A Highly Sensitive Immunoenzymometric Assay Involving "Common-Capture" Particles and Membrane Filtration


This highly sensitive immunoenzymometric assay method involves monoclonal antibodies, a common-capture microsphere, and a rapid, membrane-filtration separation step. The common-capture solid phase is monomodal anti-fluorescein antibody covalently attached to 6.5-µm-diameter latex particles. In sandwich-type assays for large-molecule analytes, the capture antibody is conjugated with fluorescein isothiocyanate and the probe antibody is conjugated with beta-galactosidase (EC 3.2.1.23). In competitive assays for small analytes, the analyte–beta-galactosidase conjugate competes with the antibody in the clinical samples for the fluorescein-labeled capture antibody. After simultaneous incubation of the reagents for 2 h, the bound and unbound reagents are separated by filtration through the bottom of each well of a 96-well plate. Substrate (4-methylumbelliferyl-beta-D-galactopyranoside) is then added to the wells, and the rate of product formation is determined kinetically for 12 min. The rate is proportional to the concentration of analyte in the sandwich assays and inversely proportional in the competitive assays. The assay results for chorionic gonadotropin, thyrotropin, digoxin, and thyroxin show the assay to be sensitive, rapid, and applicable to any size analyte. With this system, several different sandwich- and (or) competitive-type assays can be performed simultaneously on the same plate.

Additional Keyphrases: monoclonal antibodies • chorionic gonadotropin • thyrotropin • digoxin • thyroxin • beta-galactosidase • fluorometry • immunofluorometric assay

Since the development of radioimmunoassay (RIA) by Yalow and Berson (1) in 1959, numerous assay systems have been developed based on the use of sensitive detection methods for measuring antigen–antibody binding. Many of the disadvantages of the RIA—the short stability of the label, the requirements for specialized expensive equipment, and the problems associated with reagent handling and disposal—were largely overcome by the development in 1971 of the enzyme immunoassay (2, 3). The increasing use of enzyme immunoassays for various antigens and antibodies (4–6) has been reinforced by improvements in enzyme-labeling methods (7). Through the use of fluorogenic substrates, detection limits equal to or surpassing those of RIA can be attained (8, 9).

A further significant advance in immunoassay development has been the use of highly specific monoclonal antibodies in the two-site sandwich assays for various protein antigens (10). These assays, based on the binding of two antibodies directed against different distinct sites on the antigen, have increased assay specificity and sensitivity, and have simplified the assay format by allowing the simultaneous incubation of sample with both capture and probe antibodies without an intermediate wash step.

We have developed a sensitive "common-capture" immunoassay reagent and instrument system. The highly specific common-capture anti-FTIC monoclonal antibody is covalently coupled to 6.5-µm-diameter latex particles. Protein antigens are bound to the particles and detected with a combination of two monoclonal antibodies, one conjugated to FITC and the other to beta-galactosidase (EC 3.2.1.23). Hapten analytes are bound by FITC-conjugated antibody and compete with the binding of hapten–beta-galactosidase conjugate. Use of a fluorogenic substrate lends high assay sensitivity. The assays are conducted with a semi-automated instrument, which was developed to facilitate each step of the assay from reagent addition to data calculation. We describe the application of the system to immunoassays of human chorionic gonadotropin (HCG), thyrotropin (TSH), total thyroxin (T₄), and digoxin.

Materials and Methods

Materials

Instrument. The key features of our semi-automated instrument, designed and developed for use with the common-capture assays, are as follows:

The plate-loading station is multiplexed microprocessor-controlled, and the addition of samples is instrument-prompted. Movement of the plate on a heated carrier is controlled by a microprocessor-operated xy mechanism. Each pipetting step is confirmed by a light-emitting diode display. Reagent description and quantity are displayed after each reagent is dispensed. The plate incubation and processing station is fully computer-controlled. This station contains heated carriers (37 ± 1°C) with a two-plate capacity, automatic low pressure (~21 kPa), and two automatic shakers. The instrument contains a computer-controlled station for washing and substrate addition. The read station contains a microprocessor-controlled fluorimeter. Automatic gain adjustment maintains day-to-day reading stability within 2%. The kinetic fluorescence measurements and the data calculations are done automatically by the instrument.

The dynamic range of the fluorimeter is 10⁻¹¹ to 10⁻¹⁷ mol of 4-methylumbelliferone at pH 7.8. The optical and electronic precision over the entire range is 1%. Inter- and interwell precision are 1.5% and 2.0%, respectively, also over the entire dynamic range (unpublished data).

Reagents. The assay buffer contained, per liter, 50 mmol of TEA (pH 8.0), 0.5 g of MgCl₂·6H₂O, 5 g of NaCl, 2 g of

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* Nonstandard abbreviations: BSA, bovine serum albumin; AF, change in fluorescence; FITC, fluorescein isothiocyanate; HCG, human chorionic gonadotropin; PBS, phosphate-buffered saline; SMCC, succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate; TEA, triethanolamine; TSH, thyrotropin; T₄, thyroxin; "bis-tira" propene, 1,8-bis(tios(furazyl)methylamino)propene.
BSA, and 1 g of sodium azide. For the T₄ assay, the buffer also contained 1.5 g of 8-anilinonaphthalenesulfonic acid (sodium salt) and 1.0 g of salicylic acid (sodium salt) per liter. The wash buffer contained, per liter, 50 mmol of TEA, 10 g of NaCl, 2 g of BSA, 1 g of sodium azide, and 0.5 g of MgCl₂·6H₂O.

Stock substrate solution was prepared by dissolving 4-methylumbelliferyl-β-D-galactopyranoside (Sigma Chemical Co., St. Louis, MO), 20 mmol/L in N,N-dimethylformamide. We diluted the stock solution 200-fold in 0.1 mmol/l sodium phosphate buffer, pH 7.65.

We used a 300 g/L suspension of 6.5-μm-diameter particles of latex (Saragen, Indianapolis, IN). Fluorescein isothiocyanate (isomer I) was obtained from Sigma Chemical Co., beta-galactosidase (specific activity, 600 kU/g protein) from Boehringer-Mannheim, Indianapolis, IN.

The HCG, TSH, T₄, and digoxin standards were prepared with charcoal-stripped, delipidated pooled human serum.

**Filter plates.** We used 96-well Millititer™ plates (Millipore Corp., Bedford, MA), the bottom of each plate being sealed with Durapore (5.0-μm pore size) hydrophilic polyvinylidene fluoride membrane. The Millititer plate is designed to hold liquids inside the wells until air pressure is reduced.

**Preparatory Procedures**

**Monoclonal antibodies.** The preparation of hybridomas and the procedures used in screening and clone characterization were described previously (11). Monoclonal anti-FITC antibodies were obtained from fusions with spleen cells from mice immunized with FITC conjugated to keyhole limpet hemocyanin. Monoclonal antibodies from ascites were purified on a protein A-Sepharose CL-4B column equilibrated with 0.1 mol/L sodium phosphate buffer, pH 8.1. The bound antibodies were eluted with either sodium phosphate buffer (pH 5.5, 0.1 mol/L) or citrate buffer (pH 3.0, 0.1 mol/L).

**FITC-conjugated antibodies.** Conjugates were prepared by reacting 5 mg of antibody with 0.1 mg of FITC in 2.5 mL of 0.1 mol/L borate buffer at 37 °C for 2 h. The conjugation mixture was purified on a 0.7 × 10 cm column of Sephadex G-25, eluted with PBS (phosphate 0.1 mol/L, NaCl 150 mmol/L, pH 7.4). The purified FITC-conjugated antibody, eluting in the void volume, had approximately five FITC molecules incorporated per IgG molecule.

**Beta-galactosidase–IgG conjugates.** Antibody was activated for conjugation by reacting 5 mg of protein in 2.5 mL of 0.1 mol/L sodium phosphate buffer (pH 7.0) with 0.5 mg of sulfo-SMCC (Pierce Chemical Co., Rockford, IL) for 1 h at room temperature. The reaction mixture was purified on a 0.7 × 10 cm column of Sephadex G-25, being eluted with a solution of 10 mmol each of “bis-tris” propane and MgCl₂·6H₂O per liter, pH 7.4. The void volume peak was diluted to 250 mg/L with sodium phosphate buffer and reacted with 5 mg of beta-galactosidase at 37 °C for 1 h.

After quenching the reaction by adding 100 μL of 1.0 mol/L 2-mercaptoethanol solution, we purified the reaction mixture by applying it onto a Pharmacia MONO Q™ HR 5/5 prepacked anion-exchange column. Free antibody, conjugate, and free enzyme were separated by elution with a linear salt gradient for 40.1 min. The low-salt buffer contained, per liter, 10 mmol each of bis-tris propane and MgCl₂·6H₂O (pH 7.4); the high-salt buffer contained, per liter, 50 mmol of bis-tris propane, 10 mmol of acetic acid, 0.4 mol of NaCl, and 10 mmol of MgCl₂·6H₂O (pH 8.0). Free antibody was eluted after about 12–14 min, whereas the conjugate and free enzyme were eluted after about 27 and 29 min into the gradient.

**Beta-galactosidase–T₄ conjugate.** T₄ was activated for coupling to beta-galactosidase by reacting with SMCC (Pierce Chemical Co.) to produce T₄-maleimide. The conjugate was prepared by reacting 10 mg of beta-galactosidase (reconstituted in 2 mL of 0.1 mol/L sodium carbonate, pH 9.0) with 0.72 mg of T₄-maleimide for 2 h at room temperature. The reaction mixture was purified on a 1.6 × 40 cm column of Sephadex G-25 (superfine), eluted with 10 mmol of Tris buffer containing 150 mmol of NaCl and 1.0 mmol of MgCl₂·6H₂O per liter. The purified conjugate, eluting in the void volume, was diluted with an equal volume of a solution containing 10 mmol of triethanolamine buffer (pH 8.1), 10 mmol of MgCl₂·6H₂O, 0.1 mol of NaCl, 2.6 g of BSA, and 0.03 g of dithiothreitol per liter.

**Beta-galactosidase–digoxin conjugate.** Digoxin was activated by reacting with 10 mmol/L sodium periodate solution for 15 min at room temperature. To prepare the conjugate, we reacted 0.3 mg of periodate-oxidized digoxin with 10 mg of beta-galactosidase (reconstituted in 2 mL of 0.1 mol/L sodium carbonate, pH 9.0). After incubation for 2 h at 2 °C, we quenched the reaction by adding sodium borohydride and mercaptoethanol. The conjugate was purified by column chromatography and diluted in stabilizing buffer as described above for the beta-galactosidase–T₄ conjugate.

**Anti-fluorescein-coupled latex.** Anti-FITC IgG was coupled to carboxylated latex particles of approximately 6.5-μm diameter via a covalent coupling method involving glutaraldehyde. The coupled latex was thoroughly washed with PBS and finally suspended in a 50 mmol/L triethanolamine buffer, pH 8.0, containing 250 mmol of sodium chloride, 1 mmol of MgCl₂·6H₂O, and 1 g of sodium azide per liter.

**Assay Procedures**

Assay reagents, “common-capture” latex, monoclonal antibody conjugates, and sample are simultaneously incubated in 0.6-cm-diameter wells of the multi-well filter plate. The assay principle and procedure are outlined in Figures 1 and 2, and are as follow:

**Sandwich assay.** All assays for large proteins, exemplified

![Sandwich assay diagram](image-url)

**Fig. 1.** "Common-capture" assay principle: sandwich assay.
by TSH and HCG, involve use of two monoclonal antibodies that recognize two different epitopes of the antigen molecule. One antibody is conjugated with enzyme (beta-galactosidase) as a detection probe, and the other antibody is conjugated with FITC. The two antibody conjugates form a "sandwich" with the antigen when the latter is present. The immune sandwich is captured by anti-FITC derivatized latex particles. After 2 h of incubation of all the assay components and subsequent washing, the sandwich is mixed with enzyme substrate and assayed for enzyme activity. The rate of product formation by the action of enzyme on the substrate is proportional to the concentration of antigen in the sample.

Specifically, 25 μL of standard or sample, 100 μL of combined antibody-conjugates, and 100 μL of common-capture latex particles (5 mg/mL) are pipetted into each well of the filter plate. The plate is incubated for 2 h at 37°C, washed three times with wash buffer, automatically dispensed from the wash station in the instrument, then manually blotted on absorbent paper and moved to the substrate dispenser/reader station. The instrument dispenses 100 μL of fluorogenic enzyme substrate (4-methylumbelliferyl-beta-D-galactopyranoside) into each well. The plate is then mechanically moved to the reader position for the kinetic measurement of enzyme activity, in three rate measurements at 4-min intervals. The data calculated for each standard or sample well are then printed as ΔF/minute.

**Competitive assay.** Assays for small analytes (T4 and digoxin) involve only one monoclonal antibody specific to the analyte. The antibody (conjugated to FITC) and the analyte (conjugated to beta-galactosidase) are simultaneously incubated with the serum sample. The enzyme-labeled analyte competes with the analyte in the sample for the antibody. The quantity of captured analyte-enzyme conjugate is therefore inversely proportional to the concentration of analyte present in the sample. The assay procedure for the competitive assays is identical to that of the sandwich assay procedure except for two 50-μL pipetting steps to add enzyme and FITC conjugates.

**Results**

**HCG assay.** The standard curve, which was linear through 200 int. units of HCG per liter (relative to the Second International Standard for HCG), was highly reproducible: correlation coefficient 0.999, slope 0.0313, and y-intercept 0.131 for three replicate assays (Figure 3). The minimum detectable concentration of HCG, defined as that concentration corresponding to 2 SD from the mean of 10 replicate determinations of the zero standard, was 1.0 int. unit/L.

The results of within- and between-run precision studies are shown in Table 1. A correlation study of 17 samples run in our assay and in the Hybritech Tandem E-RIA gave a correlation coefficient of 0.96 (Table 2).

**TSH assay.** Standard curves, linear through 50 milli-int. units/L (relative to the WHO human-pituitary standard), were highly reproducible and had the following results: correlation coefficient 0.999, slope 0.081, and y-intercept 0.382 for replicate assays. The minimum detectable concentration of TSH in this assay was 0.3 milli-int. unit/L. The results of within- and between-run precision studies are shown in Table 1. The results of a correlation study of 85 serum samples determined by our assay and by the RIA from Clinical Assays, Cambridge, MA, are shown in Table 2.

**Total T4 assay.** A typical T4 standard curve, zero through 240 μg/L, is shown in Figure 4. The minimum detectable concentration was 5 μg/L. Replicates of a 69 μg/L sample run within one assay gave a CV of 3.3%. The T4 results agreed well with values determined with an OREIA II T4 assay (Organon Diagnostics West Orange, NJ) for 130 samples (Table 2).

**Digoxin assay.** The assay sensitivity is 0.23 μg/L. Assay precision data are shown in Table 1. Assay results correlated well (r = 0.992) with values for 12 sera assayed by use of an RIA from New England Nuclear, Boston, MA (Table 2).

**Discussion**

This immunoassay method with a "common-capture" solid phase and a membrane filtration wash method greatly increases the ease and convenience of running both sandwich and competitive enzyme immunoassays. We chose FITC as the capture hapten because it is highly immuno-
genic, eliciting high-affinity antibodies (12). Furthermore, labeling antibody with FITC is a well-established method, known not to adversely affect the activity of the haptenated antibody (13). Several anti-FITC monoclonal antibodies were generated. The optimum antibody was chosen on the basis of affinity, stability, and performance in our assay system after being coupled to the solid phase. This common-capture format greatly simplified the task of developing an assay system common for a large variety of analytes. This design eliminates the need to optimize antibody/latex coupling conditions according to the specificity of each individual antibody. The common-capture format also simplifies the simultaneous performance of multiple assays because only one capture reagent is required for all assays.

The limited capacity of an immunoassay solid phase can adversely affect both the sensitivity and the range of an assay. To overcome this problem, we use latex microspheres; their large surface area and high capacity enhance assay kinetics and range. The microspheres remain suspended for the entire incubation period, are readily washed, and are easily resuspended in the substrate.

The 96-well filter plate provides the assay system with several advantages. The area and pore size of the membrane are relatively large, allowing rapid filtration. Visibly lipelmic samples do not require more time for filtration or washing. Combining the use of a common-capture solid phase with the 96-well format enables the user to conduct several assays, sandwich or competitive, simultaneously on the same filter plate.

The use of beta-galactosidase conjugates with the fluorogenic substrate allows the development of assays with high sensitivity. A problem frequently encountered with enzyme–antibody conjugates, particularly with beta-galactosidase, is nonspecific binding of the conjugate to the solid phase (7). Assay sensitivity can generally be increased by increasing the concentration of conjugate; however, a point is reached at which this increases the nonspecific binding much more than the specific signal. This nonspecific binding is generally caused by failure to remove, from the conjugate preparation, free enzyme and conjugate-containing multimers having a high antibody/enzyme ratio. Our method of liquid-chromatographic purification of the conjugate readily separates the free enzyme, free antibody, and large multimeric complexes from the conjugate preparation, resulting in a specific reagent that produces very low nonspecific binding in the assay. The results presented for the HCG and TSH assays were obtained by using a dilution of each conjugate that yielded a similar slope for each assay. Assay sensitivity, for either assay, could have been increased by increasing the conjugate concentration.

Assay precision was maximized by using kinetic rate measurement of fluorescent product generated by the action of solid-phase-bound enzyme conjugate on the substrate. Rate measurements, unlike end-point readings, minimize well-to-well differences caused by variations in background filter fluorescence and variations in incubation periods. Adequate rates are obtained in each assay with a 10-min incubation with the fluorogenic substrate.

We have described a semi-automated instrument devel-

Table 1. Assay Precision

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Within runs</th>
<th>Between runs</th>
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<tbody>
<tr>
<td>HCG, int. units/L</td>
<td>n: 36</td>
<td>Mean: 57.65</td>
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<tr>
<td></td>
<td>SD: 4.58</td>
<td>CV: 8.40</td>
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<tr>
<td></td>
<td>n: 42</td>
<td>Mean: 14.9</td>
</tr>
<tr>
<td></td>
<td>SD: 1.04</td>
<td>CV: 6.99</td>
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<tr>
<td>TSH, milli-int. units/L</td>
<td>n: 20</td>
<td>Mean: 1.04</td>
</tr>
<tr>
<td></td>
<td>SD: 7.7</td>
<td>CV: 3.3</td>
</tr>
<tr>
<td>Digoxin, µg/L</td>
<td>n: 14</td>
<td>Mean: 1.46</td>
</tr>
<tr>
<td></td>
<td>SD: 0.113</td>
<td>CV: 7.7</td>
</tr>
<tr>
<td>Total T₄, µg/L</td>
<td>n: 12</td>
<td>Mean: 69</td>
</tr>
<tr>
<td></td>
<td>SD: 0.227</td>
<td>CV: 3.3</td>
</tr>
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Table 2. Correlation Data

<table>
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<tr>
<th>Analyte</th>
<th>n</th>
<th>Correlation method</th>
<th>r</th>
<th>Slope</th>
<th>y-intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCG</td>
<td>17</td>
<td>Tandem E-RIA</td>
<td>0.960</td>
<td>1.05</td>
<td>5.2</td>
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<tr>
<td>TSH</td>
<td>58</td>
<td>RIA (Clinical Assays)</td>
<td>0.983</td>
<td>0.87</td>
<td>0.5</td>
</tr>
<tr>
<td>Digoxin</td>
<td>12</td>
<td>RIA (New England Nuclear)</td>
<td>0.932</td>
<td>0.818</td>
<td>0.183</td>
</tr>
<tr>
<td>Total T₄</td>
<td>130</td>
<td>OREIA (Organon)</td>
<td>0.963</td>
<td>0.922</td>
<td>0.899</td>
</tr>
</tbody>
</table>
opedsimplyfys reagent handling and assay set-up. There are separate stations for reagent additions, controlled temperature incubation, automated washing and substrate addition, and kinetic reading of the fluorescent product generated. This instrument, with its specific assay reagents, provides an immunoassay system with high assay throughput and flexibility.

Our assay format is based on the use of monoclonal antibodies in preparing both the "common-capture" solid phase and antibody conjugate for each analyte specificity. The advantages of using monoclonal antibodies in immunoassays is widely recognized (10). Their use increases assay specificity and allows the simultaneous incubation of sample with both the capture and probe antibodies. Most assays require highly specific reagents, with low cross reactivity with other serum components. The TSH assay, for example, requires the use of two specific antibodies, both with low cross reactivities with HCG, folliclitolipin, and lutropin. This level of specificity is difficult to obtain with polyclonal antibodies.

Until recently, the available TSH assays, because of inadequate sensitivity, could not discriminate between euthyroid and low TSH concentrations. A reliable, highly-sensitive TSH assay would allow the identification of primary hyperthyroidism without the need for additional testing (11). The importance and utility of such an assay has led to the development of several TSH assays that can detect less than 0.1 milli-int. unit/L (15–18). Our TSH assay can detect 0.3 milli-int. unit/L in a 2-h incubation time. This response was obtained by using reagent dilutions that gave a standard curve having ΔP/min values within the same range as our other assays. Preliminary experiments indicate that, because of the high purity of our enzyme conjugates, we will be able to detect as little as 0.1 milli-int. unit of TSH per liter without increasing incubation time.

The T4 and digoxin assays exemplify the performance of our system for hapten analytes. The hapten–beta-galactosidase conjugates are easily prepared and purified and show good binding to the FITC-conjugated, analyte-specific monoclonal antibodies. The range and sensitivity of the competitive assays can be readily manipulated through the degree of hapten substitution on the enzyme and through the dilution of the FITC–monoclonal antibodies and hapten–beta-galactosidase conjugates. This system will easily accommodate the range requirements of high-analyte-concentration assays such as theophylline, as well as those requiring high sensitivity such as digoxin and T4.

This reagent and instrument system is capable of running simultaneous, multiple assays. The "common-capture" reagent system, as exemplified by assays for HCG, TSH, T4, and digoxin, is applicable to a wide range of analyte specificities.

References