix-based reference materials with long-term stability, appropriate measurand concentrations and commutability with individual human specimens are required. The Clinical and Laboratory Standards Institute (CLSI) guideline C37-A (now archived) provided guidance to prepare commutable pooled serum reference materials for use in the calibration hierarchies of cholesterol measurement procedures. Experience using the C37-A guideline has identified a number of technical enhancements as well as applications to measurands other than cholesterol. This experience is incorporated into this updated protocol to ensure the procedure will continue to meet the needs of the medical laboratory. The updated protocol describes a procedure for preparing frozen human serum units or pools with minimal matrix alterations that are likely to be commutable with individual human serum samples. The protocol provides step-by-step guidance for the planning phase, collection of individual serum units, processing the units, qualifying the units for use in a pool and frozen storage of aliquots of pooled sera to manufacture frozen serum pools. Guidance on how to perform quality control of the final product and suggestions on documentation are also provided.

Keywords: commutable reference material; off-the-clot serum; pooled serum.

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Introduction

Commutable matrix-based reference materials are required for metrological traceability of results from *in vitro* diagnostic (IVD) medical devices used in clinical laboratories to higher order references as described in ISO 17511 [1]. Such materials are also important for use as trueness controls or for external quality assessment (proficiency testing) intended to assess the agreement of results among different IVD medical devices.

The College of American Pathologists published a procedure in 1998 to prepare off-the-clot serum pools from units of blood to assess accuracy of measurement procedures participating in a proficiency testing program [2]. Based largely on the College of American Pathologists work, the CLSI C37-A guideline was published in 1999 for preparation of commutable serum pools for use in the calibration hierarchies of cholesterol measurement procedures [3]. Subsequently, the C37-A protocol for blood collection and serum preparation has been applied to prepare commutable frozen human serum reference materials for other analytes such as electrolytes, small organic molecules, hormones, enzymes, proteins and other clinical measurands. The guideline has also been used to prepare unaltered single donor serum aliquots. A detailed summary of reference materials, measurement procedure performance studies, standardization/harmonization and external quality assessment programs that successfully implemented materials prepared using the C37-A protocol can be found in Supplementary Tables 1–3 in the on-line Supplementary Data that accompanies this article.

Even though CLSI made a decision in 2018 not to revise C37-A despite the fact that it has been used for many

measurands other than cholesterol, users have developed practical improvements to the protocol to increase its efficiency and utility for various measurands, and to address safety considerations with originally used glass centrifuge bottles. CLSI has placed C37-A in an archived status that means it is available as a reference text but is no longer in the consensus review process and no longer a consensus guideline. CLSI advised those who submitted the proposal for revision "to seek other opportunities to publish the information within the global medical laboratory community". This report incorporates the fundamental principles of the original College of American Pathologists and C37-A protocols with appropriate updates based on knowledge acquired by users of the protocols over the past 20 years.

The specifications and protocols described in this report enable laboratory scientists, IVD manufacturers, external quality assessment providers, and suppliers of clinical laboratory reference materials to prepare frozen human serum pools that have minimal matrix alterations and are likely to be commutable with individual clinical samples across different measurement procedures. However, it is the responsibility of a user to verify that the prepared material is suitable for its intended purpose (e.g. as a certified reference material [CRM], trueness control or external quality assurance material) and to validate its commutability. The protocol can also be used to prepare an individual blood donor unit of off-the-clot serum. Recommendations for characterization and qualification of commutable reference materials, as well as assessment of commutability of a serum pool are not provided here but are described in other publications [4–6].

General considerations during the planning phase

Several factors could significantly impact the quality of the final product. Issues such as appropriate choice of containers throughout the process, duration of the protocol steps and type of donor units should be considered during the preparatory phase. The procedure is suitable for any analyte in serum that is stable over the time required to complete the protocol and during a single freeze-thaw cycle. It is imperative that the completion of blood collection, processing, serum pooling, dispensing and freezing are done as quickly as possible to assure the quality of the final product. Sterile containers should be used where practical in blood processing steps. In addition, aseptic handling wherever possible will minimize exposure to potential microbial contamination. Relatively short times at ambient temperature minimizes bacterial growth in the event of contamination. Metabolites can be produced by bacteria if present in the serum units during pool preparation [7], even though the final filtration will remove bacteria.

Ammonia increases as a function of time and temperature during the pool preparation because it is produced by the action of normal serum enzymes on various amino acid substrates present in the serum. These enzymatic reactions are unavoidable during the preparation and mixing of the serum pool. For this reason, the pool should be kept cold during preparation. One of the acceptance criteria for the final pool is ammonia concentration measured primarily as an indicator of time spent in the liquid state, and secondarily as an indicator of potential bacterial contamination during pool preparation [7]. The glucose acceptance criterion is also intended to detect possible contamination with bacteria during pool preparation because glucose is consumed by microorganisms causing a lowered value.

A major assumption made when preparing serum pools by combining donor units is that the behavior of the pooled sera is not adversely affected by any sample-specific influences in one or a few of the donor units. Therefore, caution should be used to eliminate donor units with known interference with measurement of the intended measurand(s) when possible. However, if a donor unit with known interference needs to be used, then the impact of the interference that might occur from a donor can be estimated by assuming that bias from an interference is proportional to the donor's serum volume fraction in the pool. If this assumption is correct, the bias introduced into the pool is the bias from the single-donor divided by the total number of donors ($Bias_{pool} = Bias_{donor}/N$). For example, if a single-donor sample had a large cholesterol bias of 30% due to interferences, a 1% bias is expected in a pool comprised of 30 donors (assuming equal volumes of single donor samples were used). If a potential bias from an interfering substance is suspected, then appropriate assessments should be performed, such as quantitation of the interfering substance as part of the qualification of units as suitable for use. If there is concern for possible presence of interfering substances in one or a few donors, using more single donors to a pool will minimize the influence of an interfering substance from any one donor within a tolerance threshold.

The material of collection or storage vessels may have an impact on the quality of the final product. Collection into plastic blood bags is an industry standard practice. However, plastic blood collection bags are suspected of contaminating serum with phthalate esters and other plasticizers. The extent of contamination depends on

concentration of the contaminant in the bag, the temperature used during collection and processing and the length of contact time with the bag. It is possible that serum left in contact with plastic blood collection bags for extended periods or at elevated temperature could alter some matrix components and potentially adversely affect the biochemical stability of the pool. In this protocol, collection with the blood bag in ice water prevents clotting and allows rapid separation of plasma from cells. Rapid separation of plasma from blood cells prior to clotting minimizes exposure to cells and plastic, thus minimizing biochemical processes and contamination from blood cellular and plastic-derived molecules. Centrifugation conditions for the cold blood bags were chosen to produce plasma with adequate platelets for clotting after aseptic transfer into centrifuge bottles.

The original C37-A protocol used glass bottles for clotting and centrifugation to recover serum. However, there is a risk that glass bottles may break during the centrifugation step. Safety considerations and practical experience support use of plastic centrifuge bottles for this step. Glass bottles are acceptable when leaching from plastic might be a source of interfering substances for a measurand. When glass bottles are used, precautions for cushioning the glass bottles in the centrifugation step should be taken to minimize the risk for breakage.

Evacuated bottles should not be used to receive the plasma because flow through the tubing causes frothing of the plasma which can introduce an undesirable random variable in the process. Polypropylene containers with inert caps and stoppers should be used for processing serum and for storing vials at –70 °C or colder. Polypropylene is a good choice to minimize contamination from surface interactions and chemical leaching. However, the choice of plastic composition of the bottles should be appropriate for the measurand. When necessary, other alternative containers can be used.

Membranes for filtration of the serum pool should be selected to minimize adsorption of proteins or other serum constituents particularly the measurand. The membranes should be tested for suitability by measuring critical analytes before and after a trial filtration. Polyvinylidene difluoride membranes typically have minimal adsorption from serum. Membranes should be washed appropriately to avoid contamination with preservative materials. An initial small volume of serum filtrate can be discarded if necessary to minimize dilution from membrane washing. Typically, individual serum units are not filtered prior to pooling in order to match as closely as possible the matrix usually used for testing in a clinical laboratory and to avoid increased risk of bacterial contamination. Not filtering individual units also minimizes the time between collection of blood and freezing of the pooled sera.

It is desirable to avoid shipping serum units from a collection center to a pool preparation center. Shipping has a potential for degradation of the serum and thus can introduce a random variable into the quality of the final product. If shipping cannot be avoided, the serum containers must be fully filled with no air space to minimize the opportunity for mechanical agitation and denaturation of protein and other constituents. Shipping conditions must be adequate to maintain temperature of all material at 2 °C–8 °C. Ideally, all components of a pooled material should be treated in the same manner. For example, the pool and its aliquots should be kept at the same temperature during the aliquoting step.

Freezing of the prepared aliquots of pooled sera is critical. Freezing conditions should be uniform for each vial. An optimal temperature range of –70 °C to –80 °C should be achieved for storage of the frozen pooled sera, or individual serum, aliquots. This temperature is necessary for complete ice formation and immobilization of water molecules in the presence of the serum matrix constituents. Complete ice formation limits molecular diffusion and interactions which can result in degradation of proteins and other molecules over time. Once the entire pool is aliquoted, freezing should be performed as uniformly as possible. Following initial freezing, vials must be handled for transfer or shipping in such a way that they do not experience greater than 1 h cumulative temperature between –70 °C and –30 °C. Any vial in which the contents become partially or fully liquefied must be immediately used or discarded. Material should not be refrozen.

Donor unit collection considerations

Donor selection

Donors should be selected according to well defined criteria and local governmental regulations [8, 9]. Institutional Review Board requirements should be followed and adequate safeguards for donor privacy must be ensured. Donors can be prescreened if necessary and organized according to desired analyte concentration levels and number of pools required. If fasting serum is specified, a minimum 8 h fast is recommended prior to blood collection. It is important to note, that if non-fasting blood is accepted, the serum may contain chylomicron levels that can cause rejection of units based on the criterion for turbidity in the final pooled serum (see considerations for a normal pool that represents

apparently healthy donors described in the section detailing acceptance criteria for the final serum pool).

General inclusion criteria for donor units

Donor units are included if they meet the pre-established acceptance criteria for the intended use. Typical considerations for acceptance criteria include negative testing for regulated infectious agents (e.g. human immunodeficiency virus, hepatitis B virus, hepatitis C virus and others) specified by the applicable regulatory body such as the World Health Organization, the US Food and Drug Administration or the Centers for Disease Control and Prevention [8]. Hemoglobin concentration should be less than 0.3 g/L (30 mg/dL). The donor serum unit should be visually clear, straw- or yellow-colored and free from particulate matter.

Additional acceptance criteria can be added depending on the intended use. For example, the units may be selected to meet desired concentration values for single donor or pooled serum; or additional specifications for potential interfering substances (such as bilirubin <25 μmol/L [1.5 mg/dL] or triglycerides <1.7 mmol/L [150 mg/dL]) can be stated. However, the final decision on the donor unit selection should be based on the intended use of the prepared material. For example, concentrations of triglycerides can be specified as required if the study intends to evaluate the influence of hypertriglyceridemia on measurement procedures for another analyte or to investigate measurement procedure performance for high concentrations of triglycerides [10]. The minimum information to be recorded for each individual donor unit is presented in Table 1.

Acceptance criteria for the final serum pool

Acceptance criteria for the final serum pool must also be specified and depends on the analyte and intended use of the serum pool. If not otherwise requested by the user of the material, the recommended target value for ammonia is <100 μmol/L (170 μg/dL) with an upper limit of <150 μmol/L (255 μg/dL) as an indicator of excessive time above 2–8 °C or excessive time before freezing. Microbial contaminates should be ruled out by standard blood agar and chocolate agar cultures that must yield growth of less than 10 CFUs per mL of pooled serum. Pool homogeneity evaluation to assess uniformity of all aliquoted vials should be performed per procedures described in the Validation of the serum pool section of this article. Commutability as described in the same section should be validated when applicable for the intended use of the pool.

As for individual donors to a pool, additional acceptance criteria can be added depending on the intended use. Typical additional considerations for a normal pool that represents apparently healthy donors can include albumin/globulin ratio >1.0 and glucose concentration >3.3 mmol/L (60 mg/dL). “Spiking” to achieve glucose levels is specifically prohibited. Absorbance at 710 nm should be less than 0.5 in a 1-cm cuvette measured against a water blank. Total protein concentration in the 58 g/L–80 g/L (5.8 g/dL–8.0 g/dL) range, bilirubin <25 μmol/L (1.5 mg/dL) and triglycerides <1.7 mmol/L (150 mg/dL) are recommended for normal serum pool that represents apparently healthy donors. Certain parameters, such as pH, that may provide additional information about the prepared pool can be recorded and kept on file. The information that should be recorded for the final pooled serum is listed in Table 2.

Table 1: The minimum information to be recorded for the individual donor units.

1. Unit ID
2. Date/time of collection
3. Name of phlebotomist
4. Relevant test results to qualify the unit as suitable for use
5. Additional characteristics specific for the unit based on the intended use

Table 2: The minimum information to be recorded for the final pooled serum.

1. Pool ID
2. Operator
3. List of units associated with the pool including the volume of each unit used to prepare the pool
4. Date/time each individual blood collection began
5. Date/time each individual blood collection ended
6. Date/time each individual serum unit was expressed from the red cells
7. Date/time each individual serum unit was centrifuged after clotting
8. Date/time pooling started
9. Date/time pooling completed (i.e. end of mixing prior to dispensing)
10. Date/time filtration and dispensing started
11. Date/time filtration and dispensing ended
12. Date/time of pool freezing
13. Results, including the measurement procedures and instrumentation used, for acceptance testing and the acceptance criteria
14. Results for additional characteristics specific for the intended use of the pool
15. Final acceptance decision

Blood collection and processing procedure

Blood collection, processing to obtain serum, pooling serum, aliquoting serum, capping, freezing, and storage of the serum pool should be completed as quickly as possible to ensure the quality of the final product. For example, a long processing time can cause degradation of analyte and accumulation of ammonia. Figure 1 presents an overview of the step-by-step procedure described below to produce a serum pool likely to be commutable with individual sera.

(a) Individual donor units of whole blood are collected using an industry-standard empty blood-pack with integral donor tube, a 15-gauge needle, and a 600-mL plastic container. The blood bag must be immersed in an ice-water slurry to ensure the correct temperature (0 °C–4 °C) during the procedure. Ice is added as needed. If the collected blood starts to clot during this step, this unit cannot be used for this protocol.

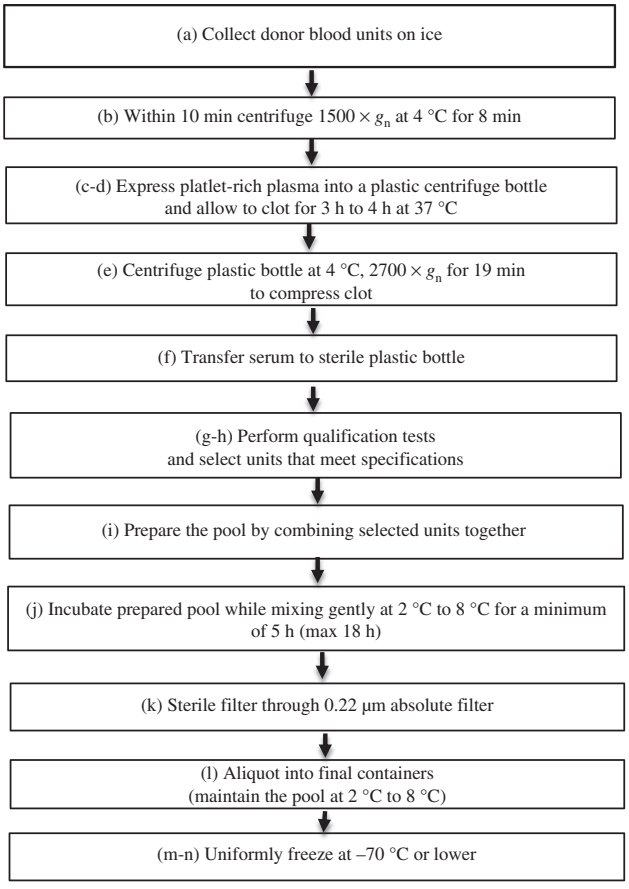

Figure 1: Flowchart for preparation of the donor units and serum pool.

(b) Within 10 min of unit collection, before the unit clots, plasma is separated from cells by centrifugation at $1500 \times g_n$ for 8 min at 4 °C. The unit should be continuously kept in an ice-water slurry prior to centrifugation. This centrifugation condition is intended to produce platelet-rich plasma that will successfully clot when other cellular components are removed.

(c) The platelet-rich plasma is aseptically expressed into a clean and aseptic 250-mL plastic centrifuge bottle with a plastic screw cap. Centrifuge bottles should be cleaned and rinsed with purified water and then with 70% ethanol (volume percent), and air dried in a bacteria free laminar flow hood. Vacuum bottles should be avoided. Plasma flow into the centrifuge bottle should be controlled to avoid spattering and frothing.

(d) The platelet-rich plasma is allowed to clot at 37 °C for 3–4 h. If the unit has not visually clotted in 3 h, 10 mL of serum from a well-clotted unit can be added, the unit mixed and incubated for an additional 1 h.

(e) Each bottle is centrifuged at $2700 \times g_n$ for at least 19 min at 4 °C.

(f) The clear serum layer is aseptically transferred to a new 250-mL container with appropriate inert cap. New plastic bottles are manufactured using a heat forming process and thus likely to be aseptic. In order to avoid contaminants and bacterial contamination, plastic storage bottles should not be washed and reused.

(g) Serum units are excluded based on specifications for the final pooled material and acceptance tests specified for the material. Units failing any of these tests should be rejected from the pool.

(h) All units are stored at 2 °C–8 °C and should not be frozen. If transportation is required at this stage, agitation of the liquid should be avoided by filling the containers as full as possible.

(i) The serum pool is prepared by combining the serum from the qualified donor units.

(j) The pool is incubated at 2 °C–8 °C for a minimum of 5 h (maximum 18 h) with constant, low-speed mixing in a borosilicate glass, or other suitable container for the intended use of the pool. Using a stainless steel container with magnetic stirring bar may produce metal filings that can contaminate the final pool. This step assures homogeneity of the final pool and allows any potential interactions and aggregate formation to occur between molecules from individual units. Possible aggregates can then be filtered out at the next step. The time can be reduced if needed based on the stability characteristics of a given analyte.

(k) The pool is sterile filtered through a 0.22 µm absolute filter (hydrophilic membrane) and filtrate is collected into an appropriate sized sterile container prior to final dispensing of the pool. During this step, the pool is stored at 2 °C–8 °C (e.g. by placing in an ice-water slurry). When possible, the filter can be rinsed with sterile fluid, preferably deionized water, to remove any preservative and the rinse liquid is discarded. A small amount of pool can be used to flush the filter to avoid dilution of the serum before filtering the bulk pool if the dilution will impact the final pool. Loss of some analytes may occur during the filtration process. Testing of the filtration process is recommended to ensure the conditions are suitable.

(l) Aseptically transfer the desired volume of pooled serum to the final containers. The pool should be maintained at 2 °C–8 °C with constant stirring throughout the dispensing process to assure homogeneity of the product. Aliquot vials should be pre-labeled prior to or during dispensing and before freezing and kept at 2 °C–8 °C prior to freezing.

(m) The aliquots are uniformly frozen so that a constant freezing rate is achieved for each vial. Styrofoam or similar insulated racks or boxes which may cause freezing gradients from the outside to the inside of the container must not be used. All vials must be frozen in an upright position. For example, one of the approaches to achieve uniform freezing is to intercalate paper boxed samples with dry ice to ensure that all samples are surrounded at all times with a uniform –70 °C to –80 °C environment. Once one layer of paper boxes with pool vials have been put in the freezer, they are overlaid with dry ice blocks. The second layer of paper boxes with pool vials are then placed on top of the dry ice layer. The process is repeated until all of the boxes of pooled vials are in the freezer in a similar freezing environment. Another approach to uniform freezing is to place all paper boxes with vials in direct contact with a metal shelf in a freezer. The vial boxes should not be stacked on one another because non-uniform heat transfer will occur.

(n) The boxes of pool vials should be stored at –70 °C or lower.

Although out of scope for the original C37 protocol, literature reports starting with the C37 protocol or very similar protocols have shown that supplementing a serum pool with a number of analytes has produced pools with elevated concentrations with acceptable commutability properties [11, 12].

Validation of the serum pool

Prepared serum pools should be evaluated for homogeneity and commutability based on the intended use. Homogeneity assessment assures that the pools demonstrate uniformity across the production batch or lot. The CLSI EP30 guideline [13] or the ISO Guide 35:2017 [14] should be consulted for a procedure to validate homogeneity.

The protocol for preparing the serum pool is intended to have a high likelihood to produce a pooled serum that is commutable with individual serum samples. However, there are manipulations in the protocol, pooling can alter interactions between matrix components in different donor units, and freezing can affect the matrix or the measurand. Commutability of the serum pool should be validated using an appropriate assessment procedure when needed for the intended use of the pool. Commutability assessment procedures are available from the IFCC [4–6]. When aliquots of the individual donors to a pool are saved, commutability can be assessed as described in Supplementary Data S4 that accompanies this report. It is important to note that analyte binding to filtration media or the presence of a binding protein for an analyte can be a limitation to this approach for commutability assessment.

Conclusions

This report provides updated information to enable the original CLSI C37-A guideline (now archived by CLSI and no longer a consensus guideline) to continue to be used by the clinical laboratory community to prepare pooled off-the-clot serum that is likely to be commutable with individual clinical samples.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
Conflict of interest: The authors declare there is no conflict of interest.
Disclaimer: The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and

Prevention/the Agency for Toxic Substances and Disease Registry. Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service and the US Department of Health and Human Services. Certain commercial equipment or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/cclm-2019-0732).