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Electrochemiluminescence Detection for Development of Immunoassays and DNA Probe Assays for Clinical Diagnostics

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Electrochemiluminescence (ECL) has been developed as a highly sensitive process in which reactive species are generated from stable precursors (i.e., the ECL-active label) at the surface of an electrode. This new technology has many distinct advantages over other detection systems: no radioisotopes are used; detection limits for label are extremely low (200 fmol/L); the dynamic range for label quantification extends over six orders of magnitude; the labels are extremely stable compared with those of most other chemiluminescent systems; the labels, small molecules (~1000 Da), can be used to label haptens or large molecules, and multiple labels can be coupled to proteins or oligonucleotides without affecting immunoreactivity, solubility, or ability to hybridize; because the chemiluminescence is initiated electrochemically, selectivity of bound and unbound fractions can be based on the ability of labeled species to access the electrode surface, so that both separation and nonseparation assays can be set up; and measurement is simple and rapid, requiring only a few seconds. We illustrate ECL in nonseparation immunoassays for digoxin and thyrotropin and in separation immunoassays for carcinoembryonic antigen and alpha-fetoprotein. The application of ECL for detection of polymerase chain reaction products is described and exemplified by quantifying the HIV1 *gag* gene.

Additional Keyphrases: *electrochemistry · chemiluminescence · polymerase chain reaction · ruthenium(II) tris(bipyridyl) label*

Electrochemiluminescence (ECL), in contrast to conventional chemiluminescence, is the chemiluminescent reaction of species that are generated electrochemically at the surface of an electrode(*1–3*).[1] Here we demonstrate how the detection of luminescence from such a reaction can be used to sensitively quantify ECL-active species and to develop immunoassay and DNA probe assays in which the ECL-active species is used as a label.

ECL processes have been demonstrated for many different molecules by several different mechanisms. In the present investigation, we use the ECL reaction of ruthenium(II) tris(bipyridyl), $Ru(bpy)_3^{2+}$, with tripropylamine (TPA) (*3*). Salts of $Ru(bpy)_3^{2+}$ are very stable, water-soluble compounds that can be chemically modified with reactive groups on one of the bipyridyl ligands to form activated species with which proteins, haptens, and nucleic acids are readily labeled. In this study, the activated form of the $Ru(bpy)_3^{2+}$ we used was $Ru(bpy)_3^{2+}$-NHS ester:

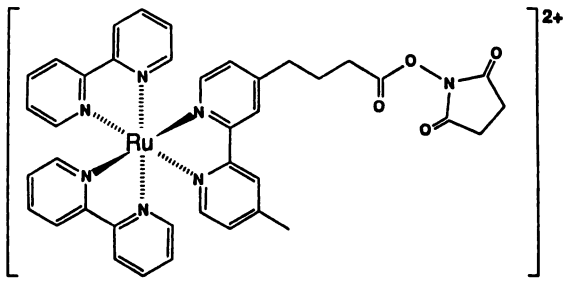

Figure 1 shows the proposed mechanism for the ECL reaction of $Ru(bpy)_3^{2+}$ and TPA. $Ru(bpy)_3^{2+}$, the label, is oxidized at the surface of an electrode, forming the strong oxidant, $Ru(bpy)_3^{3+}$. Simultaneously, TPA, which is present in large molar excess in the solution, is oxidized at the electrode to form the cation radical $TPA^{+\cdot}$, which rapidly and spontaneously loses a proton to form the radical $TPA^{\cdot}$. $Ru(bpy)_3^{3+}$, a strong oxidant, and $TPA^{\cdot}$, a strong reductant, react to form the excited state of the ruthenium complex, $Ru(bpy)_3^{2+*}$, as well as other inactive products. The energy necessary for formation of the excited state arises from the large difference in electrochemical potentials of the $Ru(bpy)_3^{3+}$ and the $TPA^{\cdot}$. The excited-state $Ru(bpy)_3^{2+*}$ decays through a normal fluorescence mechanism, emitting a photon at 620 nm. This process regenerates the original form of the $Ru(bpy)_3^{2+}$, which is free to cycle multiple times through the reaction sequence. Each ECL-active label, therefore, can emit many photons during each measurement cycle, thereby enhancing the detection of the label.

Quantification of the $Ru(bpy)_3^{2+}$ label can be readily automated with relatively uncomplicated instrumentation. Figure 2 is a diagram of the essential components of instruments under development for automated immunoassays and DNA probe assays. The heart of the instrument is the electrochemical flow-cell, containing

IGEN, Inc., 1530 East Jefferson St., Rockville, MD 20852.

[1]Nonstandard abbreviations: ECL, electrochemiluminescence; $Ru(bpy)_3^{2+}$, ruthenium(II) tris(bipyridyl); TPA, tripropylamine; PCR, polymerase chain reaction; NHS, *N*-hydroxysuccinimide; HIV, human immunodeficiency virus; and PMT, photomultiplier tube.

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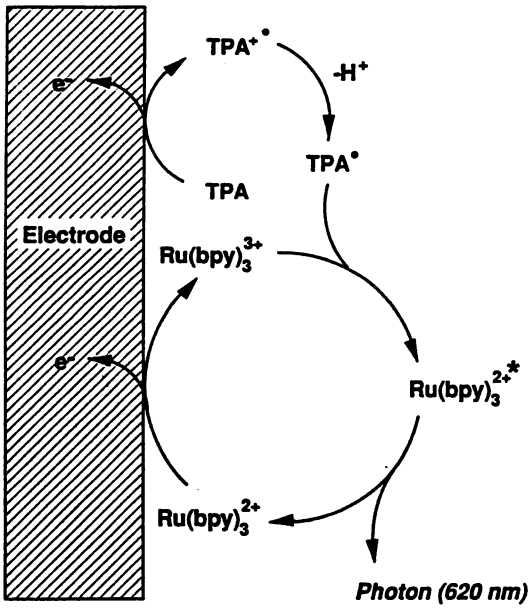

Fig 1. Mechanism of ECL excitation

$Ru(bpy)_3^{2+}$ and TPA are oxidized at the surface of a gold electrode, forming $Ru(bpy)_3^{3+}$ and $TPA^{+\cdot}$, respectively. The $TPA^{+\cdot}$ spontaneously loses a proton, forming TPA•. The TPA•, a strong reductant, reacts with $Ru(bpy)_3^{3+}$, a strong oxidant, forming the excited state of the label, $Ru(bpy)_3^{2+*}$. The excited state decays to the ground state through a normal fluorescence mechanism, emitting a photon having a wavelength of 620 nm

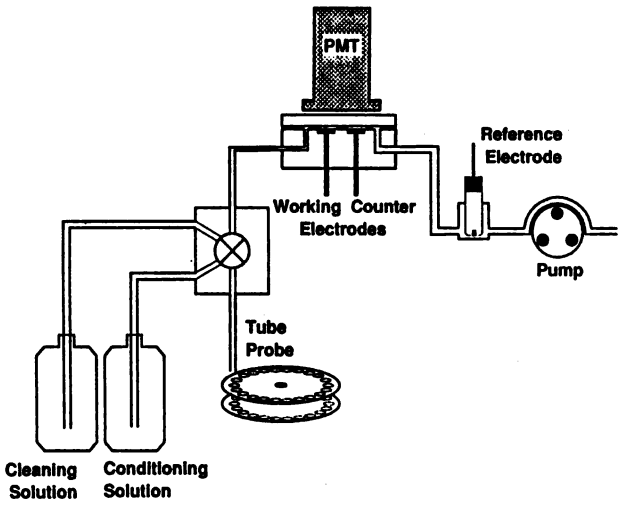

Fig 2. Principal components of the ECL analyzer

See text for description of the function of each component

the working electrodes and counter electrodes for initiation of the ECL reaction. Both of the electrodes are fabricated from gold, but other materials have been used with various degrees of success. A potentiostat (not shown) applies various voltage waveforms to the electrodes, and a single photomultiplier tube (PMT) detects the light emitted during the ECL reaction. An Ag/AgCl reference electrode is placed in the fluid path downstream from the flow cell, and a peristaltic pump is used to draw various fluids through the flow cell. In a typical sequence, the assay fluid is drawn from a test tube into the flow cell and the label is quantified by applying a ramp voltage to the electrodes and measuring the emitted light. After the measurement, a high-pH cleaning solution is drawn into the cell for an electrochemical cleaning procedure. A conditioning solution is then drawn into the cell, and a voltage waveform is applied that leaves the surfaces of the electrodes in a highly reproducible state, ready for the next measurement cycle.

The ECL reaction can be efficiently initiated by many different voltage waveforms. Figure 3 illustrates measurements of the working electrode current and the ECL intensity induced by the application of a triangle wave to the electrodes. The applied voltage as shown is actually the voltage measured at the Ag/AgCl reference electrode and includes the effects of a significant uncompensated resistance; consequently, the actual voltage applied at the working electrode is substantially less than that depicted. The triangle waveform rises from 565 to 2800 mV at a rate of 750 mV/s and then decreases at the same rate to 1000 mV. The current that flows in the cell is primarily the result of the oxidation of the TPA and of the hydrolysis of water. Oxidation of both the TPA and $Ru(bpy)_3^{2+}$ becomes evident when the applied voltage reaches ~1100 mV and produces luminescence. The intensity of the luminescence increases with the applied voltage until the TPA at the surface of the electrode is depleted, resulting in decreased intensity. The intensity of the observed luminescence is great enough that it can easily be measured with conventional PMTs operating either in photon-counting or current modes.

Immunoassays can be readily demonstrated and developed in a wide range of different formats. Nonseparation competitive assays of haptens can be formatted by using labeled hapten, which competes for antibody

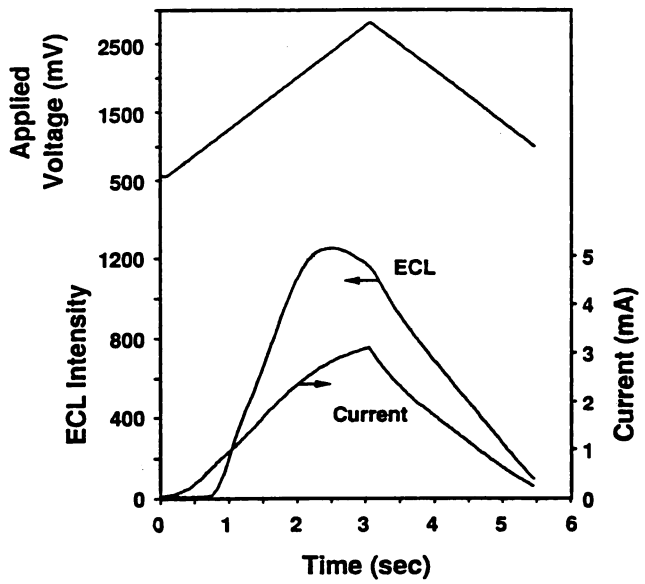

Fig 3. Electrochemical excitation waveforms and the resulting current and luminescence waveforms

The potential is applied to the counterelectrode and the waveform shown is measured at the Ag/AgCl reference electrode. The current is measured at the working electrode, and the analytical signal (luminescence) is measured with the PMT mounted adjacent to the flow cell, as shown in Fig. 2

with the analyte. The efficiency of ECL excitation differs considerably for the free and bound fractions of the labeled hapten because of steric effects and large differences in the diffusion coefficients. Therefore, the ECL intensity from the free fraction can be quantified in the presence of the bound fraction in a true homogeneous format.

Competitive assays for either haptens or large molecules can also be formatted as solid-phase assays. Microparticles coated with antigen compete with the analyte for labeled antibody. This assay format can be used for either large analytes or for haptens. The format is illustrated later with a digoxin immunoassay in which the particles are coated with an ouabain/bovine serum albumin conjugate.

Solid-phase sandwich immunoassays can also be formatted by using two antibodies specific for different epitopes of the analyte. These solid-phase assays can be formatted as either separation or nonseparation assays. Unless the particles undergo a proprietary preparatory step (*4*), excitation of ECL from the surfaces of the particles is inefficient. Whereas the unbound labeled molecules are free to diffuse to the surface of the electrode during the measurement cycle, the motion of the labeled molecules bound to the particles is largely dictated by Brownian motion of the particles. Because this phenomenon does not efficiently transport the particles to the electrode surface for excitation, the ECL from the bound fraction of labeled molecules is greatly diminished. A recent innovation, however, has allowed the efficient excitation of labeled molecules on the surfaces of particles; in fact, the signal from the bound fraction is greatly enhanced over that of the free fraction. Consequently, nonseparation assays can be performed in which the bound fraction is quantified without separation from the free fraction.

DNA probe assays can also be performed with use of ECL to detect hybridization of labeled probes to nucleic acid sequences. We will demonstrate this application here by quantifying the products of the polymerase chain reaction (PCR) (Figure 4). In this format, PCR is first used to amplify the specific genes by use of two primers, one of which is biotinylated. The double-stranded DNA is then captured on streptavidin-coated microparticles and washed with an alkaline solution to denature and separate the strands of DNA. Incubation of the particle-bound DNA with $Ru(bpy)_3^{2+}$-labeled probe is followed by washing the sample and quantifying the particle-bound label. Although the example shown here includes a wash procedure, nonseparation formats have also been used. We have also demonstrated (unpublished) the compatibility of the detection technique with other amplification schemes.

Materials and Methods

Instrumentation

For all ECL measurements we used an Origen® I analyzer (IGEN, Inc., Rockville, MD 20852). The instrument integrates a luminometer, potentiostat, electrochemical flow cell, fluid-handling components, and a 50-tube carousel. The instrument is controlled by a microcomputer via operator manipulation of on-screen menus.

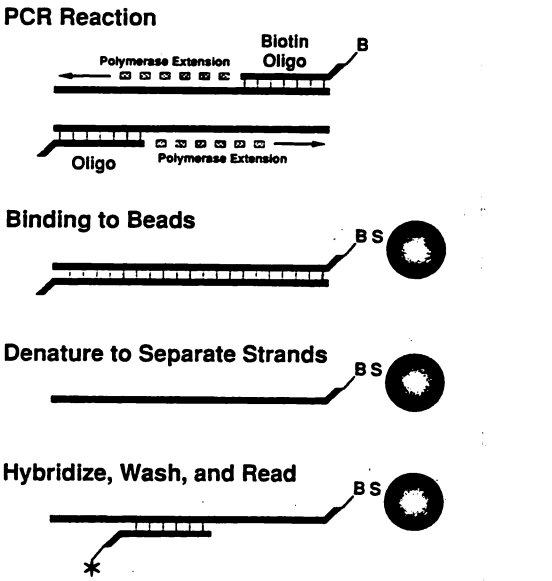

Fig 4. Diagram of the procedure for detection of PCR products in the DNA probe assay described in the text

Reagents

All solutions necessary for performance of the assays and for operation of the Origen I analyzer and $Ru(bpy)_3^{2+}$-NHS ester were obtained from IGEN, Inc. Microparticles (Dynabeads M-450) were obtained from Dynal A/S, Oslo, Norway.

Immunoassay Procedures

Microparticles were coated with protein by mixing 1 mL (30 mg) of the particles in 150 mmol/L sodium carbonate/bicarbonate buffer (pH 9.6) with an equal volume of 0.5–1.0 g/L protein solution. This mixture was incubated overnight at room temperature with mixing. The particles were then magnetically separated from the solution and removed. Next, we incubated the particles for 2 h at room temperature with 1 mL of 30 g/L bovine serum albumin solution in phosphate-buffered saline (per liter, 150 mmol of potassium phosphate, 150 mmol of NaCl) containing NaN_3, 0.5 g/L, agitating the suspension to block unreacted sites. The particles were washed five times (2 mL per wash) and resuspended in 1 mL of the same buffer for storage.

To label antibodies with $Ru(bpy)_3^{2+}$, we mixed 1 mg of the antibody in 0.5 mL of phosphate-buffered saline (pH 7.8) with 35 μL of 5.0 g/L $Ru(bpy)_3^{2+}$-NHS ester in anhydrous dimethyl sulfoxide (Aldrich Chemical Co., Milwaukee, WI 53233), while shaking. The labeling was allowed to proceed with agitation at room temperature for 30 min, then was terminated by the addition of 25 μL of 1 mol/L glycine reagent and further incubated for 10 min. The labeled protein mixture was purified by passage through a 1 × 20 cm Sephadex G-25 column

(Pharmacia, Piscataway, NJ), eluted with phosphate-buffered saline (pH 7.2) containing sodium azide, 0.5 g/L. The $Ru(bpy)_3^{2+}$-labeled protein fractions were collected and pooled.

All immunoassays can be performed by using only minor variations on the following procedure: mix 100 μL of sample, 75 μL of coated microparticles, and 75 μL of labeled antibody, and incubate with agitation for 15 min. For a nonseparation format, insert the mixture directly into the Origen I analyzer and measure the ECL. For separation assay formats, wash the microparticles by using magnetic separation before inserting them into the analyzer.

Oligonucleotides for the DNA probe assay of HIV1 *gag* gene were synthesized with a Model 380B DNA synthesizer (Applied Biosystems, Foster City, CA 94404) and functionalized with 5′ amino groups by use of amino modifiers from Clontech (Palo Alto, CA 94303). The oligonucleotides used for the specified HIV *gag* gene PCR assays were the SK38 and SK39 primers and SK19 probe (*5*). The SK39 probe was labeled with $Ru(bpy)_3^{2+}$ by mixing 0.1 μmol of the primer with 0.5 μmol of $Ru(bpy)_3^{2+}$-NHS ester in an 800 g/L solution of dimethyl sulfoxide in phosphate-buffered saline, pH 7.4. Biotinylation of the SK39 primer was performed similarly except that biotin X-NHS (Clontech) was used and the reaction was performed in 500 g/L dimethyl sulfoxide solution. The labeled oligonucleotides were precipitated with ethanol and washed to remove unreacted label. The PCR was performed essentially as described in the Perkin-Elmer Cetus product literature (*6*), with a 100-μL reaction volume containing 500 ng (50 pmol) of the biotinylated SK39 primer and 500 ng (50 pmol) of unmodified SK38 primer. The temperature cycles used with the Thermocycler (Perkin-Elmer, Norwalk, CT 06859) were 95 °C for 1 min and 60 °C for 1 min. The PCR reaction was cycled 30 times to quantify 50–2000 copies of the gene and 40 cycles for the low copy-number assay.

Results and Discussion

Figure 5 demonstrates the dynamic range for measurement of free label, $Ru(bpy)_3^{2+}$. In this Figure, as in all cases where log–log representation is used, the ECL intensity measured at zero concentration was subtracted before the log is calculated. Linearity of the label detection with label concentrations from subpicomolar to greater than micromolar was observed. Four different PMT voltages were used in this experiment because the dynamic range for the present luminometer is limited to approximately four orders of magnitude. The values obtained for ECL intensity at PMT voltages of 800, 500, and 400 V were normalized to those measured at 900 V by measuring ECL intensity for identical $Ru(bpy)_3^{2+}$ concentrations at the various PMT voltages and determining the gain correction factors. This change in PMT voltage is not necessary for performing immunoassays or DNA probe assays unless a dynamic range greater than four orders of magnitude is required. The instruments currently being developed will provide automatic

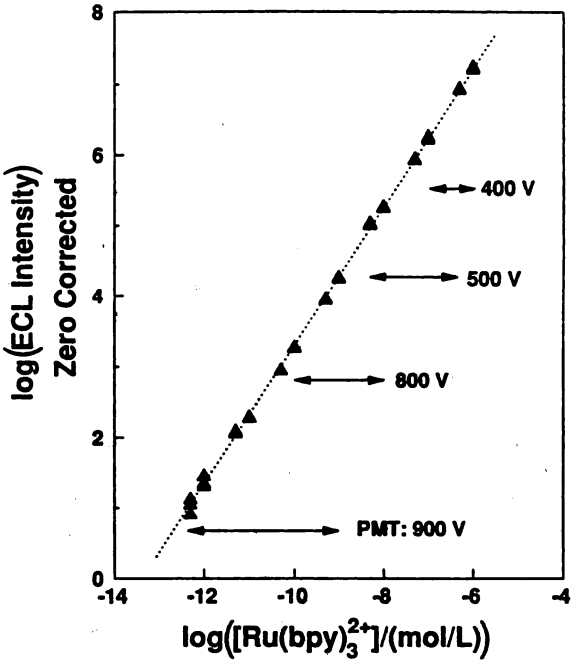

Fig 5. Demonstration of dynamic range for detection of the label, $Ru(bpy)_3^{2+}$

Four PMT voltages were used and the ECL intensity of the plotted points were all normalized to the values obtained at a PMT voltage of 900 V. Three to six measurements are plotted at each concentration

PMT voltage adjustment under microprocessor control.

Figure 6 shows the results of an experiment for determining the detection limit for the free label $Ru(bpy)_3^{2+}$. The detection limit (mean + 2.5 SD of the assay blank) is 200 fmol/L. The ECL observed from label-free buffers (0 pmol/L) is typically at the value shown in Figure 6. Although this limits the detection

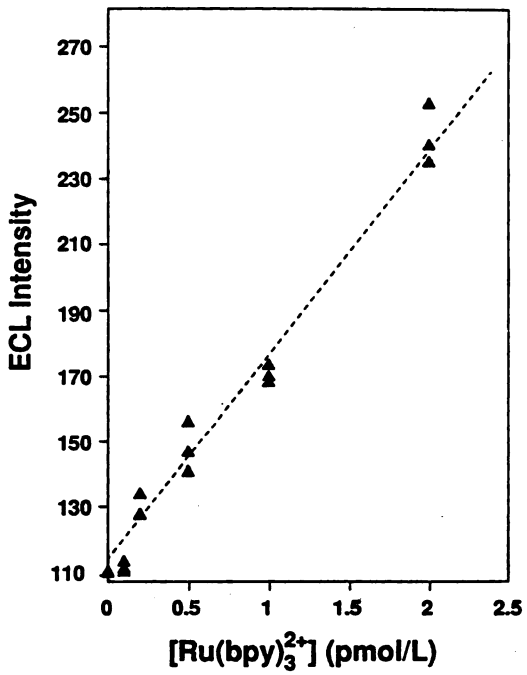

Fig 6. Detection of subpicomolar concentrations of $Ru(bpy)_3^{2+}$

Triplicate measurements were made at each concentration. The *dashed line* represents the linear regression of the data: $y = 38.1x + 110.5$; ($r = 0.991$)

limit for free label, the limitation is not evident in immuno- and DNA-probe assays because the background ECL intensity due to nonspecific binding of labeled molecules is generally substantially higher than that of label-free buffer.

Results of the separation sandwich immunoassay of carcinoembryonic antigen are presented in Figure 7. The linear calibration curve extends from the detection limit of 0.2 to 0.4 μg/L to >2000 μg/L. In Figures 7–9, duplicate measurements are plotted for each analyte concentration although the distinction between the points is not always evident. A correlation study for the ECL assay with a standard radioimmunoassay of alpha-fetoprotein (Figure 7) demonstrated a linear-regression line having a slope of 0.821 and an intercept of −2.27 μg/L (r = 0.967). For alpha-fetoprotein, the linearity of the calibration curve extends from the detection limit of <0.4 to >5000 μg/L.

Figure 8 shows the results of the nonseparation competitive assay of digoxin, in which ouabain/bovine serum albumin conjugate on the microparticles competes for the labeled antibody with the analyte. The detection limit for the assay is <0.1 μg/L, and the dynamic range extends to >5 μg/L.

The ECL nonseparation sandwich assay of thyrotropin (Figure 9) routinely shows a detection limit of <0.04 milli-int. unit/L; the linear range extends to >50 milli-int. units/L. A correlation study comparing results of this assay with those of the IMx ultra-sensitive TSH assay (Abbott Laboratories, Abbott Park, IL 60064) for 60 human serum samples gave a correlation coefficient of 0.992, a slope of 1.17, and a y-intercept of −0.30 milli-int. unit/L.

Figure 10 demonstrates the results of the DNA probe assay, in which we used ECL to quantify the PCR products, plotting the ECL intensity as a function of the

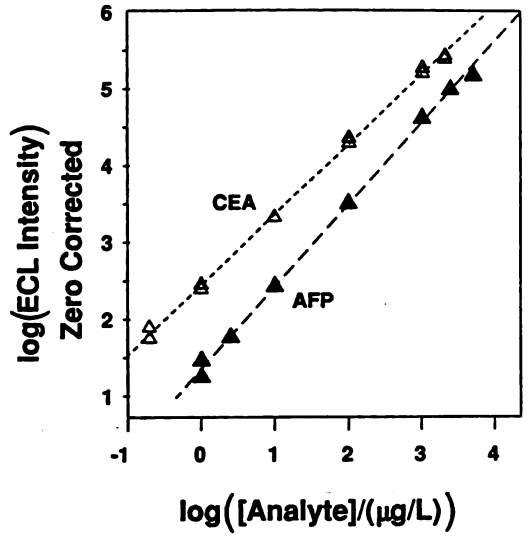

Fig 7. Calibration curves for immunoassays of carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP)

The detection limit for the CEA assay is typically 0.2 to 0.4 μg/L, and the linearity of the curve extends to >2000 μg/L. The detection limit for the AFP assay is <0.4 μg/L, and the dynamic range extends to >5000 μg/L

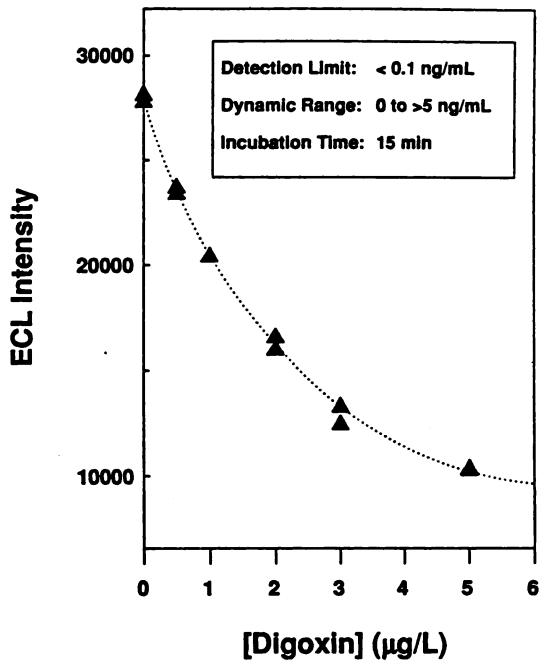

Fig 8. Calibration curve for the nonseparation competitive immunoassay of digoxin

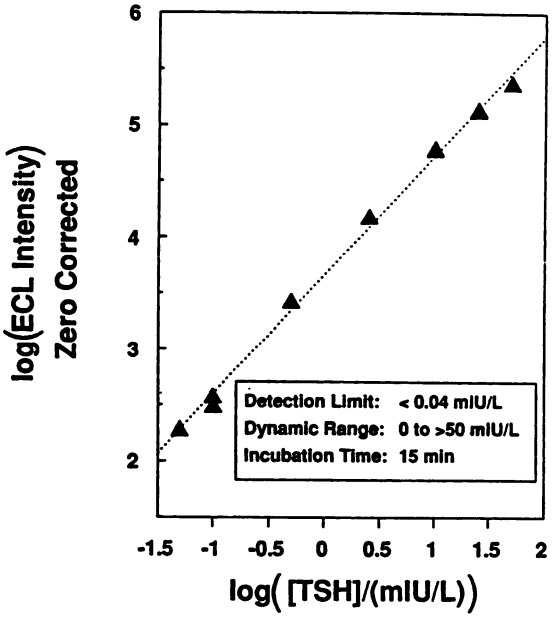

Fig 9. Calibration curve for the nonseparation sandwich immunoassay of thyrotropin (TSH)

TSH was measured in milli-international units per liter

number of HIV copies before amplification. A linear response is demonstrated over the range of 50 to 2000 gene copies. The inset to Figure 10 demonstrates the ability of the ECL-based detection to differentiate fewer than 10 copies of the HIV1 *gag* gene. Results are shown for 10 samples assayed in triplicate. Student's unpaired t-test analysis (two-tailed) gives t = 4.62; i.e., the probability that the data are from the same population is <0.002.

In conclusion, we have demonstrated the utility and versatility of ECL detection for development of immu-

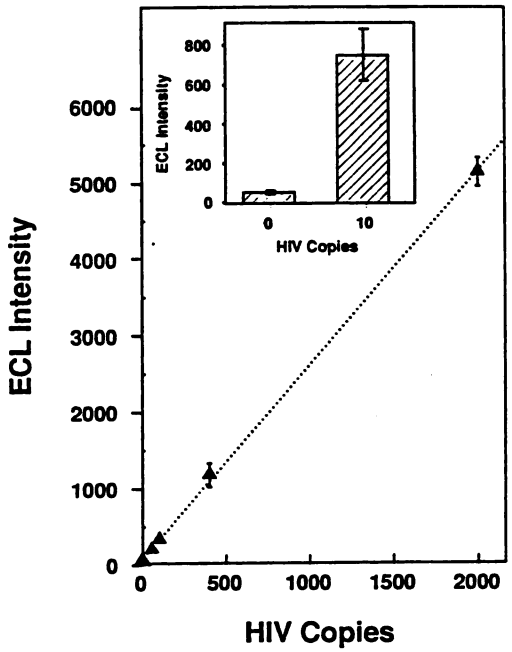

Fig 10. Calibration curve for the quantification of 50–2000 copies of HIV1 *gag* gene

The number of copies of the gene is before the PCR amplification procedure. *Error bars* show 1 SD. The *inset* demonstrates the detection of <10 copies of the gene; the *error bars* represent 1 SEM

noassays and DNA probe assays. Some of the advantages of this technique include the following:

- The method involves no radioisotopes, eliminating disposal and lifetime problems inherent in radioimmunoassays.
- ECL provides for extremely sensitive label detection at subpicomolar concentrations, as well as an extremely wide dynamic range of greater than six orders of magnitude.
- The metal–ligand labels are extremely stable, having shelf lives of longer than one year at room temperature. Labeled proteins are similarly stable if stored at 2–5 °C.
- The label is extremely versatile: it can be chemically activated with several different reactive groups to provide for easy labeling of haptens, antibodies, and nucleic acids as well as other molecules.
- Because of the label's low molecular mass and its hydrophilicity, immunoglobulins can readily be labeled with >20 label molecules per antibody without affecting antibody solubility or immunoreactivity. Similarly, the label does not affect the specificity or hybridization of DNA probes.
- Assay development is accelerated by the simplicity and versatility of the technique. Both immunoassay and DNA probe assay detection use common instrumentation and very similar assay formats. The technique is easily adaptable to the measurement of both large and small analytes with both high sensitivity and wide dynamic range. Assays can be formatted as either separation or nonseparation.
- The detection is rapid, requiring only a few seconds for quantification.
- Finally, the instrumentation required is not complicated, and there are no unique constraints on the disposable portions of the assay; these properties allow the rapid development of automated instruments for both the clinical immunoassay and DNA probe assay markets.

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