CHOLESTEROL REFERENCE METHOD LABORATORY NETWORK (CRMLN)

LDL Cholesterol Certification Protocol for Manufacturers

UPDATED: July 2023

***New additional sample requirement is added for LDLC certification from June 1, 2018.**

Website: https://www.cdc.gov/labstandards/csp/cvd.html

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1 General Information

The three reports from the National Cholesterol Education Program's (NCEP's) Adult Treatment Panel identified low-density lipoprotein cholesterol (LDLC) as the primary target of cholesterol-lowering therapy (1,2,3). The first report established specific medical decision points derived from national population studies. LDLC, as defined by NCEP, includes intermediate-density lipoprotein cholesterol (IDLC) and lipoprotein (a) [Lp(a)] cholesterol. LDLC was determined in the national population studies by either estimation using the Friedewald equation or by determination using the beta-quantification (BQ) method. The NCEP's Working Group on Lipoprotein Measurement made recommendations regarding clinical laboratory measurements of LDLC (4). This group recommended that the link be maintained between laboratory measurements and the existing epidemiologic and clinical databases. The Working Group also recommended that the basis for accuracy for LDLC be based on the current BQ reference method at the Centers for Disease Control and Prevention (CDC). The CDC BQ reference method uses a three-step procedure involving ultracentrifugation, precipitation of the bottom fraction with heparin-manganese reagent, and quantification of both the bottom fraction and the HDLC fractions with the Abell-Kendall reference method for cholesterol. Because of matrix effects when measuring processed samples, the accepted approach for accuracy transfer is a fresh sample comparison with the reference method. Therefore, the Cholesterol Reference Method Laboratory Network (CRMLN) uses a protocol for certification of LDLC methods based on a fresh sample comparison with the BQ reference method. The methods used in CRMLN laboratories are standardized to the CDC's BQ reference method for LDLC. The CRMLN believes that nationwide standardization can be achieved most effectively through the manufacturers of analytical instruments and reagents. This document provides the details of how this program works.

The NCEP Working Group recommends that laboratories perform LDLC analyses with bias

≤ 4.0% from the true value (reference method) and precision, as measured by coefficient of variation (CV), ≤ 4.0% (4). These goals for accuracy and precision suggest that, for a single measurement, the allowable total error would be ≤ 12%. Precision can be improved within each laboratory by adherence to accepted principles of good laboratory practice and quality assurance. Accuracy can be improved by evaluation of traceability to the accuracy base through a fresh sample comparison with the LDLC reference method in a CRMLN laboratory.

Although the CRMLN believes that evaluation of total error is important, it encourages manufacturers to strive to meet the NCEP's accuracy and precision recommendations separately.

The certification protocol for manufacturers is based upon the NCCLS Guideline EP9-A, Method Comparison and Bias Estimation Using Patient Samples (5). The procedures are outlined in this document. The protocol includes a comparison with the reference method using at least 40 fresh specimens. The protocol also includes analysis of a quality control (QC) material in 20 runs.

As of June 1, 2018, CRMLN requires participants to collect and measure five additional samples to address concerns about the accuracy of LDLC measurements in samples with high triglyceride levels and from patients with certain diseases. Please refer to sample collection section (section 2.3) and Appendix B for further information regarding the characteristics of these samples. The intent is to provide information to participants about the analytical accuracy in samples with these characteristics. Currently, they will not be used in the certification process. However, it is planned to add such samples in future assessments for certifications. Certificates will only be issued to those participants providing these five additional samples. The CRMLN laboratory will assign target values to these sample using appropriate reference methods.

Demonstration of absolute average bias ≤ 4% of the reference method and total CV ≤ 4% qualifies an analytical system for certification. The set points assigned by the manufacturer to that system's calibrators should be appropriate to ensure accurate analytical results on patient specimens in the hands of users. Because the NCEP

recommends that clinical laboratories achieve total error ≤ 12% on patient specimens, total error will be calculated. However, certification will be based on meeting the recommended goals for accuracy and precision.

Manufacturers must assume all responsibility for the results and should make the initial contact with the CRMLN laboratory. Manufacturers are advised to contact a CRMLN laboratory before beginning this protocol. A list of CRMLN laboratories is available from the CRMLN website at (https://www.cdc.gov/labstandards/csp/crmln_members.html). One set of fresh samples may be split and used to evaluate several applications, thus necessitating only one evaluation by the reference method.

2 The Certification Process

Anyone collecting and handling any biological material of human origin MUST observe Universal Precautions (6).

2.1 Purpose

The LDLC certification program is intended to assess the bias of the comparison method against the reference method under defined conditions. Not all samples that may be encountered in clinical laboratories are included in the scope of this program. Thus, the protocol is designed to verify the calibration, not the robustness, of the methods evaluated.

2.2 Manufacturer's Preliminary Responsibilities

Before pursuing certification through the CRMLN, manufacturers should establish that their analytical instrument 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 performed and must be in peak operating condition.
- Precision testing (such as that outlined in CLSI Guideline EP05-A3, Evaluation of Precision of Quantitative Measurement Procedures) should be done to ensure that total precision is ≤ 4% (7).

Note: Manufacturers who have the DCM or the RM set up in house may establish its traceability to the accuracy base by evaluation through the CRMLN. Although this internal verification does not substitute for certification of the manufacturer's products, manufacturers can use the DCM or RM in house to check their products before evaluation of traceability by a CRMLN laboratory.

2.3 Manufacturer's Specimen Collection

It is critically important that manufacturers contact a CRMLN laboratory before beginning this protocol. Samples for comparison with the reference method must be sent to the CRMLN laboratory while they are still fresh; frozen samples cannot be used with the reference method. This requires coordination with the CRMLN laboratory to ensure that the samples can be analyzed at the CRMLN laboratory within one day of collection. Because manufacturers must analyze the samples in 5 different runs, multiple shipments will be required.

Specimens must be fresh; fasting donors are preferred. While fresh samples are required for the reference method, frozen samples may be used with the manufacturer's test method if the manufacturer has conducted a careful freeze/thaw study and determined that use of frozen specimens in the test system would not compromise the results. The certification protocol is designed to evaluate analytical method bias only. Therefore, variation from pre-analytical 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 to the reference method. Values for all other blood matrices must be traced back 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 for the analysis of 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 patients 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 fresh specimens from patients. The LDLC concentration of these specimens should be distributed over the clinically meaningful range, as close as possible to the following target distribution:

- 20% of samples from < 100 mg/dL (2.59 mmol/L)
- 30% of samples from 100 to 130 mg/dL (2.59 to 3.36 mmol/L)
- 30% of samples from 131 to 160 mg/dL (3.37 to 4.14 mmol/L)
- 20% of samples from 161 to 400 mg/dL (4.15 to 10.35 mmol/L)

A worksheet for specimen distribution is attached as an aid.

Specimen that are collected for certification must:

- be free of interfering substances known to affect the system being tested (e.g., hemolysis, icterus, lipemia);
- be collected in sufficient quantity (see sample volume requirements for reference method testing below);
- not include samples that the product's package insert indicates should be excluded; and
- be collected using good laboratory practice (such as outlined in CLSI Guideline GP41*, Collection of Diagnostic Venous Blood Specimens* (8)).

Additional five specimen that are collected (not part of certification) must:

- **Include at least one sample from donor with Diabetes Mellitus (type I and type II)**
- **Include at least one sample from donor with Hypertriglyceridemia**
- **Include at least one sample from donor with Cirrhosis**
- **Include two additional sample types (See Appendix B)**
- **be collected in sufficient quantity (see sample volume requirements for reference method testing below).**

Please see Appendix B for further specification of these additional samples.

Participants will be required to indicate the type of samples that are provided during the comparison study. Analyze these additional samples in the same way as the samples that are used for certification.

To make the comparison process reflect reality, the protocol followed should mimic, as much as possible, the conditions in the clinical laboratory. Manufacturers are encouraged to develop package inserts that include information about time frames for storage and analysis of patient samples. Storage times and methods should be evaluated as part of the method development.

This protocol was designed to reflect analytical conditions in clinical laboratories. Balancing this is the need to have the samples analyzed by the manufacturer be as similar as possible to those analyzed by the CRMLN laboratory. Serum should be separated from red cells within 2 hours of collection. The serum samples for the analysis by the reference method should not be frozen. A fresh, unfrozen aliquot (11 mL) of each sample must be sent to the CRMLN laboratory so that it will arrive within 1 day of collection. The manufacturer should store the sample in the refrigerator until the CRMLN laboratory has received the aliquots. The CRMLN laboratory will notify the manufacturer when the samples have arrived, and analysis will begin at the same time at both facilities within

one day of sample collection. This protocol requires that samples for the CRMLN laboratory be shipped in at least five separate runs.

The minimum amount of serum needed per sample for reference method analysis is 11 mL. Specimens must be clearly identified using coded identification numbers and ***no patient identifiers***. Specimens must be securely contained in vials that prevent breakage, leakage, and evaporation.

More than one tube of blood from a single individual will be necessary to obtain enough volume for the CRMLN laboratory analyses as well as the test method analyses. Harvest the serum, combine the contents of the tubes, and mix well before aliquoting the samples. Serum must be provided to the CRMLN laboratory; a separate sample tube from the same person must be collected at the same time if the test method uses either plasma or whole blood.

Difficulty in obtaining a sufficient amount of the sample from one individual can be overcome by combining two specimens-but no more than two-from separate individuals. This combined specimen pool should be treated as an unpooled (single) sample. The combined specimen pool should be mixed well before aliquoting.

Some of the same samples used for certification of other analytes by the CRMLN may be used. Because all samples are unlikely to meet the sample distribution guidelines for all analytes, additional samples should be collected. This will ensure that the guidelines are met for all analytes.

The CRMLN strongly recommends more than 40 samples be sent to the CRMLN laboratory to avoid delays from insufficient samples, outliers or lab accident.

The CRMLN strongly recommends that manufacturers set aside and store (*at -70 °C or lower*) additional aliquots of each sample (or supernate if test method uses precipitation), prepared at the same time that the initial samples or supernates for the certification are prepared. They can be used for reanalysis if changes in calibration are required to meet NCEP criteria. When new lots of calibrators, materials, or reagents are prepared, these frozen samples can provide an important link to the accuracy base during overlap analyses if a frozen versus fresh comparison has been performed.

Collection of patient samples is the responsibility of the manufacturer. However, CRMLN laboratories may assist in this process by collecting some or all of the specimens, as long as the specimens analyzed by both methods are fresh. Alternatively, manufacturers can work with a local clinic or hospital to collect 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 CRMLN laboratory. 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 130 to 160 mg/dL (3.36 to 4.14 mmol/L). A frozen human serum pool is more representative of fresh patient samples and is preferable over the use of a processed material (e.g., lyophilized). The latter may result in higher imprecision leading to a lower error budget for bias. These materials should be analyzed in single using the complete analytical system being evaluated. The runs must include those used in the split sample comparison described below. Use the attached Quality Control Results Form for reporting the results.

2.5 Manufacturer's Specimen 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 reference method. Clinical laboratories that perform successful comparisons on behalf of a manufacturer will also receive a Certificate of Traceability for LDL Cholesterol.

Calibration is the key to achieving accuracy; therefore, these comparison runs should represent the conditions recommended to customers. Calibrators should be analyzed along with the patient samples.

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 of the fresh 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 Traceability for LDL Cholesterol.

Follow the protocol for collecting, dispensing, and storing samples as described on pages 3 - 5 of this document. Store the samples at 4° before analysis and begin analysis of all specimens at the same time that analyses are begun in the CRMLN laboratory. Analyze each sample by the test method in duplicate. Randomize the concentrations in the run sequence. Assign the first set of aliquots from each specimen sequential positions in the run. Run the duplicate measurement of each specimen in reverse order.

Include the QC material selected above in every analytical run.

Analyze the specimens over at least 5 runs, one run per day. The number of samples per run is limited by the capacity of the reference method, so this must be coordinated with the CRMLN laboratory. Two runs per day are acceptable, provided that the runs are separated by 2 hours (NCCLS definition of a run, per EP5-A).

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 instruction provided in your package labeling (i.e., do not follow in-house modifications).

If an instrument problem develops during a run or internal QC is unacceptable, specimens from that run can be retested after the problem has been identified and corrected if it has been demonstrated that storage of samples does not affect the result.

Complete the Information Form, the Quality Control Results Form, and the Results Form for each method tested. Photocopy the blank forms and save them for future comparisons. Send the recorded results for each method to the CRMLN laboratory

2.6 Shipment of Specimens to the CRMLN laboratory

Contact the laboratory first to arrange for the shipment. Ship the samples on cold packs by overnight express delivery to the CRMLN laboratory. Care must be taken to ensure that frozen cold packs do not come into direct contact with the samples. Samples should be shipped between Monday and Wednesday to ensure delivery and analysis by the CRMLN during the workweek. Before shipping, ensure that the test specimens are clearly and indelibly labeled and that the labels will remain secure during shipment and subsequent storage. Include the Information Form with the samples.

A Protocol Checklist is provided. Please refer to the checklist to make sure that all requirements in this protocol have been met.

Follow current federal and state regulations for handling, packaging, and shipping potentially biohazardous materials with regard to containment, labeling, and other procedures.

2.7 Reference Method Analysis, Data Reduction, and Cost

The CRMLN laboratory will analyze each specimen in duplicate **including the additional samples**. Manufacturers will be provided with the results of the laboratory and statistical analyses. The approximate turnaround time for reference method analysis and the data analysis is 3-4 weeks from receipt of samples but could depend on the

analytical workload at the CRMLN laboratory. If a manufacturer wishes to evaluate more than one analyses (e.g., total cholesterol, HDLC, and LDLC), the reference method analyses could take longer than 3-4 weeks.

Because the reference method is manual and tedious and must be maintained within very stringent limits for accuracy and precision the assay is costly. Refer to Appendix A for current pricing. Manufacturers may test the same set of specimens on several instruments, calibrators, or reagent systems. There is a nominal fee for evaluating each additional system to cover data analysis costs.

After meeting all the certification criteria, the manufacturer will be issued a dated Certificate of Traceability for LDL Cholesterol, stating that the analytical system (including instrument model, reagent lot, and calibrator lot), has successfully demonstrated traceability to the LDLC accuracy base under the conditions tested. The certificate will list the bias versus the reference method, the among-run CV, and the calculated total error. A separate certificate will be issued for each analytical system that successfully meets the NCEP performance recommendations. Certificates expire 2 years after the date of the fresh sample comparison. The date used on the certificate is the date that the data is analyzed by the CRMLN laboratory. Manufacturers are encouraged to maintain current certification.

Once the analytical system has been certified, conventional in-house QC procedures should be adequate to monitor the system. However, if shifts occur, another direct comparison with the CRMLN should be undertaken 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 Method Certification Protocol should be followed in this case.

The CRMLN publishes a list of manufacturers' systems that have been certified through this protocol. The list is available at https://www.cdc.gov/labstandards/csp/crmln_certified_manufacturers.html and includes all systems with a current Certificate of Traceability for LDL Cholesterol.

Questions about this protocol should be directed to a CRMLN laboratory (see https://www.cdc.gov/labstandards/csp/crmln_members.html for a list of CRMLN member laboratories). Copies of this protocol are also available at the CRMLN website.

3 Statistical Criteria used for Certification

3.1 The statistical criteria used for CRMLN LDLC certification

Parameter	Criterion	Statistical approach
r2	> 0.975	Linear regression
Bias at 100 mg/dL (2.59 mmol/L)	≤ 4%	Linear regression equation; NCEP accuracy guideline
Bias at 130 mg/dL (3.36 mmol/L)	≤ 4%	Linear regression equation; NCEP accuracy guideline
Bias at 160 mg/dL (4.14 mmol/L)	≤ 4%	Linear regression equation; NCEP accuracy guideline
Average % Bias	≤ 4%	Mathematical mean of biases; NCEP accuracy guideline
Average Absolute % Bias	≤ 4%	Mathematical mean of absolute biases; NCEP accuracy guideline

Parameter	Criterion	Statistical approach
Among-run CV	≤ 4%	CV of QC results; NCEP precision guideline
T-test of bias	Not significant at α = 5%	See below
Within-method outliers	1 allowed	EP9-A, see below
Between-method outliers	None allowed, but may eliminate one	EP9-A, see below

CRMLN members participate in surveillance to evaluate their performance versus the CDC accuracy base. They are required to meet very strict performance criteria, which are listed in the following table.

3.2 Performance Criteria for CRMLN Laboratories

Accuracy Criterion	Imprecision Criterion
Bias ≤ 2%	CV ≤ 1.5%

The bias of the CRMLN laboratory is the most important factor that needs to be controlled to make appropriate decisions about the performance of test systems. 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 LDL cholesterol, results of statistical analysis of the survey data collected from July 1999 through July 2002 show a non-Gaussian distribution. The total number of events was 203. The mean percent bias was -0.4% with a SD of the bias of 1.5%. The percent biases, by percentile, were -1.1% for the 25th, -0.2 % for the 50th (median), and 0.4% for the 75th. We have determined that the CRMLN can allow test systems an additional –0.4% to 1.1% bias. This is applied asymmetrically so that the allowable bias is -4.4% to 5.1%.

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

3.3 Performance Criteria for Manufacturers and Clinical Laboratories

NCEP Inaccuracy	NCEP Imprecision	CRMLN Inaccuracy Allowance
Bias ≤ 4 %	CV ≤ 4 %	-4.4% to 5.1%

The CRMLN has also implemented the use of a t-test to evaluate whether the test system's bias is significantly different from the NCEP goal. The variance component of the t-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 t-test is that the test system is given some benefit of the doubt. The t-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 NCCLS EP9-A (5). 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 NCCLS EP9-A (5). 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.

4 References

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5 Appendix A: LDL Cholesterol Certification Protocol for Manufacturers Fee schedule

(Using betaquantification [BQ] reference method for LDL cholesterol)

Description	**Fee**
Fresh patient sample comparison Duplicate BQ analysis of 40 patient specimens Shipping charges paid by manufacturer Data analysis for one instrument/method, material, or reagent application One repeat data analysis, if necessary	$7000.00
Additional specimens (duplicate BQ analyses)	$175.00/specimen
Data analysis for additional applications	$125.00/method

*Additional administrative charges may apply

6 Appendix B: Specifications for the additional samples used for certification of LDLC measurement

Samples from patients with the first three disease/conditions are required. Two samples with other diseases/condition listed in the table can be used. Multiple samples with the same disease/condition can be used (i.e., two samples from patients with hypertriglyceridemia). However, in such situations, samples need to be obtained from different donors.

Required	Disease/Condition	Suggested biomarker	Biomarker levels
✓	Diabetes Mellitus (type I and II)	HbA1c Glucose	>6.5 % ≥7.0 mmol/L
✓	Hypertriglyceridemia	Total Glycerides	>400 mg/dL (4.52 mmol/L)
✓	Cirrhosis	Serum albumin, prothrombin time, either of the followings: Liver biopsy result Abdominal sonography ALT AST GGT	Liver enzymes above normal range or diagnosis by biopsy or sonography
	Lipid-lowering drugs including: Statins Ezetimibe Fibrates Niacin	Medication History of lipid lowering drugs Creatinine kinase	
	Dyslipidemias: Type I Type II Type III Type IV Type V	Total cholesterol Triglyceride Lipoprotein EP	>= 300 mg/dL (7.758 mmol/L) (and/or) >= 250 mg/dL (2.825 mmol/L)
	CVD, nonspecific	Troponin T or Troponin I LDL-cholesterol Lipoprotein (a)	Troponin, >99 percentile limit and/or Lp(a) > 30 mg/dL
	Prior CVD event		
	Renal insufficiency/End Stage Kidney Disease (ESRD)	Creatinine Cystatin C	Over 3 mg/dL (Creatinine)
	Other dyslipidemias: Familial hypertriglyceridemia Abetalipoproteinemia Chylomicron retention disease LCAT deficiency Niemann-Pick disease type Erdheim Chester disease	If possible ApoA-I, A-II, A-IV, B-100, C-I, C-II, C-III, E	