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Terbium and Rhodamine as Labels in a Homogeneous Time-resolved Fluorometric Energy Transfer Assay of the β Subunit of Human Chorionic Gonadotropin in Serum

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Background: **Fluorescence resonance energy transfer (FRET) is a powerful tool in analytical chemistry. The aim of the present work was to use FRET to design a homogeneous immunoassay.**

Methods: **We used a highly fluorescent terbium (Tb^{3+}) chelate (donor) and the organic fluorochrome rhodamine (acceptor) combined with time-resolved detection of the acceptor emission in homogeneous assay format for the measurement of the β subunit of human chorionic gonadotropin (βhCG) in serum. We used two antibodies labeled with Tb^{3+} and rhodamine, respectively, recognizing different epitopes on βhCG. The close proximity between the labels in the immunocomplex permitted energy transfer between the pulse-excited Tb^{3+} donor (decay time >1 ms) and the acceptor rhodamine (decay time of 3.0 ns). The prolonged emission of donor-excited acceptor (energy transfer) was measured after the short-lived background and acceptor emissions had decayed. The emission of donor-excited rhodamine was measured at a wavelength of where the emission of unbound donor is minimal.**

Results: **The energy transfer signal was directly proportional to the βhCG concentration in the sample. The limit of detection was 0.43 μg/L, and the assay was linear up to 200 μg/L. Total assay imprecision in the range 10–185 μg/L was between 7.5% and 2.8%.**

Conclusions: **Although less sensitive than heterogeneous, dissociation-enhanced europium-based separation assays, the presented assay format has advantages such as speed and simplicity, which make the assay format ideal for assays requiring a high throughput.**

The measurement of peptide hormones, such as the β subunit of human chorionic gonadotropin (βhCG),[1] in biological samples requires a highly sensitive assay technology. Analyses routinely are performed with immunometric assays in which the combination of two specific antibodies provides the specificity and a sensitive label, such as an enzyme, radionuclide, or a fluorescent compound, allows detection.

Immunometric assays usually are heterogeneous assays in which the high excess of reagents ensures rapid bindings, efficient washing decreases nonspecific signal, and a high-specific activity label provides the sensitivity and wide dynamic range *(1)*. These performance characteristics have made immunometric assays the methods of choice in routine diagnostics of peptide hormones, cancer antigens, low concentrations of specific antibodies, and other clinically relevant indicators *(2)*. In some cases, however, the required separation is too laborious to perform (e.g., from the point of view of automation) or for other reasons undesired (e.g., need for kinetic data or low affinity binding). The screening of thousands or millions of samples in a high-throughput format requires a simple and preferably a non-separation assay design *(3)*.

Several homogeneous assay principles have been published. Only a few of them, however, are applicable to macromolecules *(4, 5)*. In diagnostics, homogeneous assays are used primarily for therapeutic drug monitoring and other low-molecular weight substances, using methods such as fluorescence polarization *(6, 7)*. Similarly to most of the homogeneous methods, fluorescence polarization assay has inherent limitations, both in terms of applicability and sensitivity. Fluorescence resonance energy transfer (FRET) is a widely used technology in

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[1] Nonstandard abbreviations: βhCG, β subunit of human chorionic gonadotropin; FRET, fluorescence resonance energy transfer; TR, time-resolved; TMR, tetramethylrhodamine; and QCA, quench correction algorithm.

biomedical studies *(8)* and has also been applied to the field of diagnostic immunoassays *(9)*.

Time-resolved (TR) fluorometry adds another dimension to homogeneous assays. Because all matrix-related components are present during the fluorometric measurement, it is only through temporal resolution that the background interference can be eliminated *(10)*. In FRET, the temporal resolution is important both in avoiding background problems and in enabling more specific detection of energy transfer-excited acceptor emission *(11)*. Because the excited state lifetime of the donor probe is longer than that of the acceptor probe or the background, the energy transfer can be distinguished from the emission emanating from direct excitation of acceptor molecules by the use of a sufficiently long delay time before gating the detection. The acceptor emission at a longer wavelength can be separated from donor emission by spectral resolution (filters).

Lanthanide chelate labels exhibiting decay times from a few microseconds to several milliseconds are perfectly suited for TR-FRET technologies *(12)*. Recently, this technology has been applied to various binding assays in the field of diagnostics and research *(13–16)*. In addition to the Eu-cryptate applied by Mathis *(13)*, several fluorescent lanthanide chelates have been developed *(17, 18)*. Donor-acceptor pairs, such as Eu^{3+} with CY-5 *(15, 19)*, and Tb^{3+} with rhodamine *(20)*, have been published. Numerous chelates have been synthesized and used in different types of assays, from heterogeneous and homogeneous immunoassays to microscopic imaging *(21)*. Highly fluorescent chelates of Eu^{3+} and Tb^{3+} are particularly well suited for TR-FRET assays. Optimization of label pairs for TR-FRET generally requires an optimally fluorescent donor chelate, i.e., a chelate with a high quantum yield, efficient light harvesting, suitable coupling system with retained biocompatibility, and a suitable emission distribution *(22)*.

We studied the use of a fluorescent Tb^{3+} chelate in a homogeneous sandwich-type immunometric assay of βhCG. The assay is based on two specific monoclonal antibodies against different epitopes on βhCG. One antibody was labeled with an energy-donating Tb^{3+} chelate and the other with an acceptor, tetramethylrhodamine (Alexa^{TM}546), a sulfonated rhodamine derivative. The resulting homogeneous TR-FRET assay was validated by comparison with a heterogeneous assay.

Materials and Methods

REAGENTS AND BUFFERS

The Tb^{3+} chelate, an isothiocyanate-activated highly fluorescent and stable Tb^{3+} chelate (W14016) was obtained from Wallac Labeling Service (Wallac Oy, Turku, Finland). The anti-βhCG antibodies, clones 2764 and 3341, DELFIA® enhancement solution and DELFIA enhancer were from Wallac Oy. The βhCG calibrators were from the DELFIA Free hCGβ Immunoassay Kit (Wallac). Isothiocyanate-activated tetramethylrhodamine (TMR) and the succinimidyl ester of Alexa546, a sulfonated rhodamine derivative, were purchased from Molecular Probes Inc. The assay buffer consisted of 50 mmol/L Tris, pH 7.4, with 9 g/L NaCl and 1 g/L bovine serum albumin.

EQUIPMENT

Absorption spectra were recorded with a Pharmacia Biochrom 4060 spectrophotometer. Fluorescence excitation and emission spectra were recorded with a Perkin-Elmer LS 5 fluorescence spectrometer. TR measurements of Tb^{3+} concentrations and energy transfer emissions were performed with a 1420 Victor multilabel counter (Wallac). The interference from quenching compounds in the samples, the Tb^{3+} signal at the acceptor wavelength, and the background of plastics material were corrected for by a prototype quench correction algorithm (QCA) from Wallac.

ANTIBODY LABELING

Anti-βhCG antibody 2764 was labeled with the Tb^{3+} chelate. The antibody at a concentration of 5 g/L was incubated with a 30-fold molar excess of the chelate in 50 mmol/L carbonate buffer, pH 9.5, overnight at 4 °C. Labeled antibody was separated from unreacted chelates by gel filtration (Sepharose 6B with Sephadex G50 overlay, 0.5 × 70 cm; Pharmacia) with a buffer containing 50 mmol/L Tris-HCl, pH 8, containing 9 g/L NaCl as elution buffer. When the labeling degree (Tb^{3+}/IgG) was determined by measurement of the Tb^{3+} concentration of conjugated antibody against a Tb^{3+} calibrator (Aldrich Chemie) with a DELFIA system *(23)*, a conjugation degree of 6 was achieved. Anti-βhCG antibody 3341 was coupled with TMR or Alexa546 using a similar technology with a 15-fold molar excess of the fluorochromes. The labeling degrees, determined photometrically according to the reagent supplier's instructions, were 2.2 (TMR/IgG) and 2.8 (Alexa/IgG). Antibodies with these labeling degrees were used in the assay if not stated otherwise. Other labeling degrees were obtained by varying the amount of Tb^{3+} or rhodamine excess in the described protocols. Labeled antibodies were found to be stable for several months when stored in Tris buffer, pH 7.4, containing 1 g/L bovine serum albumin.

ASSAY PRINCIPLE

In the immunocomplex, Tb-antibody-βhCG-rhodamine-antibody, the distance between Tb^{3+} and rhodamine is short enough to allow excitation energy to be transferred from Tb^{3+} to rhodamine through Förster-type FRET *(24)*. The decay time of the energy transfer-excited rhodamine emission is directly related to the decay time of the donor chelate and inversely related to the energy transfer efficiency. Compared with the natural decay time of rhodamine (3.0 ns), the energy transfer signal has a greatly prolonged decay time, enabling its measurement with TR fluorometry, thus avoiding the interference derived from background and the emission of rhodamine excited by

pulsed ultraviolet light. The interference from the long-lived emission of unbound Tb^{3+} donor can be avoided by spectral resolution using a high-quality interference filter at a wavelength where Tb^{3+} background is minimal (570 nm; Fig. 1). The measured energy transfer signal is directly proportional to the concentration of βhCG in the sample.

ASSAY PROTOCOL

In the assay, 50 μL of βhCG calibrator or serum sample in the wells of black 96-well microtitration plates (Nunc) were incubated with a mixture of 100 ng of Tb^{3+}-labeled antibody (donor) and 100 ng of rhodamine-labeled antibody (acceptor) in 200 μL of assay buffer. Reagent mixtures were incubated either on a plate shaker for 60 min at room temperature or on the laboratory bench after 2 min of rapid shaking. Energy transfer and Tb^{3+} emissions were measured using excitation at 340 nm, emission filters at 570 nm for rhodamine and 545 for Tb^{3+}, a delay time of 50 μs, a window time of 100 μs, and a cycling time of 1 ms. Interfering processes in the assay were corrected with QCA, using wells containing buffer only, wells containing Tb^{3+}-labeled antibody only, and wells containing the highest concentration of calibrator (200 μg/L) as external standards *(25, 26)*. QCA subtracts the donor interference seen at the emission wavelength of the acceptor and the background from plastics from the acceptor signal before calculating the acceptor-to-donor ratio. The ratio, which corrects for absorption of excitation energy by the serum sample and competing energy transfer processes (quenching), is then multiplied with the donor signal measured from the highest calibrator. This blank-corrected, normalized ratio was used as response parameter in the assay.

Results

SPECTRA

The emission spectra of the donor and acceptor, and the absorbance spectrum for the acceptor are depicted in Fig. 1. For energy transfer to occur, the emission spectrum of the donor and the absorbance spectrum of the acceptor need to overlap.

ASSAY CONDITIONS

Antibodies of different conjugation degrees (label/IgG) were tested in the assay to find the degrees that produced the highest signal-to-noise ratios (Table 1). The fluorescence signal for the calibrator containing 200 μg/L βhCG divided by the signal for the zero calibrator was used to calculate the signal-to-noise ratios. The fluorescence of calibrators ranging from 0 to 200 μg/L βhCG with signals from the acceptor and uncomplexed donor as well as QCA-corrected signals are presented in Tables 2 and 3. The energy transfer efficiency in the assay remained below 15%, indicating a relatively large distance between the labels in the formed complexes and an excess of Tb^{3+}-labeled antibody in the assay. The decay profiles of Tb^{3+} (donor) and the acceptor after energy transfer are shown in Fig. 2. The donor signal shows a clear decay of unquenched Tb^{3+} (1087 μs), which to a great extent results from uncomplexed antibodies and from the fraction of Tb^{3+} chelates at a more distant location from acceptors. The decay of the energy transfer (180–390 μs), measured at 570 nm, is clearly shorter and has a multicomponent nature that can be attributed to the different donor-acceptor distances in complexes formed of randomly labeled antibodies. The decay time, however, is long enough for efficient TR detection.

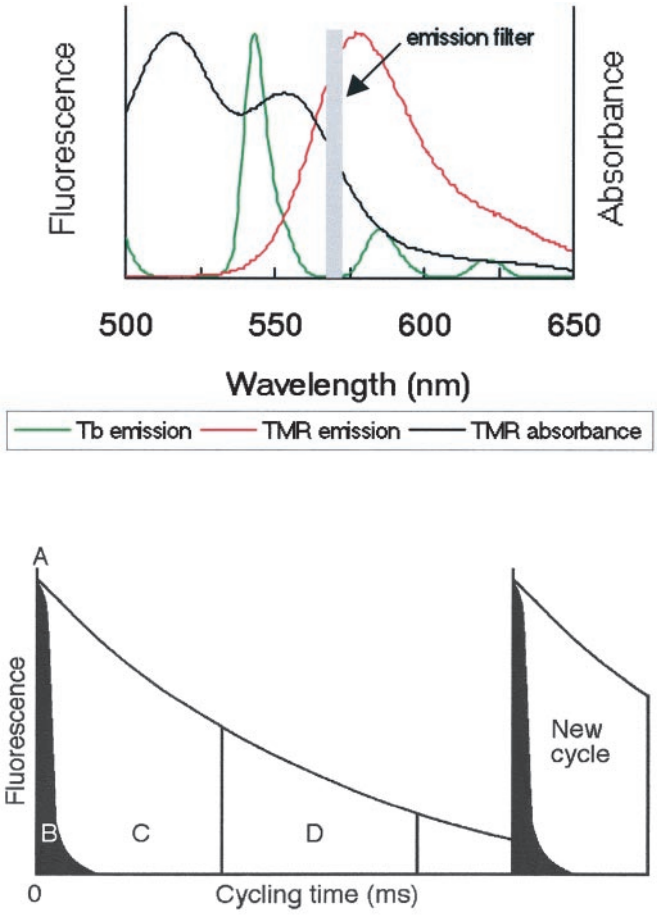

Fig. 1. Principle of TR-FRET.

(*Top*), excitation spectra for the donor-acceptor pair Tb^{3+}-TMR and absorbance spectrum for the latter. Requirements for energy transfer are dipole-dipole interaction between donor and acceptor and an overlap of the donor emission spectrum and the acceptor absorbance spectrum. (*Bottom*), in the homogeneous βhCG assay, the long-lived donor is excited with a light pulse (*A*). The emission of the energy transfer-excited acceptor is measured (*D*) after a suitable delay (*C*) to avoid interference from the matrix and directly excited acceptor (*B*). The long-lived emission of the donor is avoided by measuring the emission of the energy transfer-excited acceptor with a narrow filter at 570 nm, where the donor emission is minimal.

PERFORMANCE CHARACTERISTICS

Assay kinetics were investigated by incubating plates for up to 180 min. The binding kinetics obtained in the assay with constant shaking or no shaking are presented in Fig. 3. The reaction had almost reached equilibrium after 60 min of incubation. The detection limit, i.e., precision of the

Table 1. Effect of the labeling degree (label/IgG) on the signal-to-noise ratio.

	Tb^{3+}/IgG			
	2	4	8	10.8
TMR/IgG				
1.8	5.4	5.3	5.3	2.3
2.2	5.3	5.8	5.8	2.5
3.2	1.5	4.2	3.7	1.7
4.5	1.0	1.6	1.4	1.0
Alexa546/IgG				
2.8	13.0	14.0	14.3	3.4
4.8	12.3	12.8	14.0	3.4
8.8	8.0	8.4	9.0	2.2

measurement of the zero-dose calibrator, was 0.95 μg/L for TMR and 0.43 μg/L for Alexa546, defined as the value 2 SD above the mean of the zero-calibrator values (n = 24). According to the kit insert, the detection limit of the DELFIA Free hCGβ assay is better than 0.2 μg/L. Five serum samples were assayed four times in three different assay runs to estimate the imprecision of the βhCG assay with TMR as acceptor. The within-run, between-run, and total imprecision calculated with ANOVA *(27)* are presented in Table 4. Sixty-six serum samples relatively evenly distributed over the measurement range were assayed, and the concentrations were compared with those obtained with the DELFIA assay. The developed TR-FRET method correlated well with the DELFIA Free hCGβ assay (Fig. 4).

Discussion

Assay miniaturization and automation are major trends in diagnostics today. Homogeneous assays provide a key to adapting assays for automation because complicated coating procedures and slow surface reactions can be avoided. When developing simpler assays in a miniaturized format, however, it is important to maintain high assay sensitivity. Most homogeneous assays are compromised in terms of sensitivity and dynamic range, partly because of matrix-related problems (sample autofluorescence) during endpoint detection. In this context, time resolution is an enabling technology because it allows the measurement of a specific response without background interference *(10)*. With fluorescent lanthanide chelates as the long-lifetime labels, sensitive homogeneous assays have also been developed and applied to serum thyroxine *(28)* and steroid glucuronides in urine *(29)*. A majority of the homogeneous assay techniques are for small molecules and are less suitable for assays of macromolecules. Nonseparation and homogeneous immunoassay techniques and their applications and limitations have been discussed in detail elsewhere *(4)*.

FRET is a homogeneous assay technology that, unlike most others, is well suited for the study of the binding of large components, such as proteins in various kinds of protein-protein interaction assays *(9)*. It is not primarily the size of the proteins, but the distance between donor and acceptor that is critical in FRET assays. The shorter the distance, the more efficient the energy transfer. Therefore, it is beneficial to label proteins in a way that produces a short distance between the donor and acceptor molecules in the formed complexes and to choose a donor-acceptor pair with a high R_0 value (the distance at which the energy transfer efficiency is 50%). Probes with

Table 2. Calibrators run with the TR-FRET βhCG assay with TMR as acceptor molecule.

	Fluorescence, counts		
βhCG, μg/L	TMR[a]	Tb^{3+},[b]	Normalized ratio[c]
0	2917	378 223	0
2	3099	377 293	179
5	3479	378 068	532
20	5087	379 573	2032
104	10 995	365 567	7976
202	16 035	356 943	13 270

[a] Measured fluorescence from energy transfer-excited TMR in formed immunocomplexes.

[b] Fluorescence from Tb^{3+} on unbound antibodies and Tb^{3+} on bound antibodies not donating energy.

[c] QCA-corrected signal of energy transfer-excited TMR.

Table 3. Calibrators run with the TR-FRET βhCG assay with Alexa546 as acceptor molecule.

	Fluorescence, counts		
βhCG, μg/L	Alexa546[a]	Tb^{3+}[b]	Normalized ratio[c]
0	2473	347 870	0
2	3228	347 646	665
5	4323	347 905	1603
20	9563	348 856	6620
104	29 480	337 405	26 305
202	45 274	328 431	42 877

[a] Measured fluorescence from energy transfer-excited TMR in formed immunocomplexes.

[b] Fluorescence from Tb^{3+} on unbound antibodies and Tb^{3+} on bound antibodies not donating energy.

[c] QCA-corrected signal of energy transfer-excited Alexa546.

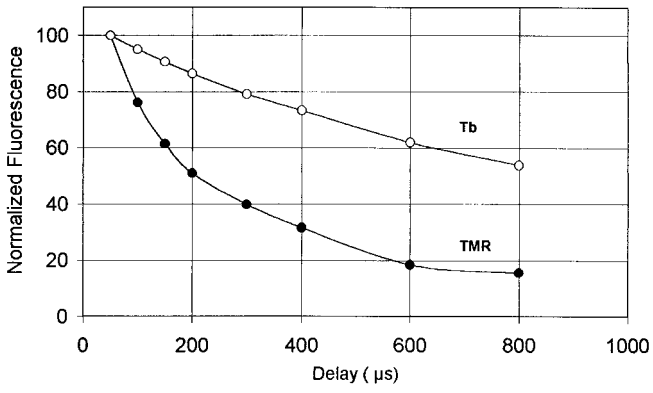

Fig. 2. Decay profiles for Tb^{3+} and TMR in the βhCG assay.

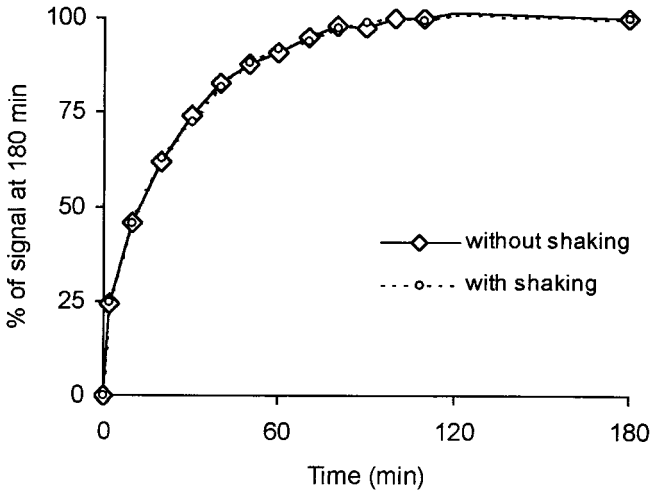

Fig. 3. Reaction kinetics for the TR-FRET assay at 202 μg/L βhCG.
Plates were incubated with constant shaking or with no shaking.

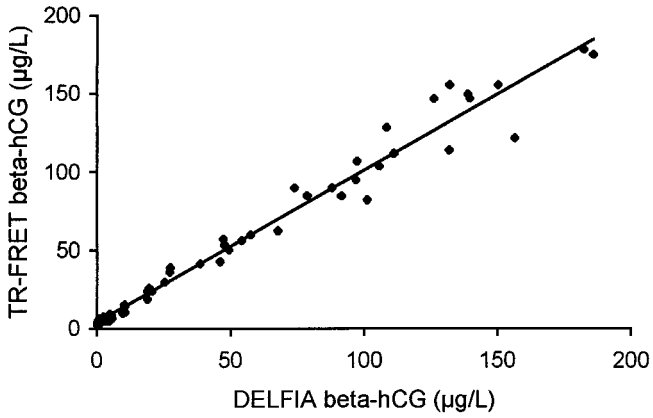

Fig. 4. Comparison of βhCG concentrations determined by the developed TR-FRET method and the DELFIA βhCG assay.
$y = 0.97x + 3.18$; $r = 0.99$; $S_{y|x} = 8.3$.

high R_0 values allow relative long between-probe distances in the assay. Addition of temporal resolution and long-lifetime donor probes can greatly improve the sensitivity and specificity of FRET *(8)*. In TR-FRET, the specific energy transfer in the complex can be distinguished from donor emission by spectral filtration and from the direct emission of the acceptor by temporal filtration. Recently, TR-FRET has been exploited commercially both in diagnostics *(13)* and as a screening tool in drug discovery *(14, 16)*.

Regardless of temporal resolution, most of the TR-FRET assays described above have limitations in both sensitivity and in dynamic range when compared with heterogeneous TR-fluoroimmunoassays. For assays requiring high sensitivity and an excess of donor-labeled reagents, the donor signal is a limiting factor. The working range is a direct function of the distribution of donor emission, i.e., to what degree the donor emits long-lived background at the emission wavelength of the acceptor. One way to alleviate the problem is to use low-quantum yield chelates *(30)*, which hence also give less background. We looked for optimal donors amongst chelates having the following major properties: (*a*) fluorescence intensity as high as possible (the product of absorptivity × quantum yield); (*b*) fully coordinated nonadentate chelating structures with no solvent perturbation; (*c*) very long lifetimes (1–2 ms); and (*d*) optimal emission distribution, i.e., chelates exhibiting major emission at donating wavelength and minimal emission at the wavelength used to measure energy transfer.

The choice between Eu^{3+} and Tb^{3+} depends on the availability of high-quantum yield chelates and respective acceptors. Highly sensitive Tb^{3+} chelates have been discovered recently, and today, stable fluorescent Tb^{3+} chelates exhibiting quantum yields >40% are available *(17, 18)*. Compared with Eu^{3+}, which emits at 613–620 nm, efficient acceptor probes are easier to find at the excitation range of 545 nm. Use of Eu^{3+} requires probes emitting in the near-infrared range, which are less common *(31)*. Tb^{3+} and rhodamine have been used in several energy transfer experiments *(20, 32)* because of the relatively high R_0 values (6–7 nm). Thus, a Tb^{3+} chelate exhibiting a high fluorescence intensity and the low-molecular weight acceptor rhodamine were used as labels in the presented sandwich assay for βhCG.

In the model assay, two monoclonal antibodies recognizing different epitopes on βhCG were labeled with Tb^{3+} and rhodamine. The antibodies were not optimized to give a short distance between the donor and acceptor, but merely to give a good binding performance. Both antibodies were randomly labeled with 1–10 label molecules/IgG. For TMR labeling, the relative insolubility of TMR and its negative effect on antibody functionality and affinity restrict optimal labeling. However, according to the experiments, on the average, 4–8 Tb^{3+} and ~2 TMR per IgG seemed to give the best response. More Alexa546 than TMR can be conjugated to proteins, probably because of lower self-quenching effects and better solubility. Alexa546 has also been reported to be more fluorescent than TMR and other spectrally compatible fluorochromes *(33)*. These observations were confirmed in this study. Alexa546 was threefold more fluorescent than TMR in the TR-FRET assay. The degrees of labeling producing the highest response were 2–5 Alexa546 and 2–8 Tb^{3+} per IgG. In the actual assay, the labels closest to each other in

Table 4. Within-run, between-run, and total assay imprecision for βhCG.

		CV, %		
Sample	Mean, μg/L	Within-run	Between-run	Total
1	10.7	5.0	5.6	7.5
2	25.4	4.9	3.2	5.9
3	51.9	1.9	3.2	3.8
4	103.7	2.3	2	3.0
5	185.4	2.4	1.4	2.8

the complex probably give the largest response, and labels in distant positions only contribute to the background

The sandwich assay for βhCG can be performed using either a direct or indirect labeling approach. The indirect way, i.e., using biotinylated antibodies and labeled streptavidin, is a more generic approach, but tends to give decreased sensitivity because of longer between-probe distances (data not shown). In FRET assays, the fluorescence decay time of the energy transfer-excited acceptor is always shorter than that of the donor. Therefore, a short delay time and counting window relative to that optimal for the measurement of free donor gives an improved signal-to-noise ratio. The shortest possible delay time depends on the phosphorescence of the plastics material used and its decay unless nonfluorescent (black) plates are applied.

The sensitivity of the developed βhCG assay compared well with the commercially available Sm^{3+}-based DELFIA βhCG assay. Bearing in mind that the Sm^{3+} label is 10–100 times less sensitive than Eu^{3+} label in DELFIA assays, it can be concluded that the homogeneous assay format is one to two orders of magnitude less sensitive than a well-optimized, Eu^{3+}-based, heterogeneous DELFIA assay *(34)*. The lower sensitivities of homogeneous assays can be attributed to the differences in quantum yields, the differences in labeling degrees, the relatively high backgrounds in TR-FRET, and to the way homogeneous assays must be optimized. Highly sensitive heterogeneous assays generally apply a strategy of reagent excess *(1)* so that the high excess of labeled antibodies guarantees rapid equilibrium, and efficient washing on the other hand ensures low background. Because of the constant background produced by Tb^{3+}-labeled antibodies and the diffusion background seen at high donor-to-acceptor concentrations, in homogeneous assays there is much less freedom in choosing the concentrations of the labeled components. It is possible to adjust the distance between labels, at least to some extent, with reagents that permit site-specific labeling. Labels in close proximity ensure an efficient energy transfer, and some of the background (interference) can be reduced because donors at distant positions, contributing only to the background, can be avoided. Heterogeneous assays frequently use antibody-coated surfaces such as beads and other solid phases to obtain convenient separation of bound and free fractions. Antibody-coated solid phases require efficient shaking or stirring of the assay mixture to speed up assay kinetics. In homogeneous assays, shaking or stirring of the assay mixture is not necessary because shaking has no effect on the reaction velocity in a homogeneous solution. The omission of solid phases and shaking means fewer assay steps, which is advantageous in automation.

Regardless of the compromises one must make when transferring a heterogeneous assay into a homogeneous format, TR-FRET has proven potential, even in two-site assay design. The assay of βhCG has a good sensitivity, and the assay correlates perfectly with respective heterogeneous assays. Homogeneous TR-FRET assays provide a tool for assay simplification and enable low affinity binding assays. They are easy to perform, rapid, involve fewer steps, and are well suited for systems requiring easy automation, high throughput, or miniaturization.

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