CHOLESTEROL REFERENCE METHOD LABORATORY NETWORK (CRMLN)- TOTAL CHOLESTEROL (TC) CERTIFICATION PROTOCOL USING TC-IDMS RMP

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1 GENERAL INFORMATION

Heart disease is the leading cause of death in the United States as well as globally. Over 17 million people die each year from cardiovascular diseases (CVDs), an estimated 31% of all deaths worldwide. Currently more than 1 in 3 adults (85.6 million) in the United States live with 1 or more types of cardiovascular disease [1,2,3,]. Blood lipid measurements are one of the cornerstones in CVD risk assessment and treatment. American Heart Association (AHA) recommends testing for cholesterol and other traditional risk factors in its recent guidelines and goals [4]. WHO Global Hearts Initiative recommends testing for high blood cholesterol [5]. To correctly estimate risk for CVDs, cholesterol and other blood lipid measurements need to be accurate and reliable.

As part of CDC's Clinical Standardization Programs, the CDC Cholesterol Reference Method Laboratory Network (CRMLN) is successfully working with assay manufacturers and laboratories to improve and maintain the accuracy and reliability of total cholesterol and other blood lipids measurements. This is done through split-sample comparisons with the reference laboratory using individual donor samples. For total cholesterol, these comparisons are performed either using the traditional spectrophotometry-based reference measurement procedure (RMP) or the mass spectrometry-based RMP. This protocol describes the procedures used for comparisons to the mass spectrometry-based RMP.

This protocol outlines the procedure for a split-sample comparison of the analytical system with the total cholesterol RMP by isotope dilution mass spectrometry (TC-IDMS) using at least 40 samples. The evaluation is based on the CLSI Guideline EP9-A, *Method Comparison and Bias Estimation Using Patient Samples* [6]. The NCEP Laboratory Standardization Panel recommended that laboratories perform cholesterol analyses with bias ≤ 3.0% from the true value (RMP) and imprecision, as measured by coefficient of variation (CV), ≤ 3.0% [7,8]. These goals for accuracy and precision suggest that for a single measurement, the allowable total error would be 8.9%. Analytical systems meeting analytical performance requirements based on NCEP's recommendation receive a Certificate of Analytical Quality.

A manufacturer or developer of assays contact one of the CRMLN laboratory before beginning the certification process. A list of CRMLN laboratories is available on the CRMLN Website [9].

2 THE CERTIFICATION PROCESS

2.1 PURPOSE

Certification procedure described in this protocol is intended to assess the bias of an analytical system against the total cholesterol RMP using isotope dilution-mass spectrometry (TC-IDMS). Not all samples encountered in clinical laboratories are included in the scope of this program. The protocol is designed to verify the calibration, not the robustness, of the analytical systems evaluated.

2.2 MANUFACTURER'S PRELIMINARY RESPONSIBILITIES

Before pursuing certification through the CRMLN, manufacturers should establish that their analytical systems meet the following standard specifications:

- Instrument system(s) must be capable of producing discrete number values.
- Instrument system(s) must have had all required preventive maintenance procedures and must be in peak operating condition.
- Imprecision testing (such as that outlined in CLSI EP5-A3, *Evaluation of Precision of Quantitative Measurement Procedures* [7] should be done to ensure that total precision is ≤ 3%.

Note: Manufacturers who have the TC-IDMS RMP set up in house may verify its traceability by certification through the CRMLN. Although this internal verification does not substitute for certification of the manufacturer's products, manufacturers may use a certified TC-IDMS method in house to check their products before evaluation of traceability by a CRMLN laboratory.

2.3 MANUFACTURER'S SAMPLE COLLECTION

Anyone collecting and handling any biological material of human origin MUST observe Universal Precautions [10].

Samples may be fresh or frozen. The certification protocol is only designed to evaluate analytical system's bias, imprecision, and total error. Therefore, variation from preanalytical sources must be eliminated or minimized. Manufacturers should carefully follow the protocol for sample collection and processing described in this section.

The recommended sample matrix is serum; however, the comparison should be performed using the sample matrix for which the analytical system is designed. Venous serum is the matrix to be used for all comparisons designed to establish traceability to the accuracy base (RMP). Values for all other blood matrices must be traced to venous serum values through paired sample comparisons. Alternate blood matrices would include all capillary samples (including serum) and all anticoagulated samples from both venous and capillary sites. For example, if a manufacturer's system is designed to analyze capillary plasma and the manufacturer wishes to be certified for this matrix, the manufacturer should collect paired venous serum and capillary plasma samples from the 40+ donors used in the comparison. The manufacturer should then analyze the capillary plasma samples and submit the venous serum samples to the CRMLN laboratory for analysis.

Collect and analyze 40 or more samples from donors. The cholesterol concentration levels of these samples should be distributed over a clinically meaningful range, to the following target distribution:

- Minimum 8 samples from 120 to 180 mg/dL (3.10 to 4.67 mmol/L)
- Minimum 12 samples from 181 to 220 mg/dL (4.68 to 5.71 mmol/L)
- Minimum 12 samples from 221 to 260 mg/dL (5.72 to 6.74 mmol/L)
- Minimum 8 samples from 261 to 400 mg/dL (6.75 to 10.34 mmol/L)

These samples must:

- be free of interfering substances known to affect the system being tested (e.g., hemolysis, icterus, marked lipemia);
- not include samples that the product's package insert indicates should be excluded;
- be collected in sufficient quantity; and
- be collected using good laboratory practice (such as outlined in CLSI H3-A6, *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture* [11]).

To make the certification process reflect reality, the protocol followed should mimic, as much as is possible, the conditions present in the clinical laboratory.

Determine the minimum amount of serum needed per sample for TC-IDMS analysis by communicating with the CRMLN laboratory**.** Samples intended for TC-IDMS RMP analysis should be dispensed into appropriate cryogenic vials and frozen at -70 °C or lower Samples need to be clearly identified by coded identification numbers with **no personal identifiers**. Samples must be securely contained in cryogenic vials to prevent breakage, leakage, and evaporation.

Difficulty in obtaining a sufficient amount of sample can be overcome by combining two samples – **but no more than two**. This combined sample pool should be treated as an un-pooled (single) sample. The combined sample pool should be mixed well before aliquoting.

Some of the same samples used for certification of other analytes (HDL cholesterol, LDL cholesterol, and total glycerides) by the CRMLN may be used. Because all samples are unlikely to meet the sample distribution guidelines for all analytes and to avoid delays resulting from insufficient sample volume, outliers, or lab accident, it is recommended that more than 40 samples be collected for certification purpose.

The CRMLN strongly recommends that manufacturers set aside and store at -70°C or lower additional aliquots of each sample (volume consistent with analytical system requirements). These samples can be used for reanalysis if changes in calibration are required to meet certification criteria. When new lots of calibrators, materials, or reagents are prepared, these frozen samples can provide an important link to the accuracy base.

Collection of donor samples is the responsibility of the manufacturer. However, the CRMLN laboratory may assist in this process by collecting some or all the samples.

2.4 MANUFACTURER'S QUALITY CONTROL

To evaluate total error, estimates of both inaccuracy and imprecision are needed. The inaccuracy can be obtained from the split sample comparison with the RMP. However, to estimate imprecision, the

CRMLN requires that manufacturers provide QC data obtained from 20 separate runs. The recommended concentration range for the QC material is 200 to 240 mg/dL (5.2 to 6.2 mmol/L). A frozen human serum pool is more representative of donor samples and is preferable to a processed (e.g., lyophilized) material. The latter may result in higher imprecision leading to a falsely high total error. The QC material must be analyzed in singlicate using the complete analytical system being evaluated. These 20 runs must be the same as those used in the split sample comparison described in 2.5.

2.5 MANUFACTURER'S SAMPLE ANALYSIS

The CRMLN recommends that manufacturers perform the comparison analyses at the manufacturing site; however, manufacturers may have a clinical laboratory using their system perform the split comparison with the RMP. Clinical laboratories that perform successful comparisons on behalf of a manufacturer will also receive a Certificate of Analytical Quality for Total Cholesterol.

Calibration is the key to achieving accuracy; therefore, these comparison runs should represent the conditions recommended to customers.

More than one system may be certified using one set of samples. If more than one lot of reagents or calibrators are to be certified, all the samples should be analyzed with each lot. Each combination of instrument, reagents, and calibrator that meets the NCEP performance recommendations will be issued a Certificate of Analytical Quality for Total Cholesterol.

Analyze each sample in duplicate. Assign the first set of aliquots from each sample's sequential positions in the run. Run the duplicate measurement of each sample in reverse order.

Include the QC material selected in section 2.4 in every run.

Analyze the samples over 20 runs, two samples per run. More than one run per day is acceptable if runs are separated by 2 hours and the analytical system is recalibrated for each run. Manufacturers of point-of-care instruments that are factory-calibrated should define "runs" similarly. Manufacturers may also choose to use more than one instrument of the same model to accomplish the runs in a shorter period of time. However, all the samples analyzed in the comparison must originate from 40 or more unique donors. If more than 40 samples are to be analyzed, divide the excess samples evenly among the 20 analytical runs. Run days need not be consecutive, but all testing should be completed within a reasonable time period (i.e., less than 1 month). Perform testing following the instructions provided in the package insert (i.e., do not follow in-house modifications). If an instrument problem develops during a run or internal QC is unacceptable, retest the samples from that run after the problem has been identified and corrected.

Complete the assay information form, results form, and QC form of the data submission template.

2.6 SHIPMENT OF SAMPLES FOR TOTAL CHOLESTEROL ANALYSIS BY IDMS RMP

When the samples have been tested by the manufacturer, provide the completed data submission template to the CRMLN laboratory along with the frozen aliquots for TC-IDMS RMP analysis.

Contact the CRMLN laboratory first to arrange for the shipment. Ship the frozen samples on dry ice by overnight express delivery to the CRMLN laboratory. Before shipping, ensure that the test samples are clearly and indelibly labeled and that the labels will remain secure during shipment and subsequent storage. Provide completed data submission template along with the samples. The CRMLN laboratory cannot analyze the samples until the completed data submission template have been received.

Follow current regulations for handling, packaging, and shipping potentially biohazardous materials.

2.7 TC-IDMS ANALYSIS AND DATA ANALYSIS

The CRMLN laboratory performs TC-IDMS analyses each sample in duplicate over a minimum of 3 runs and provides manufacturers with all results and statistical analysis.

The first replicate from one sample from each run is used to evaluate the bias. The data from all samples and replicates will be used to evaluate for outliers.

After meeting all the performance requirements (Section 3. certification criteria), the manufacturer is issued a dated Certificate of Analytical Quality for Total Cholesterol, stating that the analytical system (including instrument model, reagent lot, and calibrator lot) has successfully demonstrated traceability to the CDC CRMLN under the conditions tested. The Certificate lists the bias compared to the RMP, the total CV, and the calculated total error. A separate certificate will be issued for each analytical system that successfully meets the certification criteria. The date used on the certificate as the "Date of Comparison" is the date that the data is analyzed by the CRMLN laboratory. Certificates expire 2 years after this date. Manufacturers are encouraged to maintain current certification.

Once the accuracy of an analytical system has been validated, conventional in-house QC procedures should be adequate to monitor the system. However, if shifts occur, the manufacturer should undertake another direct comparison with the TC-IDMS RMP of a CRMLN laboratory to reset the system for optimal accuracy. Changes in lots of calibrators or reagents should be carefully checked to maintain accuracy. If the reagent or calibrator formulations or the instrumentation are substantially modified, a new direct comparison will be needed to verify accuracy under the new conditions. This Certification Protocol should be followed in this case.

The CRMLN publishes a list of analytical systems that have been certified through this protocol. The list is available at on the CRMLN Website [12] and includes all systems with a current Certificate of Analytical Quality for Total Cholesterol.

3 CERTIFICATION CRITERIA

Parameter	Criterion	Statistical Approach
r^2*	> 0.95	Linear regression, CLSI EP9
Bias prediction at 200 mg/dL*	± 3.3 %	Linear regression equation; NCEP accuracy guideline, CRMLN Inaccuracy Allowance-Appendix 5.1
Bias prediction at 240 mg/dL*	± 3.3 %	Linear regression equation; NCEP accuracy guideline, CRMLN Inaccuracy Allowance - Appendix 5.1
Average % Bias*	≤ 3.3 %	Mathematical mean of biases; NCEP accuracy guideline, CRMLN Inaccuracy Allowance-Appendix 5.1
Average Absolute % Bias*	± 3.3 %	Mathematical mean of absolute biases; NCEP accuracy guideline, CRMLN Inaccuracy Allowance-Appendix 5.1
Among-run Total Error	≤ 8.9 %	NCEP accuracy guideline
Among-run CV	≤ 3%	CV of QC results; NCEP precision guideline
Z-test of bias	Not significant at $\alpha = 5\%$	Appendix 5.2
Within-method outliers	1 allowed	EP9, Appendix 5.2
Between-method outliers	None allowed, but may eliminate one sample	EP9, Appendix 5.2

*The regression and bias parameters are evaluated using the first replicate of 20 samples (one sample from each run).

4 REFERENCES

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5 APPENDIX

5.1 CRMLN INACCURACY ALLOWANCE

Although the CRMLN has achieved a very low bias compared with CDC, some bias still exists. Statistical analysis has been used to study the distribution of the biases obtained by the CRMLN laboratories.

For total cholesterol, results of statistical analysis of the CRMLN survey data collected from December 1995 through February 2000 shows a Gaussian distribution. The total number of events (one survey material, analyzed by one lab in one survey) during this time period was 1530. The mean bias was 0.01% with a standard deviation (SD) of the bias of 0.45. The biases, by percentile, were -0.2% for the 25th, 0.0% for the 50th (median), and 0.3% for the 75th. Based on this analysis, we have determined that the CRMLN can allow test systems an additional 0.3% bias above the NCEP accuracy limit. This decision was based on the fact that 50% of the CRMLN bias lies between ± 0.3%.

The following table lists the NCEP performance recommendations, as well as the performance allowances for manufacturers and clinical laboratories, as described above.

	NCEP Inaccuracy Allowance	Additional Inaccuracy Allowance	CRMLN Inaccuracy Allowance
Bias	±3.0 %	± 0.3%	± 3.3 %

5.2 Z-TEST AND OUTLIERS

The CRMLN has also implemented the use of a Z-test to evaluate whether the test system's bias is significantly different from the NCEP goal. The variance component of the Z-test utilized in the CRMLN programs is the NCEP's maximum allowable imprecision. The rationale for using this value for the variance is that, since the CRMLN's primary goal is to evaluate accuracy, we do not want to penalize a system that has very good precision. The effect of using this Z-test is that each test system is evaluated for bias assuming that it has the maximum allowable imprecision. The Z-test is performed at various levels for alpha (α). A significant bias at α = 10% should be interpreted as a warning that the bias is very close to the NCEP criterion. A test system that has significant bias at α ≤ 5% is deemed to not meet the NCEP bias criterion and will not pass certification.

The test for within-method outliers is based on the procedure described in CLSI EP9-A [6]. Tests of absolute and relative differences are performed. For the test of absolute differences, the difference between the test method duplicates is calculated for each sample. A test limit is determined which is four times the average difference. Any sample with a difference greater than the test limit is flagged. For the test of relative differences, the difference between duplicates is divided by the test method mean. A test limit is determined which is four times the average relative difference. Any sample with a relative difference greater than the test limit is flagged. Only samples that are flagged by both the absolute and relative tests are within method outliers.

The test for between-method outliers is also based on the procedure described in CLSI EP9-A [6]. Tests of absolute and relative differences between the two methods are performed. For the test of absolute differences, the difference between the test method mean and the reference method mean is

calculated. A test limit is determined which is four times the average difference. Any sample with a difference greater than the test limit is flagged. For the test of relative differences, the difference between the test method mean and the reference method mean is divided by the reference method mean. A test limit is determined which is four times the average relative difference. Any sample with a relative difference greater than the test limit is flagged. Only samples that do not pass both tests are between-method outliers.