reagent and the sample can be analyzed first with NBT reagent and then with NBT reagent containing boronate.

Within-batch assay precision for the boronate + NBT reagent procedure was 0.86–1.62% (10 replicates) for fructosamine in the concentration range 1.18–5.07 mmol/L, and 0.41–1.23% (1.62–4.80 mmol/L) for the sample + boronate procedure. The regression equation for results obtained with the Roche RoTAG test (x) and a boronate-modified RoTAG test (sample + boronate procedure) (y) for a series of 30 serum samples was y = 0.76x – 0.55 (r = 0.93).

We also used the reaction to develop a prototype assay of whole-blood glycated protein in which a mixture of lysed blood and boronate is incubated with an immobilized glycated protein. Interfering substances and soluble boronate-glycated protein complexes are removed by washing, and residual (uncomplexed) immobilized glycated protein is reacted with NBT. If the amount of glycated protein in the blood sample is high, then most of the boronate is complexed in the initial incubation and an intense color develops on the solid phase (6).

References


Common to all heterogeneous immunoassays is the requirement for the separation of bound and free label. The precision and ultimate sensitivity of a system will be determined to a great extent by the efficiency of this separation. The separation step has proved to be the most difficult to automate. IMMULITE™ (Cirrus Diagnostics) combines well-established solid-phase technology [1/4-in. (6-mm)-diameter polystyrene beads] with an efficient and easily automated separation scheme (1). The system is built around a proprietary-design assay tube (Figure 1) containing the assay-specific antibody-coated bead. The assay tube serves as the reaction vessel for all incubations, washes, and signal development. After incubation of sample and labeled reagent, very rapid separation and efficient washing of the bead and assay tube are achieved by spinning the tube at high speed about its longitudinal axis. Sample, excess reagent, and washes are captured in a coaxial sump chamber, which is integral with the tube. The fluid contents of the tube are almost instantaneously and completely transferred to the sump, owing to the slight upward taper of the central tube. Very high centrifugal forces can be generated rapidly and without hazard because of the small mass of the tube. Four or more discrete washes of the tube and bead can be accomplished within seconds, allowing the tubes to be processed sequentially and identically with respect to timing.

The centrifugal force applied to the fluid contents of the spinning tube can be separated into two vectors: a major vector perpendicular to the tube wall, and a smaller vector in an upwards direction parallel to the tube wall. The latter vector is equal to the total relative centrifugal force at the tube wall times the sine of the draft angle of the tube. When the parallel force exceeds 1 g, the entire fluid contents are expelled into the sump chamber. The parallel force driving the fluid upward actually increases as the fluid climbs the tube wall because the diameter of the tube increases toward the top of the tube. The relative centrifugal force, in gravities, is given by the equation

\[ R.C.F. = 1.118 \times 10^{-6} \times r \times \text{rpm}^2 \]

where \( r \) = the radius in millimeters, and 
\( \text{rpm} \) = the rotational speed in revolutions per minute.

The IMMULITE assay tube has an internal radius of 4.4 mm at the bottom and a 1° draft angle. The sine of 1° = 0.017. Therefore, a spinning speed of 3500 rpm is enough to drive the fluid into the sump:

\[ 1.118 \times 10^{-6} \times 4.4 \times (3500)^2 \times 0.017 = 1.02 \text{ g} \]

The actual amount of fluid remaining in the tube depends on the speed and duration of spinning and the viscosity of the fluid. The residual fluid in the IMMULITE tube was determined during a thyrotropin assay to be \( \sim 10 \mu L \) after transfer of the reaction mixture and 3 \( \mu L \) after each wash. Therefore, the residual enzyme label not specifically bound after four 200-\( \mu L \) washes would be about two parts per billion:

\[ (10/250)(3/200)^4 = 2 \times 10^{-9} \]

IMMULITE is a bench-top instrument comprising several subsystems designed to process the assay tube. Operation of the system is very simple. After loading a carousel with alkaline phosphatase-labeled reagents for as many as 12 resident assays, the operator loads samples onto a continu-
ulous conveyer, followed in each case by as many as five different assay tubes for the tests desired on that sample. Samples can be added at any time, and "stat" samples can be inserted as the next sample to be processed with no loss in throughput. Instrument operation is initiated by pressing a "Go" button. After passing a barcode reader, the assay tubes are transferred to the incubation carousel, where sample and alkaline phosphatase-labeled reagent are simultaneously added by the pipettor to initiate the reaction. The incubation carousel is heated to 37 °C and indexed every 30 s, for a throughput of 120 tests/h. The tubes are intermittently agitated during incubation to maximize the kinetics of the reaction. Most assays require a single 30-min circuit of the carousel. For very high sensitivity tests, samples can be programmed to make two or more circuits if longer incubation is necessary.

After incubation, the assay tubes are shuttled to the spin/wash station, where bound and free label are efficiently separated as described previously (1). A chemiluminescent substrate for alkaline phosphatase (2, 3) is added and the tubes are transferred to the luminometer, which is also heated to 37 °C. After an additional 10-min incubation to develop the luminescent signal, the light output is measured with a photomultiplier tube in the photon-counting mode. High counts are automatically attenuated to extend the range of the photomultiplier tube 100-fold. Counts are converted to analyte concentration by use of stored standard curves. The curves are adjusted by lot-specific constants, which are barcoded on the reagents, and the stored standard curves are periodically recalibrated with two calibrators that are loaded just like samples.

The assay tube design enables highly efficient washing of the solid phase, thus providing low background counts. This, combined with the use of the exquisitely sensitive chemiluminescent substrate for alkaline phosphatase, results in very sensitive and precise immunoaassays. The detection limit of the thyrotropin assay, defined as the concentration equal to the mean + 2SD of the zero standard, is routinely <0.01 mU/L.

A further advantage of this assay tube is the total capture of all reagent sample and wash solutions in the device itself. This simplifies the disposal of potentially biohazardous material, an increasing concern for the laboratory.

References

Sensitive Fluorescence Method for Detecting DNA Ligation Amplification Products, Emily S. Winn-Deen and David M. Iovannisci (Applied Biosystems, Inc., 850 Lincoln Centre Drive, Foster City, CA 94404)

In 1984 Whitley et al. (1) discovered a way to enhance the specificity of a probe-based assay by using two probes that may be ligated together only when they lie immediately adjacent to one another. By applying this concept to both strands of the DNA during sequential cycles of denaturation, annealing, and ligation, one can also amplify target sequences. This amplification technique, the ligation chain reaction (LCR), became practical with the commercial appearance of a thermal-stable ligase in 1990. Application of LCR to the β-globin system was reported in 1991 by Barany (2), who used 32P as a label for following the course of the reaction. However, working with radioactivity is difficult because the oligonucleotide must be freshly labeled before the assay and because detection requires autoradiography.

We have investigated combining ligase-based amplification with sensitive fluorescence detection to detect the presence of the normal sequence or the sickle cell mutation in the β-globin gene. Each of the four oligonucleotides is labeled with a different fluorescent dye. The products of the amplification reaction are separated from the unligated oligonucleotides by electrophoresis in a fluorescent DNA sequencer, and identified by the appearance of a dual-labeled product of higher molecular mass. The amount of product formed is estimated through peak display from the laser scanner. Using this method, one can detect directly as little as 0.5 fmol of dye-labeled oligo/product. We have simplified the detection of these amplification products through the use of fluorescent labels, and demonstrate the application of this amplification technology to the differential analysis of a single-base mutation in the β-globin gene.

Oligonucleotides for the LCR assay of β-globin were chosen to bracket the site of the A → T base change for the sickle cell mutation. The four oligonucleotides were obtained from the FluoroProbe service at Applied Biosystems, Inc. (Foster City, CA) and were labeled with a rhodamine dye in red (ROX) or yellow (TAMRA) or with a fluorescein dye in blue (FAM) or green (JOE). The common oligonucleotide sequences (used for both β-A and β-S) were PO4-GGAGAGACTTCCGTATTCTG-JOE and PO4-CAGGACACCCAT-TAMRA. The β-A-specific oligonucleotides were FAM-ATGTTGACCTGACTCTGTA and CAGTACGGCAGACTTCTCCT. The β-S-specific oligonucleotides were FAM-ATGTTGACCTGACTCTGTT and CAGTACGGCAGACTTCTCCA. The 3’ end of the nonphosphorylated oligonucleotides was chosen to lie at the site of the sickle cell mutation.

LCR assays were carried out in 20 mmol/L Tris·HCl buffer, pH 7.6, containing 100 mmol of K+, 10 mmol of Mgp2+, 10 mmol of dithiothreitol, 1 mL of Triton X-100, and 1 mmol of NAD+ per liter. Each 100 μL of reaction mixture contained 1 pmol of each of the four oligonucleotides and 15 U of thermal-stable ligase (Epicentre Technologies, Madison, WI). Target DNAs consisted of cloned portions of the normal (β-A) or sickle cell mutation (β-S) sequences of the β-globin gene. Plasmids containing the β-A or β-S sequence were linearized and serially diluted in water immediately before use. To mimic the complexity of the human genome, we added 4 μg of herring sperm DNA to each reaction mixture. Reactions were carried out in 100-μL aliquots overlaid with 100 μL of mineral oil in Gene-Amp™ (Perkin-Elmer Cetus, Norwalk, CT) reaction tubes. All LCR reactions were run in a Perkin-Elmer Cetus thermal cycler for 30 cycles of 94 °C (1 min) and 62 °C (2 min), with 5-s autocycle per cycle. At the end of the cycling protocol, the reactions were cooled to 4 °C.

We precipitated with ethanol 50 μL of each reaction