

Whole-blood test for total cholesterol by a self-metering, self-timing disposable device with built-in quality control

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A whole-blood test for total cholesterol has been developed that is performed in a low-cost disposable flow device without user intervention (after sample addition). The device meters the sample, separates plasma from erythrocytes, and precisely times plasma flow into various reagent compartments, including a quality-assurance chamber. Test results are displayed as a well-defined and easily readable color bar. A quality-control window attests to the integrity of the test components. Here, we describe the assembly and individual functions of the device and report its performance characteristics. Precision and accuracy studies in four clinical studies at independent locations yielded total imprecision of <5% and an average bias of 1.35% vs the Abell–Kendall method.

INDEXING TERMS: enzymatic methods • near-patient testing

Among the first-generation solutions to the challenge of providing noninstrumented test devices for quantitative testing on whole-blood samples, the AccuMeter cholesterol test developed by ChemTrak (Sunnyvale, CA) provides quantitative results generated by two to three drops of blood deposited on a disposable cassette [1]. This cassette contains a complete reagent system that translates the enzyme-mediated oxidation of cholesterol and cholesterol esters into a visible and measurable color bar, produced by the horseradish peroxidase-catalyzed reaction of hydrogen peroxide with 3-methyl-2-benzothiazolinone hydrazone followed by diazotization of an immobilized aniline dye. The length of the colored bar is related

to concentration units in a table accompanying the system, which is used to look up the results of the assay. A different technical approach, based on analog-to-digital switching technology, has the advantages of direct read-out of results and use of dry reagents in all compartments of the device [2]. However, both approaches still require timing of the reaction steps and supplemental user intervention after the whole-blood sample is deposited on the device, and neither has provisions to prevent undersampling or includes built-in controls that attest to the integrity of the reagents.

The Analytical Chromogenic Transport (A.C.TTM; ActiMed Labs., Burlington, NJ) technique described here lends itself to the development of noninstrumented quantitative test devices that require no further user intervention after addition of the sample [3–5]. The design of such devices assures that the analytical process does not start unless sufficient sample has been added, thus preventing undersampling. Results are read directly from the device after the color of a quality-assurance (QA) window has turned green. The color change assures the user that the test is complete and that the most fragile components of the analytical cascade are intact. The resulting device lends itself to uses at alternative testing sites outside the hospital laboratory. Here, we describe the device as applied to testing for total cholesterol in fingerstick whole blood, i.e., in the ENA.C.TTM Total Cholesterol Test (ActiMed Labs.).

Principle and Device Design

The structural elements of the A.C.T cholesterol test device are shown in the exploded-view drawing (Fig. 1). The components are assembled in a completely automated, computer-controlled manufacturing process, which allows overall continued accuracy assurance through a feedback control.

To assure plasma transport from the sample well that

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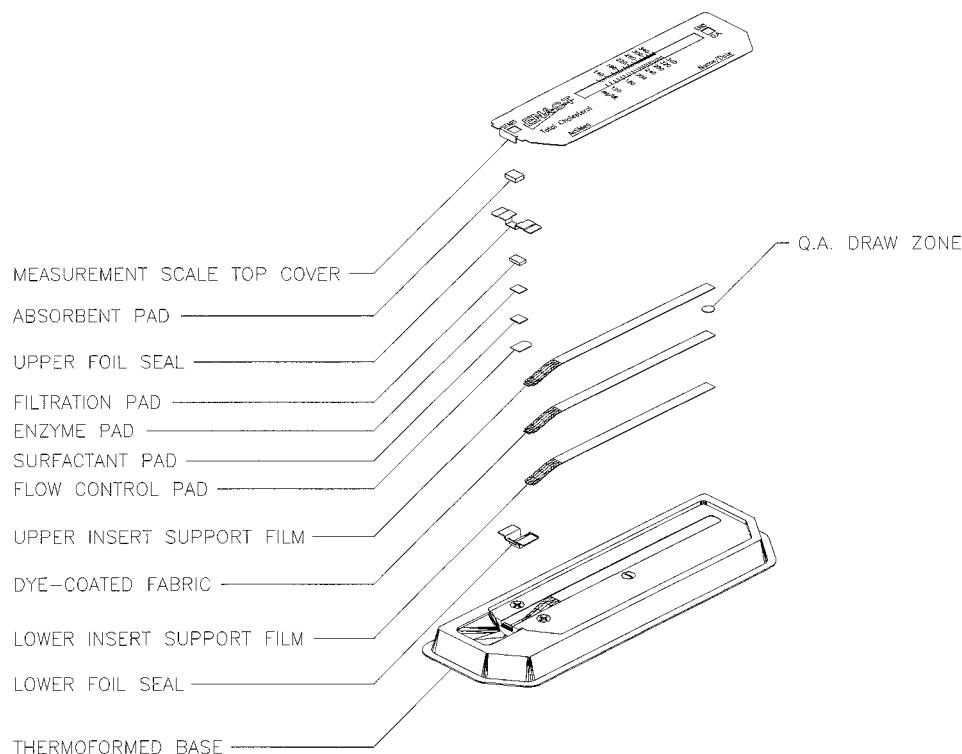


Fig. 1. ActiMed ENA.C.T total cholesterol device (exploded view).

receives whole-blood samples through to the QA draw-zone at the end of the device, the device uses a gradient of increasing surface energy established along the path of the liquid flow. To further enhance flow through the device, the design minimizes the resistance to flow, particularly in the front section, for which a stack of special filters was developed. These filters accommodate the use of blood within a wide range of hematocrit values and lipid concentrations. Other diverse functions the filters perform include separation of erythrocytes from plasma, addition of enzymes and detergents to the resulting plasma with complete mixing, retention of the plasma solution sufficiently long to ensure complete conversion of cholesterol and cholesterol esters into cholestenone and hydrogen peroxide, and subsequent release of the reacted plasma into the measurement area of the detection zone. The filters are fastened in place by a hotmelt-coated aluminum foil seal activated remotely by a radiofrequency sealer. This sealing process assures that plasma flows through the filters rather than around them.

Plasma leaving the filter stack is directed into the detection zone. In this zone a hydrogen peroxide-sensitive dual-component Trinder-type dye system is coated onto a fabric that is sandwiched between two plastic foils. This creates an enclosed flow channel through which the plasma travels, its hydrogen peroxide content generating a quantitative, visible color signal that is directly converted into concentration units via a scale printed on the cover of the device. The end of the detection zone connects with the QA draw-zone, where another cholesterol-converting reaction occurs (this time with exogenous

cholesterol) to verify the stability of key components of the reagent system that are now dissolved in the plasma sample.

Materials and Methods

REAGENTS

Cholesterol oxidase (EC 1.1.3.6), cholesterol esterase (EC 3.1.1.13), and horseradish peroxidase (EC 1.11.1.7) were purchased from Toyobo, Osaka, Japan; pancreatic cholesterol esterase was purchased from Diagnostic Chemicals, Oxford, CT. The sodium salt of 3-methyl-6-carboxy-2-benzothiazolinone hydrazone (NaCMBTH) is prepared at ActiMed. Sodium cholate, sodium chloride, lactose, and hydroxylamine hydrochloride were from Aldrich Chemical Co., Milwaukee, WI. Lectins, lithium heparin, Pipes buffer, and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO.

COMPONENTS OF THE A.C.T DEVICE

Device base. The base of the device is made of polyester thermoformed into a shape that accommodates the placement of all structural elements for the analytical processes. The surface of the sample well, an integral part of the base, is rendered hydrophilic by treatment with corona discharge followed by spray-coating with polyacrylic acid containing a heparin derivative.

Multipad front-end stack. The absorbent pad, loosely woven Orlon® (obtained from Lydall, Hamptonville, NC), sits on top of a multielement stack of porous pads. Its front end touches the upper end of a ridge that connects to the

sample well. Once the blood sample reaches this pad via the ridge, sufficient blood is aspirated to supply the device with all the plasma needed to perform the test. The pad is cut by laser from a large sheet of the material, impregnated with a proprietary agglutination solution based on lectins, and subsequently air-dried. Agglutinated erythrocytes are trapped in this pad.

The secondary separation pad for the agglutinated and free erythrocytes is positioned under the absorbent pad. It allows the plasma to drain from the agglutinate into the next lower pad, the enzyme pad, which is impregnated with cholesterol esterase (1000 kU/L) and cholesterol oxidase (750 kU/L). The enzyme pad is constructed from glass fiber material, which has a large surface area, and the enzyme reagent contains porous bulking agents that trap large amounts of air pockets inside the pads. This combination allows for rapid oxygen diffusion into the plasma to support the cholesterol oxidase-catalyzed oxidation reaction.

Supplemental reagents and additives such as sodium cholate, hydroxylamine hydrochloride, sorbitol, and buffer are added to the enzyme-enriched plasma when it enters the surfactant pad. Hydroxylamine in this pad acts to inhibit catalase, basically functioning as a competitive inhibitor.

A flow-control pad, placed at the bottom of the stack, reduces the speed of elution of the enzyme-containing plasma from the enzyme pad into the detection channel without imparting additional resistance to flow once the pad is fully impregnated with sample. This is designed to provide a 1- to 2-min incubation period for the cholesterol reactions to reach equilibrium without user intervention [6].

The stack of pads is sealed in place by a combination of two seals formed with hotmelt (National Starch Co., Bridgewater, NJ)-coated aluminum foil (activated by heat generated remotely by a radiofrequency sealer). The seals prevent the flow of plasma around the pads and direct flow across the whole surface of the pads.

Measuring channel and detection zone. The measuring channel is formed by heat-sealing the upper and lower polyethylene insert support films into a sandwich around a coated, very precisely woven fabric (Polyester PES 105/52; Saati Corp., Stamford, CT). The coating contains a derivatized aniline dye covalently linked to silica particles <3 μm in diameter (Degussa, Ridgely Park, NJ), NaCMBTH, horseradish peroxidase, and various stabilizers embedded in an acrylic/polyethylene copolymer. The polymer coating is applied in a tightly controlled process by an automated coating machine. The precision of the coating process is illustrated in Fig. 2, which shows the woven fabric and the layer of reactive coatings. The measuring channel also contains a QA draw-zone, which contains free cholesterol, horseradish peroxidase, and dye.

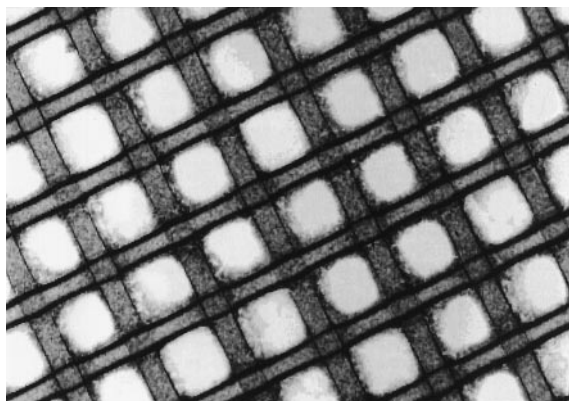


Fig. 2. Detection film: reactive coating on precision woven fabric.

In the manufacturing process, the materials of the detection zone are continuously fed into a six-element heat-sealing station. The zone is subsequently cut out by laser, automatically transferred to the thermoformed tray, and glued in place.

Top cover. The top cover of the device is placed on the tray at the end of the process. The markings of the concentration scale are printed on the cover just before the cover is placed on the fully assembled device. The distance and spread of these markings may be continually modified to adjust for small variations in the manufacturing process and assure continued accurate readings of the results. A window in the top cover allows a view of the top of the absorbent pad during the analysis.

ASSAY PROCEDURE

Fingerstick blood is drawn from an appropriately prepared individual with a single-use lancet [7]; the first drop of blood is discarded. Blood is added to the sample well of the device until the "start window" on the top cover turns red (i.e., after at least 80 μL of whole blood has been added). This event signals that blood has been transferred to the absorbent pad and that a sufficiently large sample has been obtained. No further user intervention is required until the QA draw-zone window turns green, after which the length of blue color zone may be read in concentration units.

Erythrocytes are separated from the plasma within 30 s and the plasma travels through the stack of pads until retained by the flow-control pad. Hydrogen peroxide-containing plasma enters the detection channel, where it reacts with the dye system embedded in the polymer coating wrapped around the precisely woven mesh. The length of the color bar that develops is proportional to the concentration of hydrogen peroxide, and hence total cholesterol, in the plasma. Depending on the hematocrit and the lipid content of the subject's blood, the plasma front reaches the QA draw-zone in 12 to 20 min. Because the draw-zone changes color only when all enzyme systems are still active, the results can be read off the device

in concentration units. The blue color bar remains stable for months and thus constitutes a "permanent" record.

Results

Dynamic range. The dynamic range of the ENA.C.T Total Cholesterol Test was established from calibration curves prepared by assaying both lipoprotein-supplemented samples and fresh whole-blood samples with multiple replicates of the devices. These "calibrators" were also assayed by the Abell-Kendall method [8]. The dynamic range so established was 1200 to 3600 mg/L total cholesterol (Fig. 3).

Accuracy. The accuracy of the ENA.C.T test devices was established by direct comparison with the Abell-Kendall method, both procedures being used to assay fresh patients' samples. Fingerstick samples from four clinical test sites were assayed by the test devices, whereas venous serum samples obtained at the same time were shipped to a reference laboratory (a member of the National Reference Method Laboratory Network) for Abell-Kendall analysis. Fig. 4 (top) shows the values obtained by the ENA.C.T Total Cholesterol test vs the reference Abell-Kendall method and the linear regression equation obtained. The average bias in this comparison over the analytical range of 1200 to 3600 mg/L was 1.35%.

The ENA.C.T test devices were also compared with the AccuMeter, another disposable device that accepts fingerstick whole blood. The results (Fig. 4, bottom) demonstrated that the two devices give substantially equivalent performance.

The Laboratory Standardization Panel for Blood Cholesterol Measurement has recommended that the bias of this measurement should not exceed 3%. The average bias of the ENA.C.T Total Cholesterol Test for all four clinical

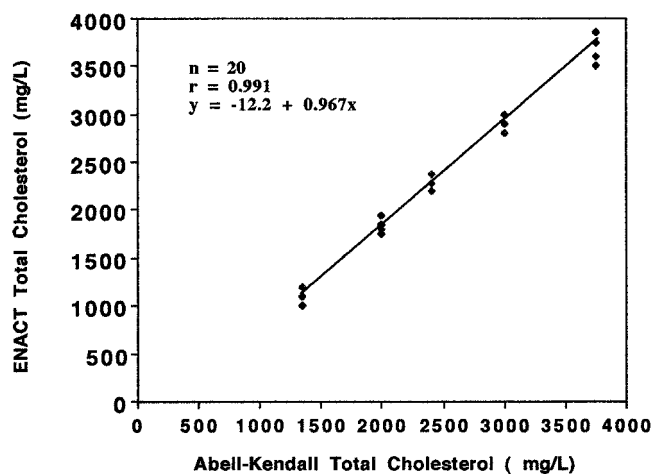


Fig. 3. Dynamic range of the ENA.C.T total cholesterol assay.

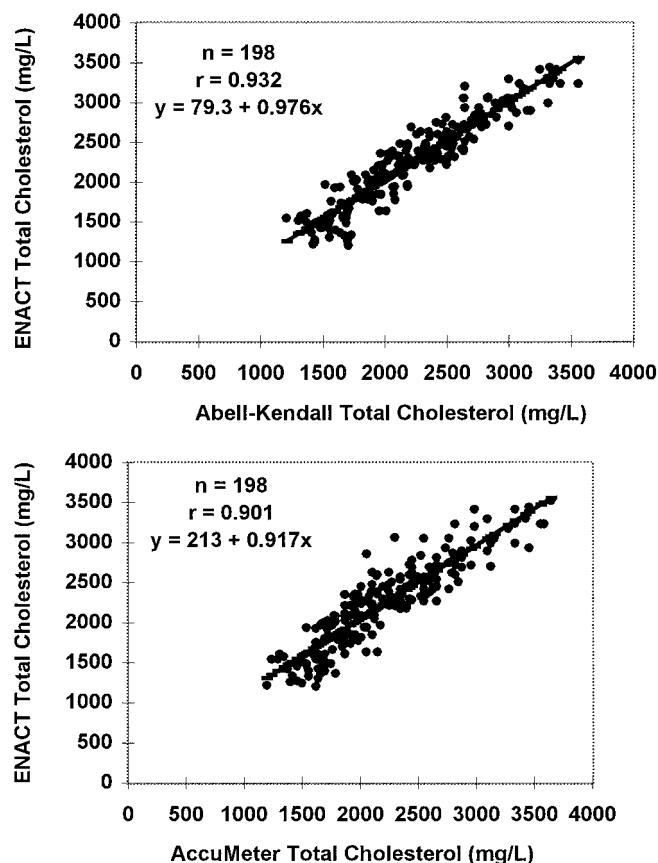


Fig. 4. Correlation between ENA.C.T (fingerstick) total cholesterol and (top) Abell-Kendall (serum) total cholesterol or (bottom) AccuMeter (fingerstick) total cholesterol.

sites combined, at the medical decision levels of 2000 and 2400 mg/L, were 1.6% and 0.9%, respectively, in comparison with the values determined by the Abell-Kendall method.

Precision. Within-run precision studies were carried out at four clinical sites with two controls containing cholesterol concentrations near the medical decision points. Run-to-run precision was established by assaying two concentrations of controls over 5 days in the field. The results ranged from 2.11% to 4.82% and from 2.58% to 5.04%, respectively (Table 1).

Heparinized whole-blood precision studies were also performed (at ActiMed Labs.). The data are shown in Table 2.

Interferences. Several biologically and chemically important compounds often present in whole blood were tested for their effect on the ENA.C.T Total Cholesterol Test. Concentrations (mg/L) of potential interferences below which there was no measurable effect on the test were as follows: ascorbic acid, 80; acetaminophen, 10; uric acid, 100; bilirubin, 150; hemoglobin, 2000; triglycerides, 8000.

Table 1. Precision for controls (n = 20).

	Within-run				Between-run			
	Site 1	Site 2	Site 3	Site 4	Site 1	Site 2	Site 3	Site 4
Control 1								
Mean, mg/L	1838	1810	1835	1780	1838	1810	1835	1780
SD, mg/L	76.6	87.3	54.8	78.3	85.5	91.4	69.0	84.5
CV, %	4.17	4.82	2.98	4.40	4.65	5.04	3.76	4.75
Control 2								
Mean, mg/L	2540	2563	2610	2555	2540	2563	2610	2555
SD, mg/L	53.6	87.3	73.3	92.9	79.5	80.2	67.3	95.1
CV, %	2.11	3.41	2.81	3.65	3.14	3.13	2.58	3.73

Discussion

The A.C.T technology was developed to generate diagnostically valid results outside the classical clinical laboratory. To minimize the frequency of errors in the use of the device, its operation had to be very simple. A design that would circumvent all timing steps and all manual interventions after sample addition was indicated. In addition, it was important to provide the untrained user with prompts and control features to signal that sufficient sample had been added, to signal when to read the concentration off the device scale, and to assure the user that the results as read are valid. For this reason we developed an undersampling safeguard to signal when sufficient sample has been added to the device and to assure the user that the test is never initiated with an insufficient amount of sample. The safeguard signal is triggered only after a sufficient amount of sample has been added to the device.

The QA draw-zone provides an independent quality check for the integrity of the reagent system. The addition of small amounts of exogenous cholesterol with an appropriate amount of cholesterol oxidase—the most labile component of the reagent system—creates an additional reaction chamber where a separate cholesterol detection reaction is performed. This occurs at the end of the analytical process when the plasma sample (from which all hydrogen peroxide has been removed through the detection process) enters the chamber. Once the activity of cholesterol oxidase has been reduced to a certain extent, the reaction rate drops precipitously (see Fig. 5). This provides a well-defined narrow range of enzyme activity below which the QA window reaction does not occur, thus providing an unequivocal message to the user. The addition of this QA feature allows the user to know whether the device has been compromised (e.g.) by heat stress.

Table 2. Within-run precision of assay with heparinized whole blood (n = 10).

	Sample 1	Sample 2	Sample 3
Mean, mg/L	1600	2240	2740
SD, mg/L	54	97	77
CV, %	3.38	4.31	2.83

Cell separation. The separation of cells from plasma had to be performed in a way that would provide minimal resistance to the continued flow of plasma through the device. This excluded any methods relying on mechanical barriers and selective adhesion of cells to surface-active materials (e.g., glass fiber paper) from which plasma is only partially drained. We instead developed an approach in which lectins attached to nucleating particles rapidly agglutinate erythrocytes to form large, well-defined aggregates of cell clusters and allow cell-free plasma to drain freely into the subsequent compartments of the device.

Flow control. Flow of plasma through the device had to be physically controlled to ensure complete reagent dilution; flow around, instead of through, the reagent pads had to be prevented. This was achieved by a novel sealing technique in which two hotmelt-coated aluminum foil strips are placed around the stack of pads. After assembly, the hotmelt layer is heated by remote control, which causes the hotmelt to flow into the rims of the pads and effectively seal them off.

Precision and accuracy. Precise and accurate total cholesterol test results are important for establishing the risk of coronary heart disease and obtaining the proper treat-

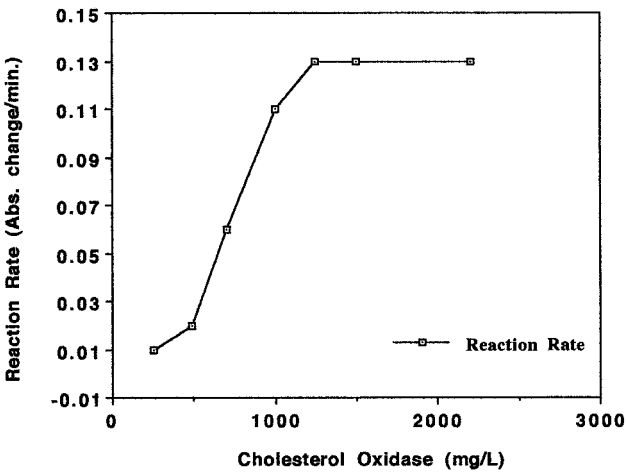


Fig. 5. QA draw-zone titration curve.

ment. The National Cholesterol Education Program risk categories for adults, established in 1988, are: <2000 mg/L total cholesterol (desirable), 2000–2400 mg/L (borderline high), and >2400 mg/L (high enough for patients to seek medical treatment) [9]. The ENA.C.T total cholesterol test has been designed to be very simple to use, and to give results as accurate as most of the major clinical analyzers that perform within the National Cholesterol Education Program analytical guidelines—i.e., that cholesterol tests should be precise (<3% CV) and accurate (<5% bias from the Abell-Kendall Reference Method) [10].

In summary, we have developed a truly one-step disposable system that is read like a thermometer, is quantitative, and is as accurate and precise as instrumented methods. The system is easy to use, requires no technical expertise or training, can be performed anywhere, and gives results in <20 min. The A.C.T test device was designed as a platform and is easily adaptable for use with other analytes. Basically, the enzyme pads can be changed and additional filter pads added to the front end; all other components of the platform remain the same. We have demonstrated working prototypes for assays of glucose, triglycerides, HDL, and LDL [11, 12].

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