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Article

Development and Analytical Validation of a Multiplex LC-MRM-MS–Based Reference Measurement Procedure for Apolipoprotein A-I and B_{total} Quantification in Serum

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BACKGROUND: Medical results from regulated in vitro diagnostic devices should be accurate. The previous reference measurement procedures (RMP) for apolipoprotein A-I (ApoA-I)/apolipoprotein B total ($ApoB_{total}$) standardization are no longer functional. A new RMP aligned with ISO15193:2009 and calibration models of ISO17511:2020 was developed using multiplex liquid chromatography–multiple reaction monitoring–mass spectrometry (LC-MRM-MS) for quantifying ApoA-I and $ApoB_{total}$ in serum.

METHODS: Serum samples underwent reduction, alkylation, lysyl endopeptidase/trypsin digestion, and proteotypic peptide purification. Optimal retention times, precursor/fragment ion *m/z* were selected using an Agilent-1290 ultra-high-performance liquid chromatography coupled to a 6495C triple-quadrupole mass spectrometer. Calibrators were indirectly traceable to WHO/IFCC SP1-01/SP3-08 (ApoA-I/$ApoB_{total}$) via the RMP, with propagated measurement uncertainty (MU), supported by replicate SP1-01/SP3-08 measurements across independent batches. Validation followed ISO15193:2009 and included linearity, recovery, uncertainty, precision, stability, preanalytical conditions, carryover, limit of blank (LoB), limit of detection (LoD), limit of quantification (LoQ), and comparability.

RESULTS: Linearity was confirmed from 0.10 to 2.97 g/L and 0.27 to 3.83 g/L (ApoA-I/$ApoB_{total}$), with <10% deviation, supporting broad analytical suitability. Duplicate MU was 2.3% for both analytes. Recovery ranged from 99.2% to 100.3%. Matrix effects were negligible except in citrate plasma (−14.1% to −15.7%). Carryover was <0.003 g/L (ApoA-I) and <0.004 g/L ($ApoB_{total}$). LoB, LoD, and LoQ were <0.001/0.002 g/L, 0.0013/0.0019 g/L, and 0.01/0.02 g/L for ApoA-I/$ApoB_{total}$, respectively. Compared with Roche immunoturbidimetric assays, data equivalence was demonstrated within allowable MU.

CONCLUSIONS: This LC-MRM-MS–based RMP for ApoA-I and $ApoB_{total}$ provides a next-generation RMP succeeding the former ELISA-based RMP. Certification of commercially available ApoA-I/$ApoB_{total}$ tests will enable continued global standardization of ApoA-I/$ApoB_{total}$ measurements, while preserving traceability of test results to the former WHO-IFCC reference materials within maximum allowable uncertainty.

Introduction

To ensure that medical tests are reliable, accurate, and comparable regardless of the time, location, or technique used, it is essential to establish robust metrological traceability chains for standardization. Medical test results should be traceable to a higher-order reference measurement system (RMS), which comprises reference materials (RMs) and a reference measurement procedure (RMP) (1, 2). RMPs are nowadays typically mass spectrometry (MS)-based, as this technology enables molecular detection of the measurand—for example, through direct measurement of proteins via enzymatic digestion and quantification of proteotypic peptides—while also allowing multiplexing, resulting in accurate and efficient quantification (3) of multiple (apolipo)-protein measurands. The RMP design is new for apolipoprotein A-I (ApoA-I) and apolipoprotein B total ($ApoB_{total}$), which are traditionally standardized using antibody-based immunoassays. Note that $ApoB_{total}$ in the RMP comprises

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both apolipoprotein B100 ($ApoB_{100}$) and apolipoprotein B48 ($ApoB_{48}$).

Current cardiovascular risk prediction predominantly focuses on classical lipid parameters—cholesterol, triglycerides, HDL cholesterol (HDL-c), and LDL cholesterol (LDL-c)—with treatment emphasis on LDL-c (4). Miller et al. (5) and, recently, Meeusen et al. (6) evaluated direct assays for HDL-c and/or LDL-c quantification and demonstrated acceptable analytical performance of LDL-c and HDL-c tests in normolipidemic samples, but inadequate recovery and shortcomings in dyslipidemic samples. In addition, it is increasingly realized that there is a forgotten majority of patients with persisting residual cardiovascular disease, even in the age of intensive LDL-c lowering therapies (7, 8). Measurements of ApoA-I and $ApoB_{total}$ have gained more attention in cardiovascular risk assessment and have been included in clinical guidelines for dyslipidemia diagnosis and lipid management (9–11). For example, the 2018 European Atherosclerosis Society/European Federation of Clinical Chemistry and Laboratory Medicine consensus statement on quantifying atherogenic lipoproteins identified $ApoB_{total}$ as a more accurate marker of cardiovascular risk and treatment response than LDL-c or non–HDL-c, particularly at low concentrations (11). As ApoB-containing lipoproteins (lipoprotein(a) [Lp(a)]), LDL, very-low-density lipoprotein, intermediate-density lipoprotein) generally carry one ApoB molecule per particle, $ApoB_{total}$ concentration is a direct measure of atherogenic particle number. Therefore, $ApoB_{total}$ more reliably predicts cardiovascular risk across ages and sexes than LDL-c alone (12, 13). ApoA-I plays a key role in reverse cholesterol transport (14). While dysfunctional HDL is associated with higher cardiovascular risk, ApoA-I–rich functional HDL is associated with lower risk. In addition, ApoA-I mimetics can raise HDL-c levels and enhance cholesterol efflux capacity (7). Still, $ApoB_{total}$ and ApoA-I do not fully reflect functional abnormalities within non-HDL and HDL particles (2). Nevertheless, quantifying apolipoproteins in atherogenic lipoproteins offers greater biological and pathophysiological relevance than assessing their cholesterol content (15).

In the last decade of the 20th century, WHO-IFCC immunoassay-based standardization efforts were initiated for serum/plasma ApoA-I and $ApoB_{total}$ through Northwest Lipid Research Laboratories (16–18). This milestone metrological legacy delivered ApoA-I and $ApoB_{total}$ measurements in g/L that were traceable up to the WHO-IFCC secondary RMs (coded by WHO and IFCC as ApoA-I: SP1-01 and ApoB: SP3-08). The former RMPs within the metrological traceability chains for ApoA-I and $ApoB_{total}$ are currently no longer operational, and the corresponding RMs are running out of stock. As a result, a next-generation RMS in accordance with ISO 17511:2020 is required (2, 17–19). The RMP's uncertainty within this RMS should account for no more than 1/2 of the uncertainty budget for each measurand (20). According to the European Federation of Clinical Chemistry and Laboratory Medicine biological variation database, the maximum allowable uncertainty (MAU) at the patient level is 7.1% for ApoA-I and 9.3% for $ApoB_{total}$. Half of this uncertainty budget therefore corresponds to 3.6% and 4.7%, respectively. Calibration and state-of-the-art RMPs for ApoA-I and $ApoB_{total}$ are needed to standardize commercial reagents and to assign values to external quality assurance (EQA) materials used in EQA schemes.

Here, we describe the development and analytical validation of the next-generation immunoassay-independent, MS-based RMP for ApoA-I and $ApoB_{total}$, calibrated with serum-based human calibrators traceable to WHO-IFCC secondary RMs SP1-01 (ApoA-I) and SP3-08 (ApoB). The method is developed as a multiplexed procedure that will enable, in the end, the simultaneous quantification of 7 clinically relevant apolipoproteins—namely apolipoprotein(a), ApoA-I, $ApoB_{total}$, apolipoprotein C-I, apolipoprotein C-II, apolipoprotein C-III, and apolipoprotein E. (2). The established RMP is compliant with the ISO 15193:2009 guideline and follows calibration models outlined in section 5.5 of ISO 17511:2020.

Materials and Methods

The RMP for ApoA-I and $ApoB_{total}$ is an extension of the previously published RMP for apolipoprotein(a) (21) and is codeveloped to also include apolipoprotein C-I, apolipoprotein C-II, apolipoprotein C-III, and apolipoprotein E (phenotype) (2). The online supplemental material provides a detailed description of the materials and methods. An overview of the essentials of the method and its validation is provided here.

PEPTIDE SELECTION AND TRANSITION DEVELOPMENT

In silico trypsin digestion of ApoA-I and $ApoB_{48}$ identified peptide targets based on these criteria: *(a)* proteotypic peptides; *(b)* no tryptophan, methionine, or cysteine; *(c)* low susceptibility to polymorphisms or posttranslational modifications; and *(d)* length between 7 and 15 amino acids. Three peptides for ApoA-I and 5 present in $ApoB_{48}$ were selected and transitions optimized (Supplemental Table 1). The 5 $ApoB_{48}$ peptides account for $ApoB_{Total}$ since these are also found in $ApoB_{100}$. Precursor ion *m/z* and retention times were set from intact mass scans, and 3 fragment ions per peptide were chosen for specificity and abundance. Collision energies were optimized. The liquid chromatography gradient was adjusted for a minimum 20 ms

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dwell time, resulting in a 20-min run to ensure sensitivity and precision.

SEMI–AUTOMATED SAMPLE PREPARATION

Serum samples were processed in a semiautomated format using the BRAVO 96-channel liquid handling system (Agilent Technologies). Eight microliters of serum (diluted 1:20 in 100 mmol/L ammonium bicarbonate) were combined with a reagent mixture containing 0.40% (w/v) sodium deoxycholate, 1.15 mmol/L tris(2-carboxyethyl)phosphine, and stable isotope-labeled peptides (final concentration approximately 30 μmol/L). Proteins were denatured and reduced at 56°C for 30 min, followed by alkylation with 4.6 mmol/L iodoacetamide at room temperature for 30 min in the dark. Digestion was performed sequentially with lysyl endopeptidase (1:700 w/w) for 1 h at 37°C, followed by trypsin (1:47 w/w) for 3 h at 37°C. Digestion was quenched using 0.6% formic acid (v/v) in 5% methanol, precipitating sodium deoxycholate. After centrifugation (2000*g*, 30 min), supernatants were subjected to solid-phase extraction (Oasis HLB, Waters) using a positive-pressure 96-well processor. Peptides were eluted with 55% methanol, dried under nitrogen at 37°C, and reconstituted in eluent A prior to LC-MS analysis.

LIQUID CHROMATOGRAPHY–MULTIPLE REACTION MONITORING–MASS SPECTROMETRY

Peptide analysis was performed using an Agilent 1290 ultra-high-performance liquid chromatography system coupled to an Agilent 6495C triple quadrupole mass spectrometer (Agilent Technologies). Samples (10 μL) were injected onto a Zorbax SB-C18 column (2.1 × 50 mm, 1.8 μm), with separation achieved using a binary gradient of Eluent A (5% methanol, 0.05% formic acid in water) and Eluent B (95% methanol, 0.05% formic acid in water). The gradient progressed from 92% A to 40% A over 15 min, followed by a rapid shift to 5% A for column washing and then reequilibration to initial conditions.

MS was conducted in dynamic multiple reaction monitoring mode using collision-induced dissociation and a 500 ms cycle time. Transitions were scheduled within a 1-min retention window. For each peptide, either doubly or triply charged precursors were monitored along with 1 quantifier and 2 qualifier transitions at unit mass resolution.

CALIBRATION

External calibration for ApoA-I and $ApoB_{total}$ was performed using 5 value-assigned native human serum samples prepared according to CLSI protocol C37-A and stored at −80°C (22, 23). Concentrations of these calibrators were traceable to the certified WHO/IFCC secondary RMs SP1-01 for ApoA-I (1.50 ± 0.08 g/L) and SP3-08 for ApoB (1.18 ± 0.028 g/L), through the use of the RMP described here (Fig. 1). Assigned concentrations of the calibrators were 1.07, 1.70, 1.71, 1.85, and 2.33 g/L for ApoA-I and 0.60, 0.73, 0.78, 0.80, and 1.82 g/L for $ApoB_{total}$. Calibration curves were generated using 1/x weighted linear regression and constrained through the origin.

DIGESTION EVALUATION

Digestion efficiency was assessed in triplicate using native serum samples collected at 0, 1, 2, 3, 5.5, 21, and 24 h of trypsin incubation. Stable isotope-labeled standards were added at the start of sample preparation. Peptide responses were measured by LC-MS to monitor digestion kinetics.

DATA PREPARATION AND ANALYSIS

LC-MS/MS data were processed using Mass Hunter Workstation Quantitative/Qualitative Analysis software version 10.0. The concentrations, peak areas, ion ratios, relative responses, retention times, full width at half maximum values, and peak symmetry results were exported as separate comma-separated values files for each batch, enabling further data evaluation using an in-house custom R script (R version 4.4.0). All transitions (both quantifying and qualifying) were evaluated individually. Concentrations were reported in g/L for both ApoA-I and $ApoB_{total}$.

QUALITY ASSURANCE

System Monitoring. System suitability testing was performed as described by Reijnders et al. and extended here (24). A peptide mix (0.15 μmol/L, unlabeled and labeled peptides in 5% methanol) was injected 5 times before and after each batch, each followed by a blank. System suitability testing assessed sensitivity, specificity, accuracy, retention time, and carryover (Fig. 2).

Internal Quality Control. Two single-donor serum samples (CLSI C37-A) stored at −80 C were analyzed in triplicate per batch. Results were compared to long-term targets using modified Westgard rules. Rule violations triggered batch or sample reprocessing based on predefined criteria (Fig. 2).

Interpeptide Agreement. Protein-level selectivity was verified using ≥3 peptides per protein. Agreement between qualifying and quantifying peptides was assessed to detect sequence variants (Fig. 2).

System Performance Monitoring. LC-MS/MS performance was tracked via calibration metrics (R^2, slope), retention times (±2.5%), full width at half maximum (±3 SD), ion ratios (±20%), and internal standard

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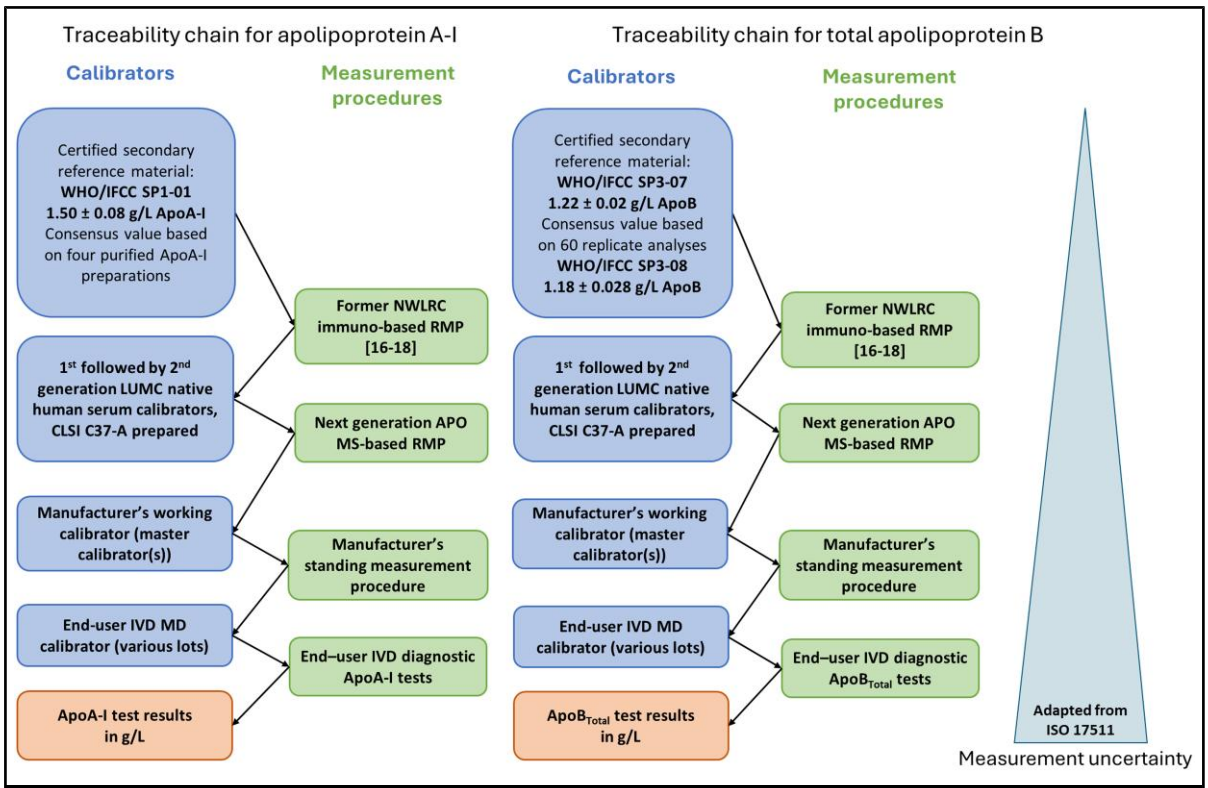


Fig. 1. Metrological traceability chains for serum ApoA-I and B_{total}, consisting of an unbroken sequence of calibrators and measurement procedures, including the IFCC WG APO MS RMP, used to relate measurement results to certified secondary reference materials (ApoA-I: SP1-01; ApoB: SP3-07/SP3-08). Abbreviations: ApoA-I, apolipoprotein A-I; $ApoB_{total}$, apolipoprotein B total; IFCC WG APO MS, IFCC Working Group for Standardization of Apolipoproteins by Mass Spectrometry; RMP, reference measurement procedure; MAU, maximum allowable uncertainty; LUMC, Leiden University Medical Center. Color figure available at clinchem.org.

intensities (±3 SD). Deviations flagged issues such as ion suppression or detector drift (Fig. 2).

ANALYTICAL VALIDATION

Analytical validation encompassed linearity (CLSI EP06 [25]), recovery, MU (JCGM 100:2008, (26)), precision (CLSI EP05-A3 [27]), carryover, limit of blank (LoB), limit of detection (LoD), and limit of quantification (LoQ; CLSI EP17-A2 [28]), as well as stability, preanalytical conditions and interferences, interpeptide agreement, and analytical specificity (see Table 1). The supplementary information provides a detailed description of methods for analytical validation.

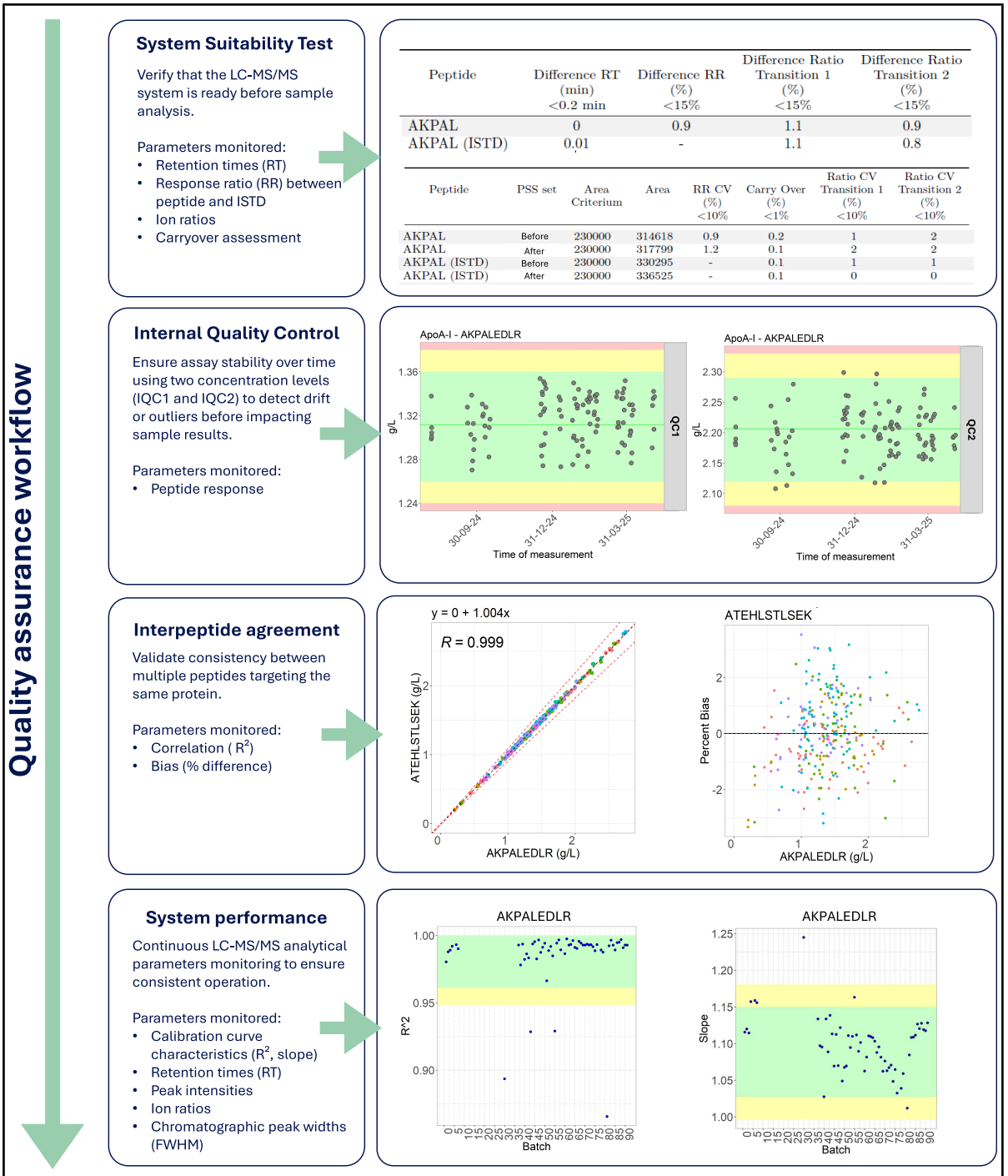
Results

METHOD DEVELOPMENT

The measurands, or quantities intended to be measured, were defined as "mass concentration of ApoA-I in native human serum" and "mass concentration of total apolipoprotein B_{total} in native human serum" expressed in g/L (34). The traceability chain, showing the position of the RMP in the RMS, together with the value assignment of native human serum calibrators currently in use, transfer of assigned values to working and lot-specific calibrators used in routine assays, and propagation of associated MU at each step, consistent with ISO 17511 principles, is depicted in Fig. 1. The calibration hierarchy is designed to ensure that end results remain within MAU.

Peptide Selection. Digestion of ApoA-I and $ApoB_{total}$ standards was performed to identify candidate peptides for targeted liquid chromatography–multiple reaction monitoring–MS analysis based on being empirically distinguishable in serum. Selection criteria included proteotypicity, absence of certain amino acids (W, M, C), low likelihood of mutations and post-translational modifications, and peptide lengths between 7 and 15 amino acids. Three peptides were selected for

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Peptide	Difference RT (min) <0.2 min	Difference RR (%) <15%	Difference Ratio Transition 1 (%) <15%	Difference Ratio Transition 2 (%) <15%
AKPAL	0	0.9	1.1	0.9
AKPAL (ISTD)	0.01	-	1.1	0.8

Peptide	PSS set	Area Criterium	Area	RR CV (%) <10%	Carry Over (%) <1%	Ratio CV Transition 1 (%) <10%	Ratio CV Transition 2 (%) <10%
AKPAL	Before	230000	314618	0.9	0.2	1	2
AKPAL	After	230000	317799	1.2	0.1	2	2
AKPAL (ISTD)	Before	230000	330295	-	0.1	1	1
AKPAL (ISTD)	After	230000	336525	-	0.1	0	0

Fig. 2. Schematic overview of the LC-MS/MS quality assurance workflow, illustrated for apoA-I peptide AKPALEDLR. First, system suitability is verified through retention time, response ratio, ion ratios, and carryover of synthetic peptides. Then, long-term internal quality control performance is evaluated to remain within predefined limits, and interpeptide agreement with ATEHLSTLSEK shows excellent correlation ($R = 0.999$). Finally, batch-wise system performance monitoring evaluates calibration characteristics (R^2, slope) and system metrics (retention time, full width at half maximum, ion ratios, internal standard intensities) to detect potential analytical drift. Abbreviation: apoA-I, apolipoprotein A-I. Color figure available at clinchem.org.

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Table 1. Analytical performance characteristics of the reference measurement procedures for serum apolipoprotein A-I and apolipoprotein B.

Analytical performance characteristic	Materials and methods	Desired performance criterion	Results
Linearity	CLSI EP06 (25), evaluated via weighted least squares regression (ApoA-I: 0.10–2.97 g/L and $ApoB_{total}$: 0.27–3.83 g/L)	Allowable deviation from linearity <10%	Linearity confirmed. Linear measuring range: 0.10 to 2.97 g/L for ApoA-I and 0.27 to 3.83 g/L for $ApoB_{total}$ with ADL <10%
Recovery	Quantitation of SP01-01 (ApoA-I) and SP3-08 (ApoB)	Mean recovery of 100% +/– MU based on target concentrations: WHO/IFCC SP1-01 1.50 ± 0.08 g/L ApoA-I and WHO/IFCC SP3-08 1.18 ± 0.028 g/L ApoB	ApoA-I (1.50 ± < 0.01 g/L): recovery 100.0–100.3%, CV < 1.2%. ApoB (1.18 ± < 0.01 g/L): recovery 99.2–100.0%, CV < 4%.
Measurement uncertainty	JCGM 100:2008 (26)	33% of the allowable MU at the patient level. This is defined at 2.37% for ApoA-I and $ApoB_{total}$, for reproducibility <50% of MAU at the patient level.	For AKPALEDLR (ApoA-I): Median CVr = 1.59% (SD 0.40), median CVwl = 1.9% (SD 0.36). %rU = 3.2; consequently, duplicate measurements are needed to fulfill the MAU. CVbl = 3.30%, which is within the available MU budget for reproducibility. For FIIPSPK ($ApoB_{total}$): Median CVr = 1.63% (SD 0.43), median CVwl = 2.12% (SD 0.39). %rU = 3.3; consequently, duplicate measurements are needed to fulfill the MAU. CVbl = 3.28%, which is within the available MU budget for reproducibility.
Precision	CLSI EP05-A3 (27) (20 × 20 × 2 design; Evaluated across 20 samples over 20 days, in duplicate)	CV ≤ 3.6% (ApoA-I) or 4.7% ($ApoB_{total}$) across runs and days	For AKPALEDLR (ApoA-I): 1.6% (SD 0.4 g/L), mean CVwl = 1.9% (SD 0.4 g/L). For FIIPSPK ($ApoB_{total}$): Mean CVr = 1.6% (SD 0.4 g/L), median CVwl = 2.1% (SD 0.4 g/L).
Storage conditions	Controlled storage conditions (29): Repeated measurements	No significant difference between 1 x F/T and alternative storage	ApoA-I remained stable across all tested conditions, including freeze/thaw

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Table 1. (continued)

Analytical performance characteristic	Materials and methods	Desired performance criterion	Results
	of samples stored under different conditions	condition based on ANOVA analysis (adjusted $P \geq 0.05$)	cycles (adjusted $P = 0.417$) and fresh/frozen comparisons (adjusted $P = 0.126$). $ApoB_{total}$ showed small but significant changes with repeated freeze/thaw cycles (adjusted $P \leq 0.005$) but no differences in fresh/frozen samples. These results confirm ApoA-I's robustness and ApoB's sensitivity to freeze/thaw cycles.
Preanalytical conditions	Evaluation of different blood collection tubes (30)	No significant matrix-induced bias: no difference vs the serum reference (adjusted $P \geq 0.05$)	Matrix effects for ApoA-I and $ApoB_{total}$ showed a negative bias in sodium citrate plasma (–14.1% to –15.7%), while other matrices (CAT, LiHe gel, EDTA) showed minimal bias, similar to serum gel. Consequently, the quantification of ApoA-I and $ApoB_{total}$ by the RMP is validated for the serum matrix only.
Carryover	Tietz Textbook of Clinical Chemistry chapter "Automation"; EP Evaluator v12.3 (31)	Max deviation ≤ ± 0.1% of high sample mean	Sequence-based analysis (e.g., L3-H1-H2-L4, etc.); Carryover was <0.1% of high concentrations, corresponding to <0.003 g/L for ApoA-I and <0.004 g/L for $ApoB_{total}$, indicating an acceptable minimal carryover effect.
Limit of blank	CLSI EP17-A2 (28)	95th percentile of blank samples. Ideally <0.005 g/L	The LoB for ApoA-I (AKPALEDLR) is <0.001 g/L and for $ApoB_{total}$ (FIIPSPK) 0.002 g/L

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Table 1. (continued)

Analytical performance characteristic	Materials and methods	Desired performance criterion	Results
Limit of detection	CLSI EP17-A2 (28)	LoB + 1.645 × cp * pooled SD (parametric approach). Ideally <0.005 g/L	The LoD for ApoA-I (AKPALEDLR) is 0.0013 g/L and for $ApoB_{total}$ (FIIPSPK) 0.0019 g/L
Limits of quantitation	CLSI EP17-A2(28)	Ideally <0.01 g/L. lowest concentration where upper 95% CI intersects threshold: CV ≤ 2.37% (ApoA-I) or 3.1% ($ApoB_{total}$).	The LoQ for ApoA-I (AKPALEDLR) is 0.01 g/L. The LoQ for $ApoB_{total}$ (FIIPSPK) is 0.02 g/L.
Analytical specificity	Ion ratio monitoring (32), Interpeptide agreement	Interference-free signal in qualifying transitions. Ion ratio should not deviate >20%. Agreement R > 0.975 and slope > 0.9 and < 1.1	Qualifier ion ratios monitored; Difference in ion ratio between endogenous and SIL peptides is <20%. ApoA-I: R > 0.998 and slopes of 0.994 and 1.005 $ApoB_{total}$: R > 0.996 and slopes between 0.982 and 0.998
Method comparison	CLSI EP09; LC-MRM-MS against ITA, OLS regression (33) Samples were selected to ensure coverage of the entire analytical measuring range, as defined by the RMP.	High correlation between methods: R > 0.95. bias below MAU	ApoA-I, (AKPALEDLR) against ITA: slope of 1.052 and bias of −0.1%. For $ApoB_{total}$ (FIIPSPK): slope of 0.992 and bias of 2.6%, demonstrating concordance. R for multiple Immunoassays >0.95. It is consequently likely that the RMP is suitable for standardization of ApoA-I and $ApoB_{total}$ measurements.

Abbreviations: ApoA-I, apolipoprotein A-I; $ApoB_{total}$, apolipoprotein B total; ADL, allowable deviation from linearity; CVr, within-run coefficient of variation; CVbl, between-laboratory coefficient of variation; CVwl, within-laboratory coefficient of variation; %rU, percent relative uncertainty; CAT, sodium citrate plasma tube; LiHe, lithium heparin; LoB, limit of blank; LoD, limit of detection; LoQ, limit of quantitation; SIL, stable isotope–labeled internal standard; LC-MRM-MS, multiplex liquid chromatography–multiple reaction monitoring–mass spectrometry; ITA, immunoturbidimetric assays; OLS, ordinary least squares; MAU, maximum allowable uncertainty; RMP, reference measurement procedure.

ApoA-I (AKPALEDLR, ATEHLSTLSEK, and THLAPYSDELR) and 5 for $ApoB_{total}$ (FIIPSPK, AAIQALR, GFEPTLEALFGK, SVSLPSLDPASAK, and TEVIPPLIENR) (Supplemental Table 1). The sample preparation used combined lysyl endopeptidase/trypsin digestion to enhance efficiency for lysine-rich proteins (35), anticipating future multiplex applications. Digestion kinetics in native serum showed stable plateaus for all 3 ApoA-I and 5 $ApoB_{total}$ peptides after 1 h (Fig. 3).

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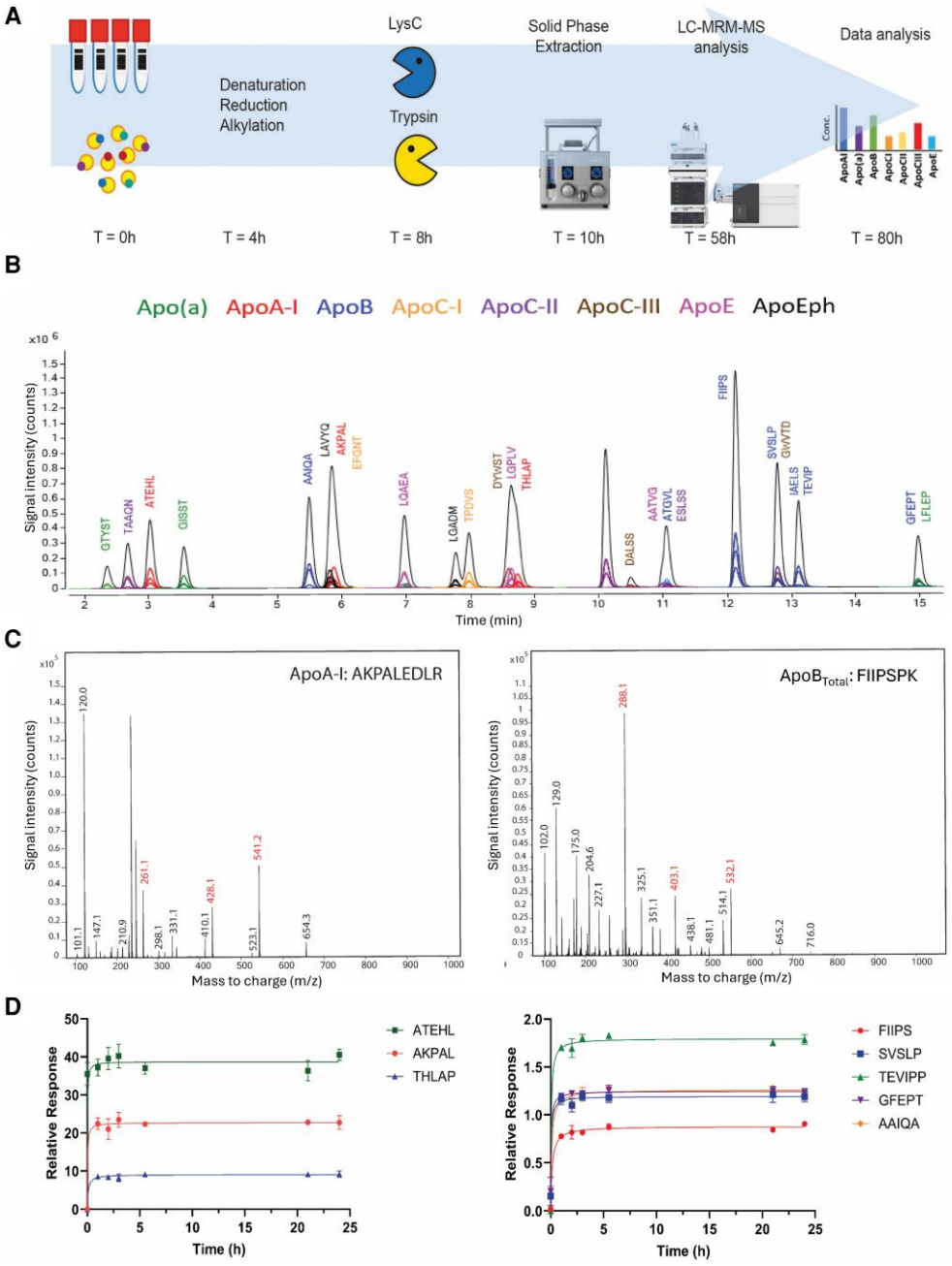


Fig. 3. Overview of the experimental workflow of the RMP, including addition of ISTD, protein denaturation, reduction, alkylation, digestion with Lys-C and trypsin, solid phase extraction, LC-MRM-MS analysis, and data analysis, as published previously (21) (A), LC-MRM-MS chromatogram showing representative peptide signals for multiple apolipoproteins, including Apo(a), ApoA-I, ApoB$_{total}$, ApoC-I, ApoC-II, ApoC-III, and ApoE; (B), Representative MS/MS spectra of selected peptide transitions; (C), Analysis of digestion kinetics for ApoA-I and ApoB$_{total}$; (D), Abbreviations: RMP, reference measurement procedure; ISTD, internal standard; Lys-C, lysyl endopeptidase; LC-MRM-MS, multiplex liquid chromatography–multiple reaction monitoring–mass spectrometry; Apo(a), apolipoprotein(a); ApoA-I, apolipoprotein A-I; ApoB$_{total}$, apolipoprotein B total; ApoC-I, apolipoprotein C-I; ApoC-II, apolipoprotein C-II; ApoC-III, apolipoprotein C-III; ApoE, apolipoprotein E. Color figure available at clinchem.org.

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ANALYTICAL VALIDATION

Linearity. The linearity of the ApoA-I and $ApoB_{total}$ measurement procedure was evaluated in accordance with CLSI EP06 guidelines (25). All samples met the allowable deviation from linearity of ±10%. Pearson correlation coefficients of $R \geq 0.9992$ (ApoA-I) and $R \geq 0.999$ ($ApoB_{total}$) indicated a strong linear relationship between measured and expected concentrations. The linear measuring range for ApoA-I was determined to be 0.10 and 2.97 g/L and for $ApoB_{total}$, 0.27 and 3.83 g/L.

Recovery. The native serum calibrators used in the RMP are indirectly traceable to RMs SP1-01 (ApoA-I) and SP3-08 (ApoB). Value assignment was achieved via first and second-generation native human serum calibrators measured in 5 subsequent batches containing bilevel internal quality control ($n = 3$ each) (Fig. 1) (unpublished observations). SP1-01 and SP3-08 were quantified to determine recovery. Quantification was based on the peptides AKPALEDR (ApoA-I) and FIIPSPK ($ApoB_{total}$), which showed recoveries of 100.0% and 100.3%, respectively. For ApoA-I, the additional peptides ATEHLSTLSEK and THLAPYSDELR showed similar recoveries of 100.2% and 100.3%, with CVs consistently below 1.2%. For $ApoB_{total}$, the confirmatory peptides AAIQALR, GFEPTLEALFGK, SVSLPSLDPASAK, and TEVIPPLIENR demonstrated recoveries between 99.2% and 100.0%, all with CVs below 4%. For the peptides AKPALEDR and FIIPSPK, measured concentrations (mean ± SE) were 1.50 ± < 0.01 g/L ($n = 12$) and 1.18 ± < 0.01 g/L ($n = 12$), respectively. The 95% CI for these peptides were 1.489 to 1.511 g/L (AKPALEDR) and 1.169 to 1.191 g/L (FIIPSPK), indicating that the mean concentrations did not differ significantly from the target values.

Precision. The precision profile was assessed in accordance with CLSI EP05-A3 (Appendix A) using 20 samples spanning concentrations of 1.03 to 2.19 g/L for ApoA-I and 0.47 to 1.63 g/L for $ApoB_{total}$, covering the respective clinical ranges of 0.90 to 1.98 g/L (ApoA-I) and 0.54 to 1.47 g/L ($ApoB_{total}$) (27, 36). Using the ANOVA-based SDs and %CVs, precision profiles were established for each peptide. Precision of both ApoA-I and $ApoB_{total}$ quantitation was concentration independent. For AKPALEDR (ApoA-I), the mean repeatability (CV within-run [CVr]) was 1.6% (SD 0.4 g/L), and the mean within-laboratory precision (CV within-laboratory [CVwl]) was 1.9% (SD 0.4 g/L). For FIIPSPK ($ApoB_{total}$), the mean CVr was 1.6% (SD 0.4 g/L), and the mean CVwl was 2.1% (SD 0.4 g/L) (Supplemental Tables 5 and 6).

Measurement Uncertainty. As $\%ru_{m,3} = \%ru_{Rw.p.3.}$ according to ISO 17511:2020 clause 5.5, the repeatability (CV_r) of the RMP was determined for both ApoA-I and $ApoB_{total}$ using a CLSI EP05-A3 precision study. CV_r was found to be 1.6% for ApoA-I (AKPALEDR) and 1.6% for $ApoB_{total}$ (FIIPSPK). Based on these repeatability estimates and applying a coverage factor $k = 2$, the unadjusted MU for single measurements was calculated as: $MU = \%rU_{rw} = k * CV_r = 2 \times 1.594 = 3.2\%$ for ApoA-I, and $MU = \%rU_{rw} = k * CV_r = 2 \times 1.627 = 3.3\%$ for $ApoB_{total}$.

The predefined analytical performance specification for the RMP for ApoA-I and $ApoB_{total}$ can be defined based on biological variation. Specifically, the minimal allowable MAU can be defined as $MAU_{rmp} = 0.33 \times 1.5 \times$ the within-subject biological variation (CVi). For ApoA-I (CVi = 4.7%), this results in an MAU_{rmp} of 2.37% and for $ApoB_{total}$ (CVi = 6.2%) the MAU_{rmp} = 3.10%.

While the expanded MU for ApoA-I and B_{total} exceeds the allowable uncertainty, the performance of duplicate measurements brings the percent relative uncertainty below the allowable MU through division by $\sqrt{2}$ ($\frac{3.18}{\sqrt{2}} = 2.25\%$ for ApoA-I and $\frac{3.26}{\sqrt{2}} = 2.31\%$ for $ApoB_{total}$). Consequently, for value assignments to fulfill the analytical performance specification for the RMP, duplicate measurements should be conducted.

ANALYTICAL INFLUENCE QUANTILES

ApoA-I and $ApoB_{total}$ stability under various storage conditions (48 h retention time, 7 days refrigerated, up to 7 freeze–thaw cycles) was assessed using linear mixed-effects models followed by ANOVA with Bonferroni correction. For $ApoB_{total}$ peptides, significant variation was observed across freeze–thaw cycles (adjusted $P \leq 0.005$), indicating sensitivity to repeated freezing. No significant differences were found in the fresh–frozen comparison (adjusted $P = 1.00$). ApoA-I showed no significant changes across any condition, including freeze–thaw (adjusted $P = 0.417$) and fresh–frozen (adjusted $P = 0.126$). ApoA-I peptides demonstrated stability across all tested storage conditions, including multiple freeze–thaw cycles and suboptimal storage conditions. In contrast, $ApoB_{total}$ showed significant variability after repeated freeze–thaw cycles, indicating sensitivity to such handling. Therefore, to ensure reliable analysis of $ApoB_{total}$, minimizing freeze–thaw cycles is essential, whereas ApoA-I peptides appear robust under diverse storage conditions. Because $ApoB_{total}$ showed significant variability after repeated freeze–thaw cycles, all frozen human serum calibrators were aliquoted and subjected to only a single freeze–thaw cycle prior to analysis to minimize handling-related effects.

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The effects of different sample collection tubes (all from Becton Dickinson) on ApoA-I and ApoB quantification were evaluated using replicate measurements, with serum gel tubes as the reference. ApoA-I peptides showed a consistent negative bias in sodium citrate plasma (−14.1% to −14.3%), while serum, lithium heparin (LiHe) gel, and EDTA tubes exhibited minimal bias. For $ApoB_{total}$, citrate plasma showed a pronounced negative bias (−14.8% to −15.7%); sodium citrate plasma tube and LiHe gel, minimal bias; and EDTA tubes, moderate negative bias. These results confirm serum as the preferred matrix for accurate apolipoprotein quantification, though LiHe gel tubes may be a suitable alternative with minimal bias.

Carryover. The carryover was assessed in samples with high ApoA-I (2.81 g/L) and $ApoB_{total}$ (3.64 g/L) concentrations that were measured concurrently with samples with low ApoA-I (0.09 g/L) and $ApoB_{total}$ (0.21 g/L) concentrations. Carryover was <0.1% of the high concentration: <0.003 g/L for ApoA-I and <0.004 g/L for $ApoB_{total}$.

LoB, LoD, and LoQ. The LoB for both ApoA-I and $ApoB_{total}$ was evaluated in protein solutions composed of albumin-IgG in PBS or albumin. The LoB for ApoA-I is <0.001 g/L for quantifying peptide AKPALEDLR. For $ApoB_{total}$, the LoB for FIIPSPK is 0.002 g/L. The LoD is 0.0013 g/L for ApoA-I (AKPALEDLR) and 0.0019 g/L for $ApoB_{total}$ (FIIPSPK), with proportions of false positives and false negatives below 5%. The LoQ for ApoA-I (AKPALEDLR) is 0.01 g/L, based on a fitted precision model $y = 0.390x^{-0.404}$, corresponding to an imprecision target of CV = 2.37%. For $ApoB_{total}$ (FIIPSPK), the LoQ is 0.02 g/L, based on the model $y = 0.082x^{-0.822}$ and an imprecision target of CV = 3.1%.

Method Comparison. The RMP was compared to Roche immunoturbidimetric assays (ITAs) for ApoA-I and $ApoB_{total}$ to evaluate its standardization potential. Roche ITAs were performed on the Cobas e602 and c502 modules of the Cobas® 8000 modular analyzer series. Results from 140 (ApoA-I) and 143 ($ApoB_{total}$) samples were analyzed (Fig. 4 Q–T), excluding samples below the ITA LoQ (0.2 g/L). Strong linear correlations were observed (Pearson *r*: 0.996 for ApoA-I, 0.992 for $ApoB_{total}$). For ApoA-I (peptide AKPALEDLR), the slope was 1.052 with −0.1% bias; for $ApoB_{total}$ (peptide FIIPSPK), the slope was 0.992 with 2.6% bias. These findings guarantee the continuation of historic standardization.

Discussion

We here introduce a successor MS-based RMP for continuing standardization of commercial reagents of ApoA-I and $ApoB_{total}$ according to state-of-the-art metrological traceability, ensuring ISO guidelines (ISO 17511, ISO 15193). Metrological traceability to the former WHO/IFCC certified secondary RMs SP1-01 (ApoA-I) and SP3-08 (ApoB) is maintained by first establishing continuity through the Northwest Lipid Research Laboratories legacy RMP and first-generation Leiden University Medical Center native human-serum calibrators, after which 5 second-generation native human-serum calibrators were value-assigned by the successor MS-based RMP. Value assignment was performed in five subsequent batches using bilevel internal quality control ($n = 3$ per level per batch) (unpublished observations). The RMP shows CV_{wl} of 1.9% and 2.1%, a linear measuring range of 0.102.97 g/L for ApoA-I and for $ApoB_{total}$ 0.273.83 g/L, as well as a LoQ of 0.01 g/L and 0.02 g/L, thus fulfilling the acquired analytical performance specifications.

The RMP is MS-based, with a bottom-up proteomics strategy, in which proteins are proteolytically digested using enzymes into peptides, which are then quantified as representatives of the protein they represent. MS offers several advantages for the detection and quantification of proteins, as measurements may be performed with high analytical specificity at the molecular level through the combination of retention times, internal standards, and distinct precursor and fragment ions. This is contrary to immunoassays, for which analytical specificity depends on the binding specificity of antibodies. In this RMP, specificity is enhanced by selecting at least 3 proteotypic peptides per apolipoprotein, each monitored with 3 distinct transitions. This strategy ensures robust protein identification and precise molecular-level definition of measurands.

Currently, the RMP reestablishes traceability to the former reference measuring system, developed in the 1990s. Notwithstanding standardization of the immunoassays for ApoA-I and $ApoB_{total}$ is adequate (37), continuity of reference services is needed. In practice, we observe equivalent results because the Roche assays also remain traceable to the IFCC-WHO RMs. Similarly, EQA materials can be value-assigned with traceability to the former RMs and used to evaluate bias of laboratory results.

Despite its name, MS is intrinsically a method for the molar (amount of substance) quantitation of biomolecules, with the signal proportional to the number of ions hitting the detector. Since proteins exist as diverse proteoforms lacking a defined molecular weight, mass-based quantification is imprecise. Therefore, RMPs for

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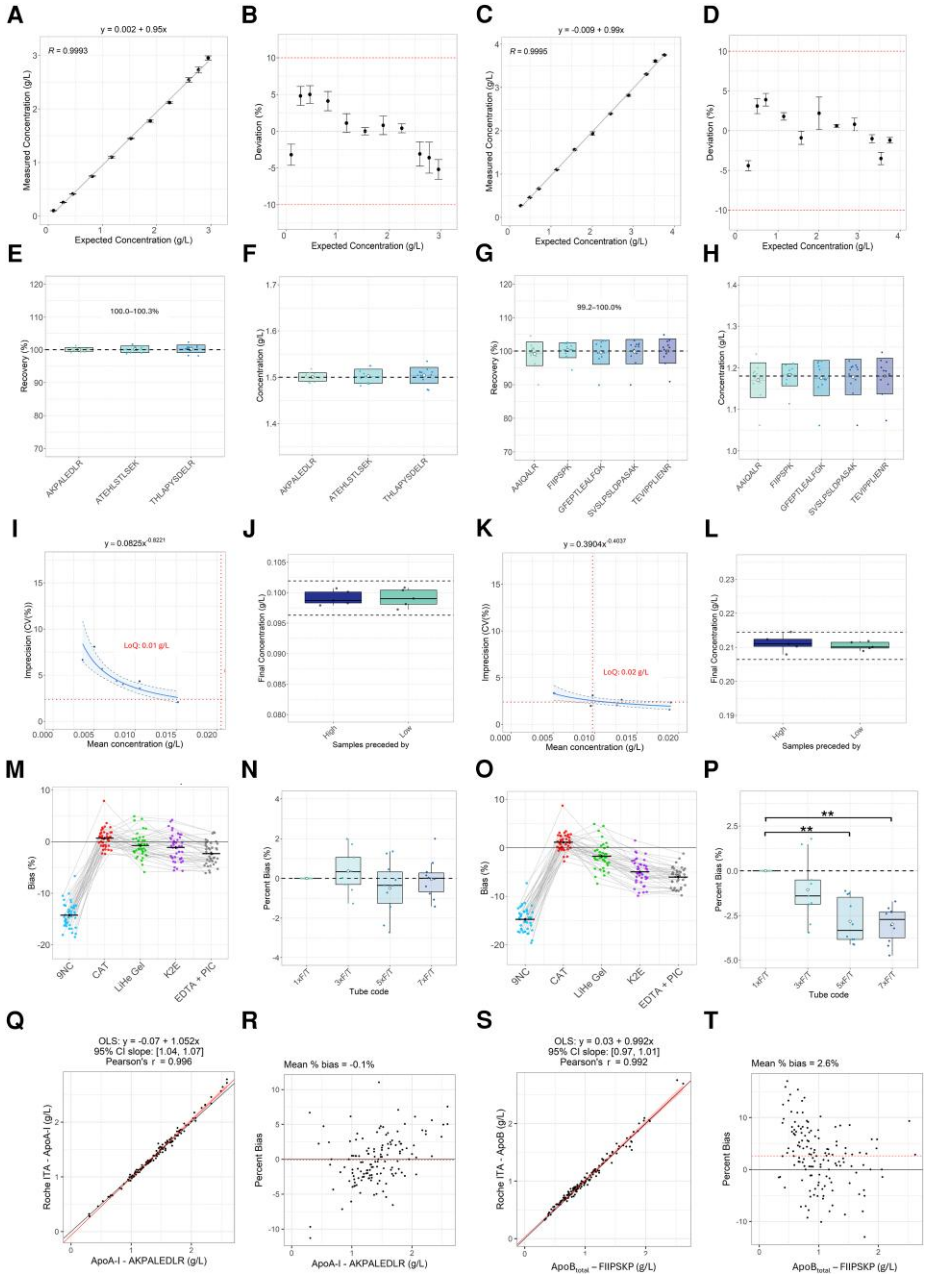


Fig. 4. Analytical validation of the LC-MS/MS assays for ApoA-I and $ApoB_{total}$ illustrating the results reported in Table 1. The left-hand figure represents apoA-I, the right-hand $apoB_{total}$. Linearity and percent deviation from linearity across the validated concentration ranges (A–D). Red dashed lines represent ±10% allowable deviation. Percent recovery and measured concentrations (E–H). Dashed lines indicate 100% recovery and mean concentration. LoQ profiles (I, K) and carryover assessment (J, L). Dashed and dotted lines denote acceptance thresholds for %CV and false positive carryover signals, respectively. Bias and percent bias observed in different blood collection tubes and matrix types (M–P). Significance levels: $*P < 0.05$, $**P < 0.01$. Method comparison between LC-MS/MS and Roche ITA (Q–T) with ordinary least squares regression. Abbreviations: ApoA-I, apolipoprotein A-I; $ApoB_{total}$, apolipoprotein B total; LoQ, level of quantification; ITA, immunoturbidimetric assay. Color figure available at clinchem.org.

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ApoA-I and $ApoB_{total}$ define measurands as amount-of-substance concentrations, via calibrators value-assigned in molar units and ideally being traceable to primary RMs. We recommend expressing $ApoB_{total}$ concentrations in molar units rather than mass units in the future, enabling direct comparison of particle numbers and harmonization with other apolipoprotein measurements (38). This, in turn, facilitates direct estimation of the proportion of Lp(a)-ApoB [%ApoB in Lp(a)] compared to $ApoB_{total}$, providing a clearer understanding of the atherogenic lipoprotein burden and the Lp(a)-ApoB contribution. In addition, quantifying multiple proteotypic peptides per apolipoprotein, without calculating the average concentration, provides an internal selectivity check: loss of signal in a (qualifying) peptide against stable signals in the others flags a potential genetic variant.

In conclusion, the validation of the MS-based RMP for ApoA-I and $ApoB_{total}$ in serum demonstrates its potential as a second-generation RMP for serum/plasma ApoA-I and $ApoB_{total}$ standardization. These RMPs represent a next-generation successor to the longstanding antibody-based ELISA methods and accuracy base established by Marcovina et al. at Northwest Lipid Research Laboratories (17, 18). The method complies with ISO 15193 requirements and fulfills the analytical performance requirements to be fit for purpose. Creating awareness of the availability of the multiplexed MS-based RMP for ApoA-I and $ApoB_{total}$ is needed for adoption and implementation by in vitro diagnostic manufacturers and other stakeholders in the traceability chain. Notably, measuring molecular-defined and robust apolipoproteins is metrologically superior to measuring cholesterol in undefined macromolecular lipoprotein complexes of LDL and HDL (39), implying more precise pathophysiological insight into the type of dyslipidemia. This multiplexed RMP will contribute to the continued standardization of ApoA-I and $ApoB_{total}$ tests, guaranteeing sustainable cardiovascular disease risk management with respect to these biomarkers.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: RMS, reference measurement system; RMs, reference materials; RMP, reference measurement procedure; MS, mass spectrometry; ApoA-I, apolipoprotein A-I; $ApoB_{total}$, apolipoprotein B total; $ApoB_{100}$; apolipoprotein B100; $ApoB_{48}$, apolipoprotein B48; HDL-c, HDL cholesterol; LDL-c, LDL cholesterol; Lp(a), lipoprotein(a); MAU, maximum allowable uncertainty; EQA, external quality assurance; MU, measurement uncertainty; LoB, limit of blank; LoD, limit of detection; LoQ, limit of quantification; CVr, CV within-run; CVwl, CV within-laboratory; CVi, the within-subject biological variation); LiHe, lithium heparin; ITA, immunoturbidimetric assay.

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