Dual Fluorometric/Colorimetric Detection System for an Automated Random-Access Instrument Utilizing Standard Polystyrene Test Tubes as Precision Cuvettes

Mark E. Astill, LaVell R. Johnson, Gale H. Thorne, Gary H. Krauth, Roger E. Smith, Randy W. Smith, and Thomas R. Witty

To attain the optical precision necessary to precisely quantify fluorescent or colorimetric signals, analytical systems have typically included quality-controlled cuvettes, flow cells, or dual-beam reference systems. We describe a system where a fluorescence or transmittance signal is quantified in single, standard, 12-mm-diameter polystyrene test tubes. Tube-to-tube variation is minimized by referencing the primary signal to a second reference signal. The tube is carefully oriented within a positioner that allows for the precise placement of the tube within a light path 7.6 mm in diameter. The detection system allows for use of either four pairs of fluorescence excitation/emission wavelengths or eight transmittance wavelengths, which are selected by using specific interference filters. The impact of temperature, tube imperfections, surface flaws, and distortions is minimized by using a reference ratio. Fluorescence is measured with an orthogonal photomultiplier tube, and transmittance with a photodiode; both are illuminated with an ordinary long-life tungsten-halogen lamp. This system is used with the Becton Dickinson AFFINITY™ system, an automated random-access analyzer with analyte-specific unit-package reagents. The polystyrene tube of the reagent package, which has an antibody-absorbed surface, serves as both the cuvette and the separation medium. Use of the reference ratio method reduces intertube imprecision of fluorometric or transmittance signals, for more precise quantification of various analytes.

**Additional Keyphrase:** quantification by reference ratio

Diagnostic instrumentation has always been concerned with the need to quantitatively differentiate the desired, specific signal from that of ancillary noise, or nonspecific, signal. This requirement has generally been addressed by, among other options, carefully controlling the optical quality of the detection cuvette, using dual-beam referencing compensation (1), or using flow cells, where the optical characteristics remain constant for all the samples determined (2–5). The limitations to such systems are twofold: (a) the costs of these types of control systems, which require sophisticated and expensive detection systems, are generally quite high, and (b) the critical kinetic or endpoint reactions are performed elsewhere and the reagents are transferred to the optical system for quantification. Becton Dickinson has developed a system designed to address these issues and provide highly precise measurements of both fluorescence and transmittance. Designated the AFFINITY™ system, this bench-top instrument utilizes a round 12-mm-diameter polystyrene test tube as both the reaction chamber and the cuvette for quantifying the optical signal. Moreover, some of the tubes may have specific antibody absorbed to their surface for use in heterogeneous immunoassays or may serve as a reaction vessel for homogeneous immunoassay determinations. These assay protocols are made possible by the following integral parts of the system.

- All the nonlyophilized reagents necessary to perform a specific test are packaged ready to use in a discrete test unit, a 9.0 × 1.8 × 3.7 cm five-well polypropylene container. This unit is sealed with specific bar-code identification. The polystyrene tube/cuvette is also contained within this package.
- The instrument precisely performs all fluid transfers, and temperature control, for both transferred reagents and incubating reactions, providing a constant reaction environment.
- All scheduling and timing of the system is microprocessor-controlled, according to the specific, bar-code-identified test unit with which the system is presented; scheduling is updated as new test units are loaded.
- For optical quantification, a device positions the polystyrene tubes, aligning each tube for fluorometric or colorimetric determinations, or both, by constraining the tube against a fixed upper stop.
- The orthogonal photomultiplier tube (PMT) detects fluorescence through as many as four filter pairs. The colorimetric signal is determined with a photodiode, through one of eight selectable filters. The light source is a long-life (16 000-h) tungsten–halogen lamp.
- The utility of a round, ordinary polystyrene tube is made possible through the detection of two distinct signals—the first associated with the specific wavelength for the reaction being performed, the second being either transmittance at a wavelength distinct from the absorption profile of the reporter signal (for a colorimetric product) or fluorescence of a second fluorescent dye that is not affected by the primary, specific reaction. For analyte quantification, the ratio between the reporter and the reference signal is compared with that from a previously established calibration curve.

Here we describe the detailed function of this optical system and report the typical precision obtained, especially for fluorometric signals, in the 12-mm-diameter polystyrene test tubes.

**Materials and Methods**

**Reagents**

Sulforhodamine 101 (SR101) and 8-methoxypyrone-1,3,6-trisulfonic acid, trisodium salt (MPT), were purchased from Molecular Probes, Junction City, OR 97448. Sulforhodamine B (SRB) was from Eastman Kodak, Rochester, NY 14650, and Oxazine 725 perchlorate and Nile Blue perchlorate were from Exciton, Dayton, OH 45431. Methyl alcohol

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Becton Dickinson, 810 N. 2200 West, Salt Lake City, UT 84116.

1 Address correspondence to this author.

Received April 10, 1987; accepted June 9, 1987.

2 Nonstandard abbreviations: SR101, sulforhodamine 101; MPT, 8-methoxypyrone-1,3,6-trisulfonic acid, trisodium salt; SRB, sulforhodamine B; PMT, photomultiplier tube.
and dimethyl sulfoxide were obtained from Burdick and Jackson Laboratories Inc., Muskegon, MI 49442; glycerol was from Aldrich Chemical Co, Milwaukee, WI 53201.

Detector Hardware

The illuminator lamp is a ANSI Standard ENL integral reflector, tungsten/halogen-filament lamp (Britton Electric, Salt Lake City, UT). The lenses were industry quality glass (Rolyn optics, Covina, CA 91722). The interference filters, three-cavity, 10-nm band pass, minimum 45% transmission, were acquired from Corion Corp., Holliston, MA 01746, as were the broad-band absorption filters (neutral density filters). The Model RO28P PMT was obtained from Hamamatsu, Middlesex, NJ 08846; the Model VTB-11138 photodiode from Vactec, St. Louis, MO 63132. For tubes/cuvettes, we used standard 12 × 35 mm polystyrene tubes from Spectrum Corp., Ansonia, CT 06401.

Procedures

The detector configuration is shown in Figure 1. The broad-band white light emitted from the lamp is brought to a primary focus by the illuminating pinhole in the integral reflector. The light traversing the hole is collimated by an aspheric lens. The collimated light traverses the filter stack (D) and is focused by a lens (E) to form a uniform illumination of 7.6 mm diameter at the sample tube. Transmitted light is measured by a photodiode diametrically opposite the illumination side.

Nonabsorbed emitted fluorescent light is measured orthogonally. The emitted fluorescent light is collected and collimated by another lens (H). Collimated light traverses the detection filters and is refocussed by lens onto the detector pinhole. Light that passes through the detector pinhole is detected by another PMT (M). The filters can be processor-selected by rotating the conical filter wheel with a directly coupled 200-step per revolution stepper motor.

Photon counting is used to measure light amplitude over a wide dynamic range. The responses of the instruments are roughly matched through the selection and use of neutral density filters (L). PMT response is specifically matched by placing a neutral density filter between the PMT and the pinhole. Differences in fluorescent detectability are matched to the photon-counting range by use of a neutral density filter in the detection filter stack (I).

The effects of ambient (stray) light are obviated through the use of high illumination intensities, so that the neutral density filters can shield the PMT.

The filter sets are installed in a single filter wheel to supply as many as four discrete fluorescent illuminator detector pairs or eight transmission wavelengths for the sample and the reference beam. The filters have been selected for spectral matching, chemical compatibility, and temperature coefficients. The dye pairs and the corresponding filters used are as follows:

<table>
<thead>
<tr>
<th>Fluorescent dye</th>
<th>Filter wavelength, nm</th>
</tr>
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<tbody>
<tr>
<td>SRB</td>
<td>560–590</td>
</tr>
<tr>
<td>Oxazine 725 perchlorate</td>
<td>630–660</td>
</tr>
<tr>
<td>Nile Blue perchlorate</td>
<td>590–630</td>
</tr>
<tr>
<td>SR101</td>
<td>400–430</td>
</tr>
<tr>
<td>MPT</td>
<td>400–430</td>
</tr>
</tbody>
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The SRB and mixed oxazine dyes (see below) are dissolved in de-ionized H2O. The SR101 and MPT pair are dissolved in a solution of glycerol, methanol, and dimethyl sulfoxide.

Primary fluorescence and (or) transmittance is measured for 1 s, after which the filter wheel is rotated to the next processor-selected filter position for the next measurement. To monitor the effects of electrical noise, each 1-s measurement is made in 50-ms increments and analyzed for expected distribution before it is accepted by the microprocessor. Any signal that varies from the expected value by >6 SD is rejected.

Results and Discussion

Ratiosing techniques, commonly used for transmittance determinations (6), can be even more valuable for fluorescence measurements, because the quantum efficiencies of fluorors are typically more sensitive to various environmental factors, e.g., temperature (7). Fluorescent pairs can be selected and matched to minimize the effect of temperature variation on the ratio of fluorescence emission. Because SRB (excitation filter 560 nm, emission filter 590 nm) has a large negative temperature coefficient (Figure 2), we examined numerous fluorors to find one with a similar temperature dependence. Although we were unable to identify a single fluor with a suitable temperature coefficient, the equimolar mixture of Oxazine 725 perchlorate and Nile Blue perchlorate (referred to subsequently as "mixed oxazines")—excitation filter 630 nm, emission filter 660 nm—closely matched the SRB temperature profile. That match is confirmed by the lack of ratio variation with changes in temperature.

![Fig. 1. Schematic illustration of the Affinity detector assembly](image)

![Fig. 2. Profile of fluorescent signal vs temperature for SRB (A), mixed oxazines (B), and the ratio of SRB/mixed oxazines (C)](image)
To assess the effect of the position of the filter wheel, we measured any concomitant variation in light intensity while advancing the filter wheel in single steps through the centerline position of a single filter. As Figure 3 shows, the measured fluorescence of SRB and mixed oxazines varied through the measured rotation. Also shown is the compensation achieved with the combination of SRB/oxazine. The SRB/mixed oxazines ratio is essentially insensitive to position over eight offset positions and to the associated variations. Despite this apparent latitude, the filter wheel is controlled to a single step, which is confirmed by the motorshaft encoder.

Figure 4 illustrates the within-run precision of concentration measurements with SRB and SRB/mixed oxazines ratio. The CV range with SRB only is 1 to 3%. The corresponding precision of the ratio is <0.5% over an SRB concentration range of 5 to 200 nmol/L.

Sensitivity to optical variations in tubes was examined by reading various tubes oriented at 90°, 180°, and 270° longitudinal offsets from 0°. Comparing the SRB results from the rotated tubes with those at the initial position showed a variance of 1.5% to 4.8%. The mean SRB/oxazine ratio measured for variations of all positions varied <1% from the initial measurement, again confirming the utility of this technique.

Although the SRB and the mixed oxazine fluors show relative temperature independence, the defined AFFINITY system provides a constant temperature environment of 37°C. We also have identified a second dye pair, having greater storage stability: SR101 (excitation filter 590 nm, emission filter 630 nm) and MPT. With temperature-dependent variations thus accounted for, we could now focus on tube variation and effects of tube/cuvette positioning. Consequently, we measured tube-to-tube variation in 30 different tubes (Figure 5). The range of variation for MPT was 0.6%, SR101 0.4%, and the ratio for MPT/SR101 was 0.3%. Tube variations were purposely increased by scratching the optical surface. Orienting these scratched surfaces in the light path reduced the fluorescent signal by approximately 4% and the transmitted signal by approximately 12%. The intertube imprecision was now 1.6% for MPT and 1.2% for SR101, but use of the ratioing technique improved this imprecision to 0.6% for MPT/SR101. We noted a similar improvement in transmittance readings obtained with the same scratched tubes.

Several analytical system combine detection capability

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**Fig. 3.** Mixed oxazines fluorescence (■) and SRB/mixed oxazines fluorescence ratio (○) measured as a function of filter-wheel position. Bars indicate the error range ± 1 SD

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**Fig. 4.** Precision of SRB (○) and SRB/mixed oxazines ratio fluorescence (■) measured at SRB concentrations of 0.8 to 200 nmol/L for both fluorescence and absorbance (8–11) to extend the utility of the instrument. The design objectives of the detector system in the AFFINITY instrument were to achieve reliability with a single reaction-reading tube. Optical surface characteristics of a round polystyrene tube, subjected to scratches, suggested potential errors that could not be minimized by physical improvements alone. The use of a soluble reference dye in the unit package improves precision when the ratio of specific signal to reference signal is measured. Factors critical to the function of this system are as follows:

- reduction of the effects from scratching or otherwise marred optical surfaces
- lack of sensitivity to tube position or rotation
- insensitivity to filter positioning/light variation
- insensitivity to minor temperature variation

These factors are especially significant in the design requirements for a relatively low cost, random-access, bench-top diagnostic instrument capable of utilizing economical disposable test packages. The AFFINITY system has been designed to utilize the detector described here, which addresses these factors.

**References**


