Enzyme Immunoassay System for Panel Testing

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An immunoassay system based on enzyme immunoassay technology has been developed for quantitative panel testing. The system includes test card disposables, reagents, and an instrument. Patients’ samples are processed semi-automatically in the instrument with minimum user intervention. The test card has multiple test areas at individual locations on a membrane solid phase so that simultaneous determinations from a single specimen are possible. Each panel also includes positive and negative reagent procedural controls. Factory-determined calibration curves for each analyte are provided in barcode form with each test kit. The reagents include a specimen dilution buffer, enzyme conjugate, and precipitrogenic substrate. Up to 10 test cards at a time can be processed in random-access and continuous-access modes, with automated agitation of sample and reagents over the solid phase, temperature-controlled incubation, and membrane washing and reading, data reduction, and printout of results. The optical reader measures diffuse reflectance and features source intensity and wavelength compensation.

Additional Keyphrases: nitrocellulose membrane • reflectance photometry

One of the more significant trends in diagnostic testing technology is the move from large centralized sites to de-centralized sites, e.g., the smaller laboratories, physicians’ group practices, hospital-immediate care centers, and physician’s offices. Advances in electronics, mechanical systems, and chemistry have allowed automation of the critical and tedious functions of performing blood analysis. Another benefit of rapid advances in electronics and mechanics is that, even though system complexity goes up, component costs are decreasing, so that system designers can use sophisticated modules while still keeping cost in check.

Quantitative enzyme immunoassay (EIA) methods, being largely manual techniques with long incubation times, laborious wash and transfer steps, and poor precision, have not migrated to the alternative care sites. We have developed an instrument/reagent system that allows the user to run a panel of quantitative EIA tests in a simple, cost-effective format. The instrument is designed to be small, automated, and easy to use, with minimized critical operator functions.

Principle

The assays of the EIA panel are performed in a test card on a membrane solid phase, embossed to provide individual reaction sites for each assay in the panel. Each capture protein is applied to its specific area on this array so that a panel of specific antibody assays is constructed. Anti-human immunoglobulin antibody, anti-conjugate antibody, and membrane-blocking material are applied to other sites, to provide the following additional information: an assay to indicate total antibody, a procedural control to confirm that the reagents have been added and in the correct order, and a negative control to detect nonspecific binding. The assay protocol typically has four steps (Figure 1): sample and buffer incubation, conjugate incubation, substrate incubation, and dry & read. The analyzer in this system performs these steps automatically after reading the barcodes on the test card to determine assay protocol.

In the sample incubation step, the prepared solid phase is reacted with the patient’s sample. During this timed incubation, specific antibodies in the sample bind to their corresponding antigens on the solid phase as well as to the total antibody site. Little binding occurs at the procedural and negative control sites. At the end of this incubation, the instrument automatically carries out a wash cycle and alerts the user to add conjugate. Because the membrane is in a washed state at this stage, it is not critical for the user to add reagent immediately.

In the second step, conjugate is manually applied to the test card through a port in the instrument door. During the ensuing incubation, the enzyme-labeled conjugate binds to any human antibody that was bound to the solid phase during sample incubation. As before, the instrument times this incubation and performs a wash cycle at its conclusion.

The substrate incubation step proceeds in the same manner as the conjugate step. The user adds a colorless substrate in solution, which is acted upon by an enzyme of the bound conjugate to produce a colored product that precipitates at the site of reaction.

After the wash cycle, the instrument alerts the user to remove a lid from the test card. The membrane is then dried in the instrument and the optical reflectance is read. The location of color development identifies the specific antibody, and the color density is proportional to the amount of antibody bound. From the density readings and the internally stored calibration information, the instrument determines and provides a printout of the antibody quantity present.

Materials and Methods

Apparatus

General description. The analyzer (Figure 2) is 43.2 × 56.0 × 25.4 cm (l × w × h) and weighs 14.5 kg. It consists of the following major subsystems: random-access positioning mechanism for carousel, optics boom, and wash boom; carousel chamber temperature control (at 35 °C); carousel; wash/waste fluid handling; handheld barcode decoder; human interface, including display, speaker, keypad, and printer; optical calibration reference; power and microprocessor electronics; and user-changeable software module. The analyzer is designed to operate within an ambient temperature range of 15 to 30 °C and a relative humidity range of 10% to 90%.

Traditionally, EIAs have been performed with use of a
polystyrene bead, tube, or microtitre plate as the solid phase. We use instead a flat piece of membrane (nitrocellulose) that has been embossed by an ultrasonic horn to form multiple isolated islands. Typically, the scoring pattern is a $5 \times 6$ array of 2.5-mm-diameter circles to which the capture protein can be applied. The surface of the nitrocellulose provides high-affinity binding of the proteins, as well as a uniform surface for scanning with the diffuse reflectance reader. The capture proteins are applied and dried. To prevent nonspecific binding on the areas of the membrane not treated with a protein, a "blocking" step is performed. The array is soaked in horse serum at 37 °C, then washed in a Tris-buffered saline and left to dry. The solid phase is then assembled into a plastic (acrylonitrile-butadiene-styrene) test card.

The reagent kit typically contains three solutions: Reagent A, the sample dilution buffer (equine serum in buffer with azide); Reagent B, the conjugate (an anti-human antibody labeled with alkaline phosphatase with azide); and Reagent C, the substrate (alkaline phosphatase-responsive chromogen with azide).

Carousel and mechanical positioning system. The carousel and mechanical positioning system are designed to maximize automated sample handling by providing the capability to agitate the reagents during incubation, wash the test card automatically, and perform optical reads without removing the cards from the carousel. The key features of this system are drive mechanisms that allow random-access positioning for the carousel, optics boom, and wash boom; the 10° tilt of this assembly to the horizontal plane of the benchtop; and the tightly controlled parallelism of the optics boom plane of travel to the plane defined by the test-card surfaces.

The carousel is driven by a stepper motor and associated gear train. Positional feedback is provided by "flags" molded into the carousel that cut an optosensor beam to produce an "interrupt" to the microprocessor. The carousel can be rotated clockwise or counterclockwise, continuously or incrementally. The optics boom is driven by a stepper motor and swings in a plane horizontal to the carousel. It has one "flag" to define its "home" position. Its range of motion extends from the reference post (where the optics calibration target is located) to approximately the center of the carousel. The wash boom is attached to the optics boom so it also is free to swing horizontally. In addition, it has its own stepper-motor drive and "home flag" for vertical positioning. This allows the wash probe to be driven through the port and into the test card, to dispense wash and aspirate waste.

Tilting the entire chassis assembly by 10° (the rear being higher than the front) allows for gentle agitation of the reagents and wash fluid as the carousel rotates. Gravity pulls the fluid toward the wall that is on the downside of the slope (Figure 3). During incubations, the carousel rotates continuously counterclockwise at approximately 8 rpm.

For the results on the cards to be accurately read on the carousel, the mechanism must precisely locate the test card.
in three dimensions to ensure that the optics boom plane of travel is parallel to the plane defined by the test-card surfaces. Figure 4 shows the details of a mechanism that is molded in the carousel to provide precise three-dimensional positioning of the test-card. The locating tabs and ribs precisely define the test-card position. A series of springs forces the test card against the locating features.

**Temperature control system.** The air temperature of the carousel chamber is controlled by a thermistor in the air inlet to the chamber and a thermistor in the exhaust stream, such that the temperature of the fluid in the test card is maintained at 35 ± 0.5 °C.

**Wash/waste delivery system.** To perform a wash, the wash boom is placed over the port in the test-card lid, lowered through the port, and the waste pump is activated to remove the residual sample or reagents. The wash pump is turned on to deliver 1 mL of wash solution. The probe is then retracted and the carousel will rotate for several minutes to agitate the wash solution. The aspirate/delivery cycle is then repeated twice more before the final aspiration. The expected life of the tubing on the peristaltic pumps under normal use is greater than six months.

**Diffuse reflectance reader.** The action of the enzyme on the substrate causes a precipitate to form on the surface of each analyte area. The precipitate causes a change in the color of the site. By illuminating the island, then measuring the light diffusely reflected from the surface, an analyte concentration can be calculated. A high-intensity (nominal 3000 mcd) light-emitting diode (LED) is used as the light source, at a nominal wavelength of 660 nm and with a 25-nm bandwidth. In an otherwise dark environment, the LED light is focused onto the analyte island. Light that is reflected in a specular pattern (i.e., the angle of reflection is equal to angle of incidence) is rejected by the optics, to avoid signal changes due to surface morphology.

The angle between the illuminating light axis and the detector axis was chosen as 30°, to balance the need for specular light rejection (for which the ideal angle is 45°) and the need for reasonable access to the viewing area within the walls of the test card sample well.

The secondary detector, used to monitor the intensity of the LED source, is mounted behind the illuminating LED, in an aluminum holder. Light exiting the front of the LED must pass through a cylindrical opening, which tends to diffuse the light and provide uniform target illumination when focused by the biconvex lens onto the

target area. A spring supports the LED holder so that it can be adjusted at the factory to optimize the spot image. At the same time, the spring, which is blackened, prevents stray light from the walls of the light path from being focused around the primary target area. The biconvex lens creates an image of the LED light that is approximately 0.8 mm in diameter at the test-card target.

Light reflected diffusely from the target is collected by a plano-convex lens and focused through an ambient light filter to a field-limiting aperture in front of the detector. The filter, a long-pass filter, has its 50% transmission point at 610 nm, making it virtually transparent to light from the LED. The filter is held in place by a two-piece baffle assembly, which keeps off-axis light from reflecting off the tube walls to the detector. A hybrid detector package, containing a photodiode detector and preamplifier, is mounted above the aperture. Signals from both detectors are converted to digital signals on the circuit board mounted in the optics assembly, and the signals are sent to the system computer for processing.

The chromophore used determines the spectral content (light intensity vs wavelength) of the developed island. Variation in the wavelength of the illumination can introduce error; sources of wavelength variations are LED-to-LED variation (±15 nm, range) and variations in operating temperature and drive current. The temperature stability of the optics head is adequate to eliminate temperature as a variable. Also, the range of drive current has been limited to cause less than a 2-nm shift. To minimize the effect of LED-to-LED variation, we measure the output of each LED at 35 °C and 20 mA drive current, then store the peak wavelength in the analyzer’s nonvolatile memory. The measured value can then be used later to calculate a correction factor.

The instrument is calibrated at the factory with a special target card containing a set of light-stable and uniform reflectance targets with values ranging between 10% and 86%. These calibrated targets are used to generate a linear calibration curve for percentage reflectance, which is used to calibrate an on-board reference target that remains inside the instrument and is used as a working standard every time readings are taken. All reflectance readings are taken by first generating a new calibration line between the zero (no light) signal and the reference target signal, which has an assigned reflectance value. Sample targets are read and the reflectance values assigned from this two-point working curve. The on-board target can be checked in the field by comparing it with the multi-target calibrator card, supplied with the instrument. Use of the on-board reference post as a secondary reflectance standard with values assigned by standards traceable to a single primary standard ensures accuracy; all analyzers are traceable to the same standard within a 1.0% reflectance tolerance. The optics are stable over time because a two-point optical calibration is performed before any series of readings. These two points are a dark (LED source turned off) reading and a light reading performed over the reference post. This calibration will correct for any drift of the LED output over time.

**Assay calibration and data reduction.** The system for assay calibration was developed to reduce the work burden on the customer in calibrating an entire panel. Because the optics are both accurate and stable, analyzer-to-analyzer differences are minimized. The primary reason for calibration is to overcome any lot-to-lot differences in the test-card preparation.
If the panel of tests is large (>10) and there are four to six calibrating parameters per analyte, the amount of factory calibration data becomes rather large for manual entry. To solve this problem, we devised a scheme to load the data by using the barcode wand. Once the factory has determined the panel parameters, these are formatted and printed in barcode on sheets shipped with the test cards. Each set of panel parameters is identified with a unique "link I.D. number," which is in human-readable form as well as in barcode. The link I.D. number is also printed on the test card in a proprietary barcode format that can be read by the optics boom. The user who receives the test cards loads the calibration data from the sheets via the barcode wand. The software will store the data in a structured database located in battery-backed RAM memory. To ensure data integrity, the data are check-summed, record by record, in the database. When an assay cycle is initiated, the barcode on the test card is read via the optics head. The software, informed of the link I.D. number, will search the database for the proper parameters for performing the data reduction and reporting results.

Results and Discussion

Optics performance. The analyzer specifications call out a dynamic range of 1% to 100% reflectance at 660 nm. The linearity error is less than 0.5% reflectance throughout the range, with accuracy equal to ±1.0% reflectance, and precision such that the standard deviation of 20 readings is less than 0.3% reflectance.

The dynamic range, linearity, and accuracy are tested when the analyzers are calibrated. Nine reference calibration targets tested in triplicate with four analyzers gave a linear-regression slope of 0.9999 with an intercept of 0.0019, and a correlation coefficient of 1.0000.

Precision was tested by reading three reference targets in triplicate with four instruments over five days. The worst case over all the instruments during the five days was a standard deviation of 0.23% reflectance on a target with an assigned reflectance of 98.5%.

Pump performance. Delivery precision of the wash pump was determined by delivering 1 mL of wash solution from four instruments to 10 test cards over five days, and weighing the volume dispensed. The mean volume delivered was 0.99 mL (total CV = 2.2%). A special test card and software routine has been designed to allow the customer to monitor pump performance.

Assay performance. To establish a baseline for instrument performance, we performed a correlation study, evaluating total IgE with the Abbott Quantum II and the panel EIA system. Only one analyte (total IgE) was spotted on the solid phase. We assayed 46 human serum specimens with IgE ranging from 2.60 to 95.8 int. units/mL, by both systems. Figure 5 shows a correlation plot of the results. Assay reproducibility was determined by testing an IgE control in duplicate over 10 days with one instrument; results were calculated from an analysis of variance. The grand mean was 50.2 int. units/mL; the within-day CV was 4%, between-day 8.3%, and total 8.6%.

A panel EIA has been modeled with several unique antigens bound individually to the solid phase, common liquid reagents, and a single assay protocol. The reagents and protocol are optimized to meet the demands of a full test panel. Preliminary results indicate that new cross-reactions have not been introduced as a result of combining individual assays into a panel. In addition, intra-assay precision is typical of single-analyte EIAs. The interassay imprecision is not significant.

We are at present developing immunoassay panels for allergy and immunology diagnostics. The details of these panels will be presented in forthcoming articles.

References