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## Thermochemiluminescence Immunoassay for hCG

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## Chapter 15

**THERMOCHEMILUMINESCENCE IMMUNOASSAY FOR hCG**

Theo M. Luider, Jan C. Hummelen, Jan N. Koek, D. Oudman, and Hans Wynberg

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## I. INTRODUCTION

Since the development of the radioimmunoassay for a soluble protein (insulin) by Yalow and Berson in 1959,<sup>1,2</sup> the immunoassay field has continuously progressed to more sensitive and reproducible assays. This progress made it possible to trace compounds which are relevant for all kinds of biological processes, e.g. , diseases, pregnancy, and poisoning. One of the possibilities to improve these immunoassays further and to open new clinical research areas is the application of a new type of label. Such a new label must have a unique set of characteristics, such that it is preferable over existing labels used in immunoassay.

In 1981, we prepared a new type of label, a functionalized stable 1,2-dioxetane.<sup>3</sup> Later, we reported the first results of immunoassays in which stable 1,2-dioxetanes were used as so called thermochemiluminescent (TCL) labels.<sup>4,5</sup> In the present chapter, the development and optimization of two types of solid-phase sandwich TCL immunoassays (TIA and FTIA) for human chorionic gonadotropin (hCG) using the same type of 1,2-dioxetane label are described. The results are compared to an existing sensitive hCG enzyme immunoassay, in which the same set of antibodies is used.

Although well over 200 1,2-dioxetanes have been prepared to date, the majority of these chemiluminescent compounds is of no use as label for immunoassay, since they are too unstable at room temperature. As an exception, adamantylideneadamantane 1,2-dioxetane and its derivatives such as (1), shown in Figure 1, are extremely stable (the half-life of the parent compound at 20°C is 16,000 years)<sup>6,7</sup> and therefore (1) is suitable as a TCL label for immunoassay. The chemiluminescence signal of this compound (1) is obtained by thermal activation; the half-life of the label in our thermochemiluminescence detection apparatus<sup>5</sup> is about 25 s at 240°C.

The chemiluminescence efficiency of (1) is  $5 \cdot 10^{-5}$ . This efficiency can be increased using a fluorescent acceptor (3), which is covalently attached to the TCL-labeled protein (2),<sup>9</sup> No self quenching is observed upon coupling of more than one label (1) to a protein, since there is no overlap between the weak UV absorption and the chemiluminescence spectrum of this label. Hence, proteins labeled with (1) and (3) result in conjugates with a high chemiluminescence efficiency ( $4 \cdot 10^{-2}$ ). Such dually labeled proteins (antibodies, antigens, or protein-protein conjugates thereof) can be competitive with previously known labels (enzymes, isotopes, fluorescent labels, chemiluminogenic labels)<sup>10-13</sup> and their antibody (or antigen) conjugates.

## II. RESULTS

### A. hCG ENZYME IMMUNOASSAY

In order to compare the thermochemiluminescence immunoassay with an existing sensitive immunoassay, we chose the hCG enzyme immunoassay (Organon Teknika). This immunoassay is based on the well-known sandwich principle.<sup>14</sup> The assay was performed with two monoclonals specific for two different determinants of the hormone. The combination of these two monoclonals was chosen so that cross-reactivity with strongly related hormones (LH, TSH, FSH) was prevented.<sup>15</sup> In Figure 2A the result of the hCG enzyme immunoassay, performed on polystyrene microtitration strips, is shown. The detection limit was at 1 IU hCG per liter, the sensitivity was 0.007 absorbance units per IU hCG per liter and the mean precision over the range measured was 4.7%,\* The detection limit was determined using the following formula:

$$DL = A_0 + \frac{t_{0.95} * SD}{\sqrt{n}} \quad (1)$$

\* Sensitivity = tangent of the slope of the immunoassay curve (signal/[antigen]); precision = reproducibility of a measurement; detection limit = a limiting value which can be distinguished from the zero value.

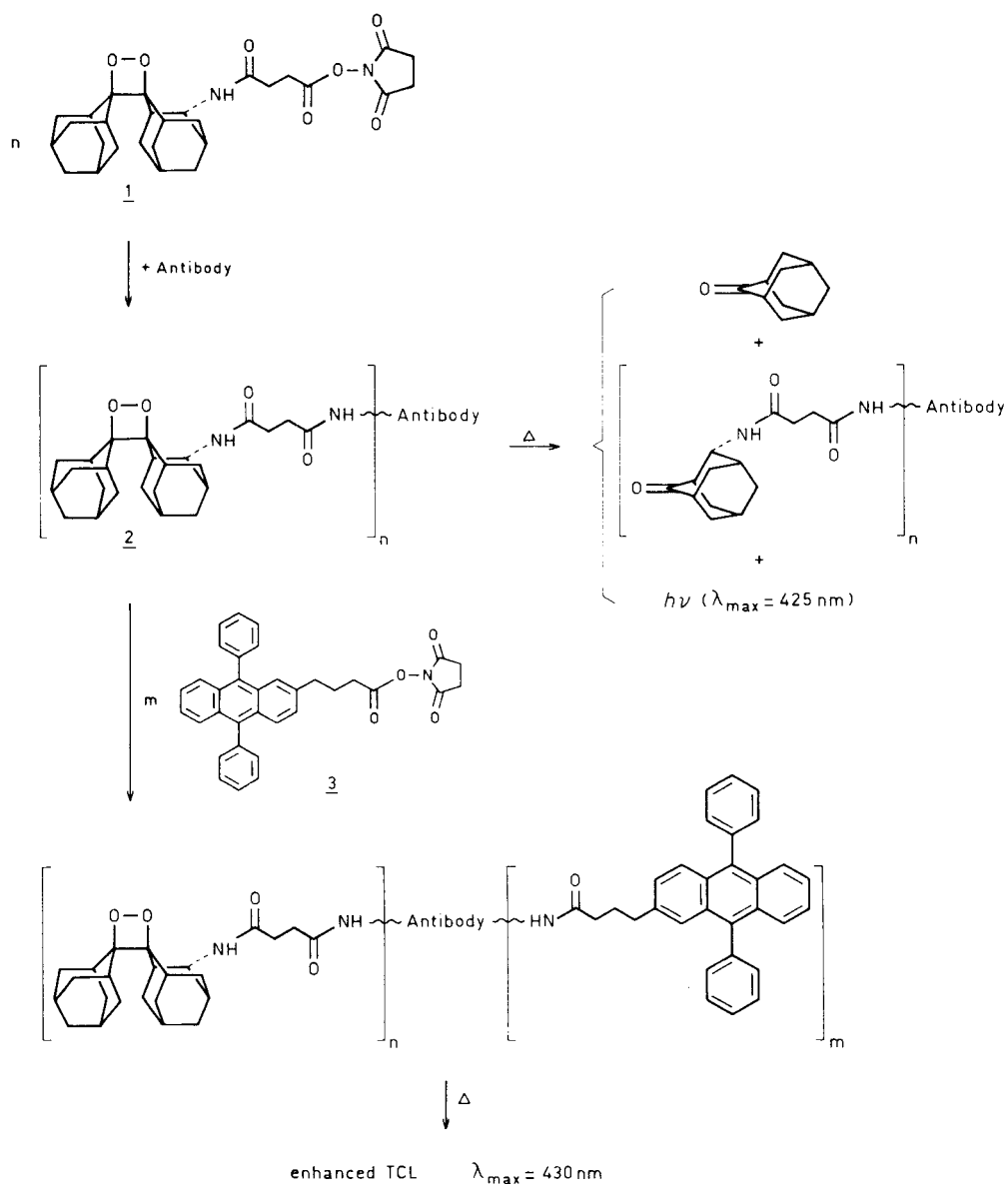


FIGURE 1. Compound (1) can be coupled to proteins in a simple one-step procedure. Upon heating, the product decomposes quantitatively into two adamantanone fragments. During this process photons are released with an efficiency of  $5.10^{-5}$ . After coupling of (1) to an antibody, the conjugate (2) can be applied in a thermochemiluminescence immunoassay. By way of (intramolecular) Förster type energy transfer to an appropriate fluorescent acceptor, e.g., 2-(*N*-succinimidylpropyl)-9, 10-diphenylanthracene (3),<sup>8</sup> the chemiluminescence efficiency is greatly enhanced.

in which DL = detection limit,  $A_0$  = background value at doses zero, and  $t_{0.95}$  = value from a student  $t$  distribution which indicates that a value larger than  $t_{0.95}$  will occur only with a chance of 5% (the value of  $t_{0.95}$  is dependent on the number of measurements), SD = standard deviation ( $\sigma_{n-1}$ ), and  $n$  = number of measurements.

Because the signal of the stable 1,2-dioxetane label is detected at a high temperature (240 to 250°C), the solid phase for this type of immunoassay must be heat resistant. A suitable heat resistant solid phase for immunoassay is Kapton 500H, a polyimide. In order

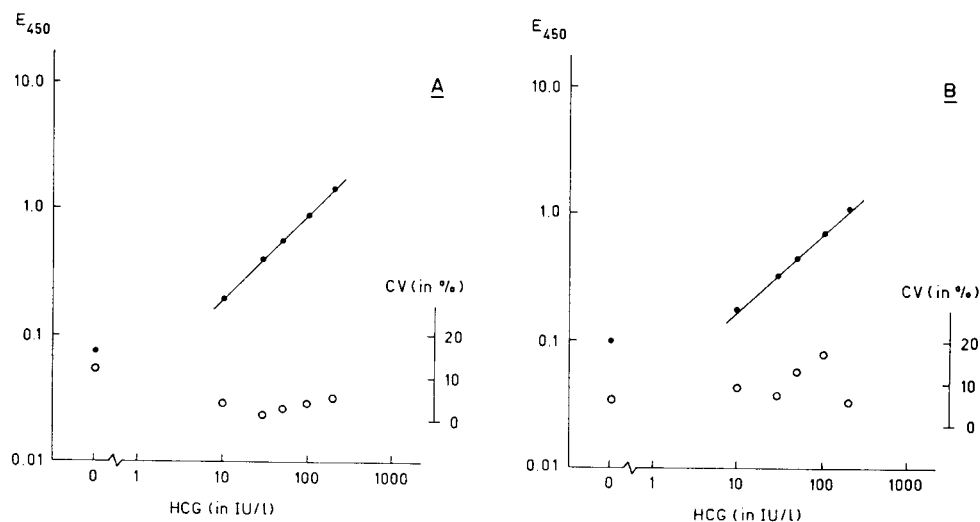


FIGURE 2. (A) hCG-enzyme immunoassay performed on polystyrene microtitration strips. The incubation of antigen and labeled antibody was performed in one step (incubation period 30 min). (B) An identical assay performed on Kapton 500H disks. (●) Each point represents the mean value of four measurements. (○) Coefficient of variation of each series of measurements.

to compare the hCG enzyme immunoassay with TCL immunoassay, the hCG enzyme immunoassay was also performed on disks of Kapton 500H (Figure 2B). Clearly, the assay on Kapton 500H is of equal quality under the chosen circumstances, which are optimal for the titration plate (see experimental part). Hence, Kapton 500 H can be used as the solid-phase material in a comparative study concerning the enzyme, and a TCL immunoassay for hCG.

### B. THERMOCHEMILUMINESCENCE IMMUNOASSAY (TIA)

All thermochemiluminescence immunoassays reported in this chapter were performed on Kapton 500H. In Figure 3, the chemical structure, the thermoluminescence characteristics of this material (between 100 and 250 °C), and a typical TCL curve in time from an aliquot of a solution of a 1,2-dioxetane labeled protein on this polymer are shown.

A TIA with monoclonal anti-hCG, labeled with ten residues ( $\underline{1}$ ) (specific activity  $6.10^5$  photon counts per microgram antibody, measured on Kapton 500H with the standard TCL reader,<sup>5</sup>  $\phi_{app} = 1.4 \cdot 10^{-3}$ ), was performed (Figure 4). Pretreated Kapton disks, which were physically coated with monoclonal anti-hCG were used as the solid phase. The circumstances of the immunoassay correspond with the hCG enzyme immunoassay on Kapton 500H, except for the incubation volume (Figure 2).

It can be concluded that (i) the dynamic range of the immunoassay is large (over three orders of magnitude), (ii) the sensitivity is  $\approx 5$  photon counts per International Units hCG per liter, (iii) the detection limit is 10 IU hCG per liter (determined by formula 1), and (iv) the mean precision over the total range measured is 15.5%. The measurements were performed with an automated TCL reader.<sup>5</sup> Table 1 shows the intra-assay precision of this immunoassay, the influence of the conjugate concentration, and the influence of the incubation time. It can be concluded that (i) no large differences between measurements on 2 separate days are observed, (ii) at a higher concentration of the conjugate (86.5  $\mu\text{g/ml}$  instead of 18.4  $\mu\text{g/ml}$ ), an increased background luminescence and an increased sensitivity (by factor 1.4) is observed, and (iii) after an incubation period of 1 h, the antibody-antigen binding reaction is already in equilibrium.

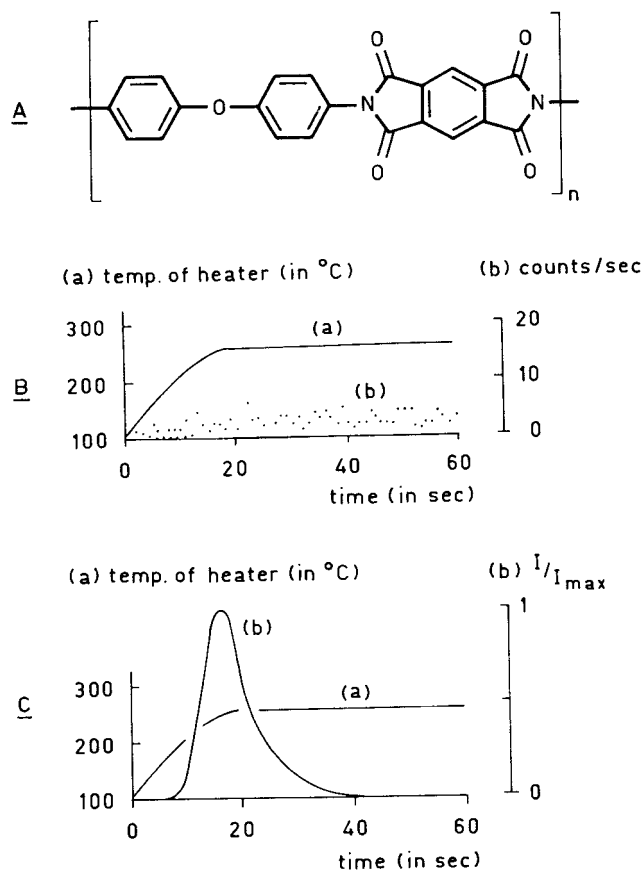


FIGURE 3. (A) Chemical structure of Kapton 500H. (B) Thermoluminescence (background) of cleaned Kapton 500H at 100 to 250°C under an atmosphere of nitrogen. (C) Curve in time of a typical TCL measurement of a 1,2-dioxetane labeled protein (bovine serum albumin) on a Kapton disk at 100 to 250°C.

In order to improve this TIA, the specific activity of the labeled antibody was increased by covalently attaching high numbers of label residues per antibody molecule. The results of such conjugates (labeled with respectively 20 and 26 residues of  $\underline{1}$ ) in TIA are shown in Table 2. An increase in sensitivity was observed especially for the antibody labeled with 26 residues ( $\underline{1}$ ). The detection limit was not lowered significantly and the precision was equal to the above mentioned TIA.

The immunoreactivity of the three TCL conjugates, relative to that of the native antibody, was determined using a competitive solid-phase sandwich immunoassay. Thus, the immune reactivity of the antibody, labeled with 10, 20, or 26 residues ( $\underline{1}$ ), was found to be 54%, 21%, and 11%, respectively. A sample of urine of a pregnant woman, measured accordingly, showed a signal indicating an hCG concentration of far above 2000 IU hCG per liter.

From these TIAs it can be concluded that:

1. A sensitive immunoassay can be performed using stable 1,2-dioxetanes as the sole label, attached to an antibody; compared to the hCG enzyme immunoassay, however, the TIAs still show a poorer precision and a detection limit.
2. The dynamic range of the TIAs is much larger than that of the corresponding enzyme immunoassay.

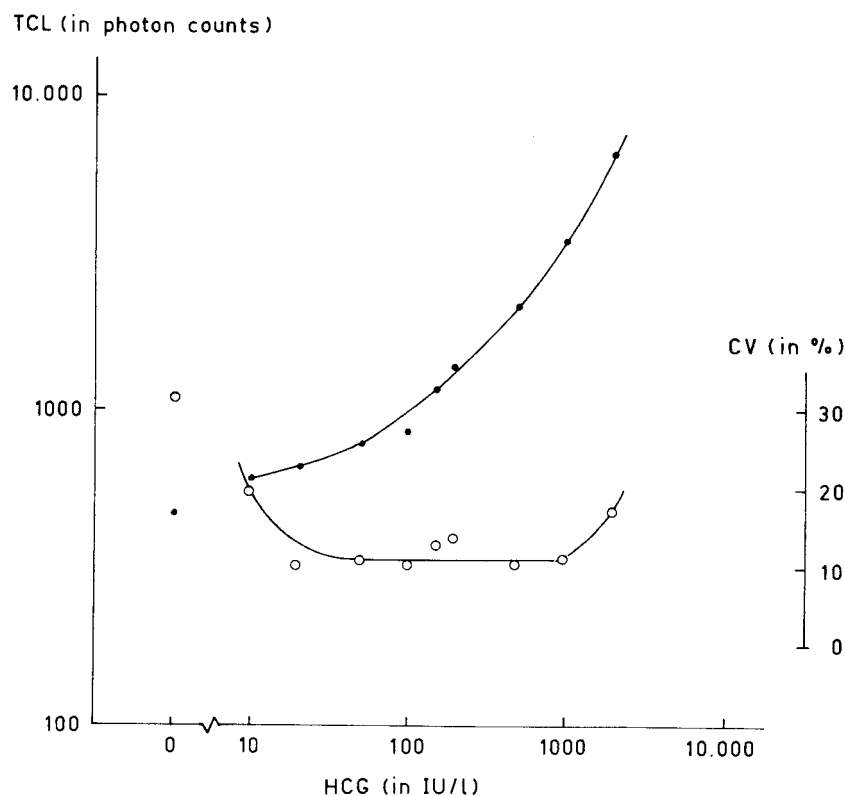


FIGURE 4. hCG-TIA on antibody coated Kapton 500H disks. The conjugate was labeled with 10 residues (○). (●) Each point represents the mean of five measurements. (○) Precision (CV).

**TABLE 1**  
**TCL Immunoassay: Intra-Assay Precision; Influence of Incubation Time and Conjugate Concentration**

		TCL (in Photon counts; CV in %) <sup>a</sup>			
		hCG concentration (in IU/l)			
		0	200	2000	n <sup>b</sup>
<b>Intra-assay precision<sup>c</sup></b>					
Day 1		598 (17.9)	1,201 (12.8)	7,283 (12.8)	6
Day 2		483 (32.5)	1,397 (14.5)	6,630 (17.1)	6
<b>Incubation time</b>					
1 h		608 (5.1)	1,577 (7.6)	7,204 (8.1)	3
16 h		598 (17.9)	1,201 (12.8)	7,283 (12.8)	6
<b>Conjugate concentration</b>					
1-h incubation					
	18.4 µg/ml	608 (5.1)	1,577 (7.6)	7,204 (8.1)	3
	86.5 µg/ml	1,140 (23.9)	2,364 (19.7)	11,886 (9.8)	4
16-h incubation					
	18.4 µg/ml	598 (17.9)	1,201 (12.8)	7,283 (12.8)	6
	86.5 µg/ml	793 (21.8)	1,988 (9.9)	12,156 (17.5)	7

<sup>a</sup> CV = coefficient of variation.

<sup>b</sup> Number of measurements.

<sup>c</sup> Concentration conjugate 18.4 µg/ml. Immunoassays shown were performed on Kapton 500H (temperature 240°C, measuring time 2 min).

**TABLE 2**  
**Influence of the Number of Residues of Label (1), Covalently Coupled to Monoclonal Anti-hCG, on TIA**

Entry	Conjugate conc. (in µg/ml)	Number of (1) in ab <sup>b</sup>	TCL (in photoncounts; CV in %) <sup>a</sup>			Urine of a Pregnant Woman <sup>c</sup>
			hCG concentration (in IU/l)			
			0	200	2000	
1	29.4	20 <sup>d</sup>	696 (10.3)	1,359 (21.1)	8,798 (2.0)	
2	86.4	20	870 (45.0)	1,995 (25.9)	5,566 (22.6)	
3	29.4	26 <sup>c</sup>	1,460 (4.9)	5,061 (7.3)	33,650 (8.8)	
4	86.4	26	3,279 (49.4)	6,258 (40.9)	26,887 (50.3)	76,897 (25)

<sup>a</sup> TCL measurements (n = 3) were performed at 240°C for 2 min. CV = coefficient of variation.

<sup>b</sup> Number of residues (1) covalently coupled to antibody.

<sup>c</sup> 12th week.

<sup>d</sup> Specific activity 1.3\*10<sup>6</sup> counts/µg antibody (on Kapton 500H).

<sup>e</sup> Specific activity 1.7\*10<sup>6</sup> counts/µg antibody (on Kapton 500H).

**TABLE 3**  
**Specific Activity of Monoclonal Anti-hCG Labeled with Different Numbers of Residues of (1) and (3)**

Entry	Molar ratio antibody: (1):(3) during labeling	Number of (1) bound to antibody	Specific activity (in photon counts *10 <sup>6</sup> /µg) <sup>a</sup>	Light units (1) per antibody molecule <sup>b</sup>
1	1:25:0	10	0.6	10
2	1:50:0	20	1.29	20
3	1:100:0	26	1.77	27
4	1:25:89	10	6.0	91.5
5	1:50:89	20	10.8	165
6	1:100:89	26	18.7	285
7	1:200:500 <sup>c</sup>	n.d. <sup>d</sup>	50	762
8	1:180:89 <sup>c</sup>	n.d. <sup>d</sup>	10.1	154

<sup>a</sup> TCL measurements were performed on Kapton 500H. The antibody was dissolved in borate buffer (100 mM; pH 8.5).

<sup>b</sup> TCL is expressed in units chemiluminescence of (1). The chemiluminescence of (1) is amplified by (3) and hence the number of residues of (1) and (3) is substantially lower than the indicated CL units.

<sup>c</sup> Label (1) and (2) were covalently coupled to the antibody in a one step procedure.

<sup>d</sup> Not determined.

- The use of antibody conjugates, containing more than ten residues of label (1), results in an increased sensitivity, but does not improve TIA with respect to the detection limit.
- The presence of either urine or serum in a sample submitted to TIA does not dramatically quench the 1,2-dioxetane signal.
- Labeling with (1) does not dramatically reduce immune reactivity of the antibody.

### C. FLUORESCENT THERMOCHEMILUMINESCENCE IMMUNOASSAY (FTIA)

The specific activities of the TCL conjugates can be increased by concurrent labeling of monoclonal anti-hCG with (1) and the highly fluorescent singlet energy transfer acceptor (3). The specific activities of different conjugates labeled with varying numbers (1) and (3) are shown in Table 3. The standard labeling reaction of this antibody with (1) resulted in a labeling efficiency of 26 to 40% (determined by TCL measurement). Subsequent labeling



**TABLE 4**  
**Fluorescent Thermochemiluminescence Immunoassay**

Entry	Conjugate (molar ratio antibody 1:3 during labeling)	Conjugate concentration (in µg/ml)	TCL (in photoncounts; CV in %)		
			hCG concentration (m IU/l) (n = 3)		
			0	200	2000
1	1:25:89	29.4	1,767 (35.6) <sup>a</sup>	3,209 (39.3)	23,297 (9.9)
2	1:25:89	86.4	4,198 (9.7)	5,392 (12.1)	32,547 (22.6)
3	1:50:89	29.4	2,712 (27.4)	7,462 (1.1)	65,234 (17.3)
4	1:50:89	86.4	6,031 (33.3)	13,707 (12.0)	96,667 (39.1)
5	1:100:89	29.4	26,090 (7.7)	31,527 (15.7)	314,238 (16.6)
6	1:100:89	86.4	67,071 (18.6)	62,380 (74.1)	287,310 (44.7)
7	1:200:500	7.5	7,228 (2.6)	15,415 (12.3)	72,476 (26.3)
8	1:200:500	20.0	14,994 (30.1)	26,053 (6.6)	84,197 (17.1)
9	1:180:89	3.0	576 (19.9)	2,891 (26.6)	17,249 (32.9)
10	1:180:89	6.0	908 (9.6)	4,121 (16.4)	25,830 (27.9)
11	1:180:89	1.5	588 (3.4)	4,599 (15.5)	14,701 (21.3)

<sup>a</sup> CV = coefficient of variation in percent.

of the conjugates with (3) (entries 4, 5, and 6) resulted in a TCL amplification of a factor ≈10. A one pot labeling reaction of the antibody with (1) and (3) (entries 7 and 8) resulted in conjugates with CL efficiencies up to 1 to 4% (the incorporated number of residues of (1) and (3) in the product of entry 7 is estimated to be 50 and 15, respectively).

All conjugates were screened for their performance in a solid-phase sandwich immunoassay analogous to the above-mentioned TIA. In Table 4, the immunoassay (FTIA) results for the quantification of three concentrations of hCG using these conjugates are shown. The conjugate concentration was varied as well, in order to search for the optimal condition for each conjugate.

In Figure 5, FTIAs are shown using different conjugates at optimal conjugate concentrations. The antibody, which was brought in reaction with 25 equivalents (1) and 89 equivalents (3) (Table 3, entry 4), was chromatographed over Sephadex<sup>®</sup> PD-10 (Pharmacia) once before use, in order to remove traces of (3) still present after dialysis. This reduces blank values of the immunoassay. Compound (3) is very hydrophobic and is poorly soluble in aqueous buffer. Removal of noncovalently bound (3) from a crude conjugate solution was rather difficult. Even the dialyzed and Sephadex PD-10 purified conjugate showed noncovalently bound (3) in a SDS-polyacrylamide gel electrophoresis.

The immune reactivities of dually labeled proteins of entries 4, 5, and 6 (Table 2) were 36%, 20%, and 3% of that of the unlabeled antibody, respectively. Surprisingly, labeling of antibody with large numbers of label (1) and (3) does not affect immune reactivity dramatically. Even the conjugate "1:200:500" (entry 7, 8; Table 4) can be used in a TCL immunoassay.

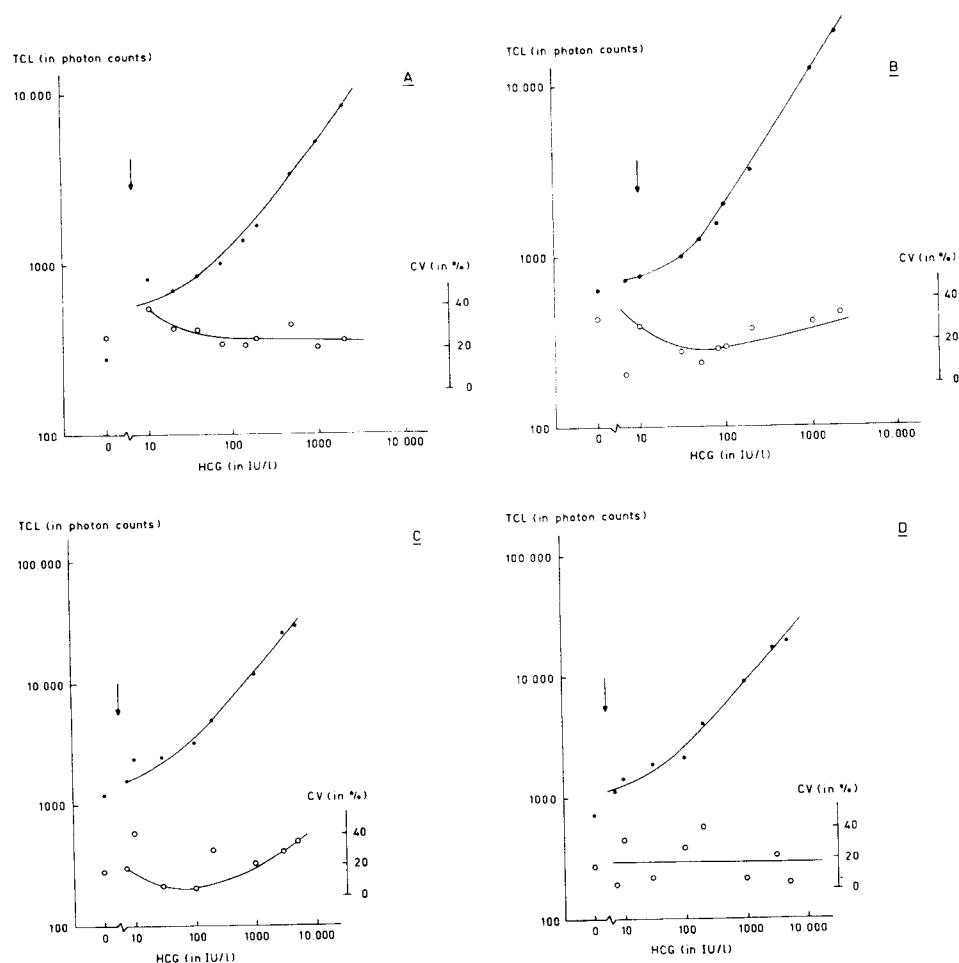


FIGURE 5. FTIA using the conjugates of Table 3, entries 4 (A;  $n = 6$ ), 6 (B,C;  $n = 3$ ), and 8 (D;  $n = 6$ ). Conjugate concentration: A, 3  $\mu\text{g/ml}$ ; B, 14  $\mu\text{g/ml}$ ; C, 7  $\mu\text{g/ml}$ ; and D, 3  $\mu\text{g/ml}$ . The arrows in the graphs indicate the detection limit (calculated according to formula (1)).

All measurements were performed with an automated TCL reader. A typical example of the presentation of these data is shown in Figure 6. The TCL curves (intensity vs. time), obtained from samples containing 0, 200, and 2000 IU hCG per liter, all have the same shape because the thermal decomposition of the 1,2-dioxetane residues is a first order process. This allows a standard heating program and a standard period of time for all measurements.

Application of antibodies, labeled with both (1) and (3), resulted in an increased blank value in FTIA compared to the corresponding TIA. The hydrophobicity of (3) is likely to cause an increased tendency to aspecific binding of the conjugate to the solid phase. However, since both the relevant signal and the blank value increased (more than was expected from the increased specific activity of the antibody), the net result of the FTIA was superior to that of the TIA. The major improvement was obtained in sensitivity of the assay. Furthermore, a minor improvement of the detection limit was reached. Although counting statistics play a diminished role in FTIA, a net improvement in precision was not observed.

It is surprising that very heavily labeled conjugates (entries 7 and 8; Table 3) still can be used in a FTIA (entries 7 to 10; Table 4). However, such a heavily labeled antibody gives rise to a further increase in the blank value of the immunoassay; a compromise must be found between maximal specific activity and minimal background luminescence due to aspecific binding.

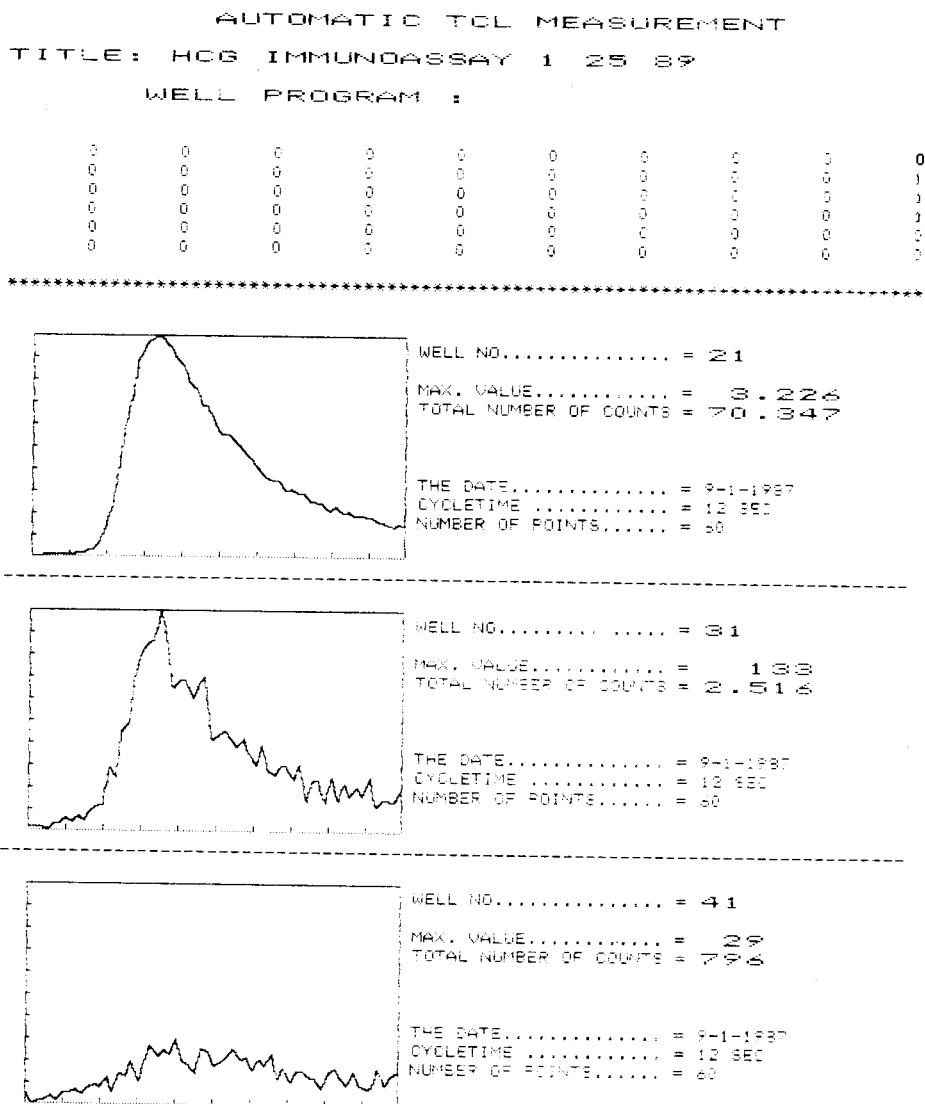


FIGURE 6. A typical example of the presentation of data of an hCG-TCL immunoassay. Wells no. 21, 31, and 41 show the immunoassay result for 2000, 200, and 0 IU/l hCG in human serum, respectively. At the left side, the TCL curve in time is shown and at the right side, the maximum number of photon counts per time interval (1.2 s) and the total number of photon counts of the TCL measurement (72 s) are printed. At the upper side, a presentation is given of the wells, which will be measured by the automated TCL reader (0 = not measured; 1 = measured by the automated TCL reader; well 1 at bottom right, well 60 at upper left side).

#### D. QUENCHING PHENOMENA IN A THERMOCHEMILUMINESCENCE IMMUNOASSAY

A remarkable observation in the TCL immunoassays shown was the relatively high signal at high concentrations of hCG (> 200 IU hCG per liter) and the relatively low luminescent signal at low concentrations of hCG (<200 IU hCG per liter). This fact (which is not observed in the corresponding enzyme immunoassay) can be explained by a quenching phenomenon playing an increasingly important role at lower levels of antibody conjugate present on the Kapton 500H solid phase. In Figure 7 the TCL of Kapton 500H disks, coated with bovine serum albumin labeled with (1) and washed with borate buffers of varying

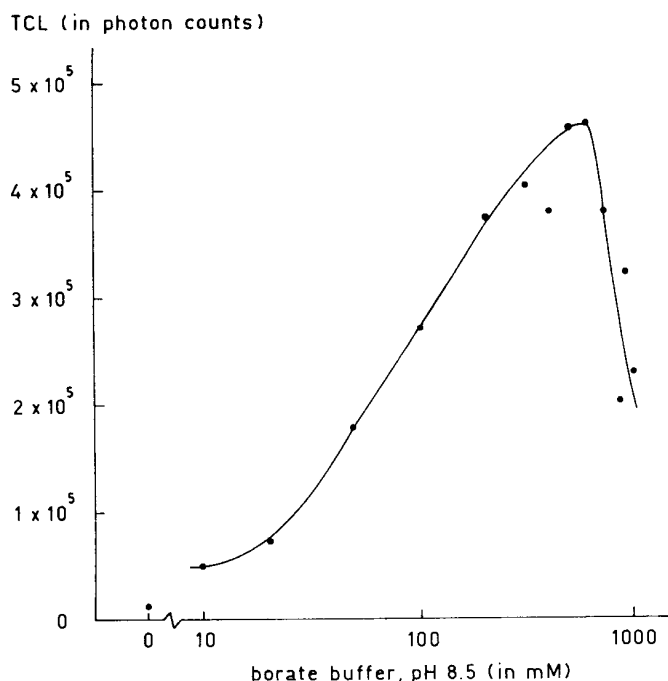


FIGURE 7. Influence of wash procedures using borate buffer of different molarities on the TCL of Kapton 500H disks coated with BSA labeled with both (1) and (3). Vertical axis: photon counts from Kapton 500H disks, coated with TCL labeled BSA (each dot represents the mean value of six disks). Horizontal axis: molarity of borate buffer used in the wash procedure after coating.

molarities, is shown. When coated disks were washed with distilled water, only a minor fraction (2%) of the 1,2-dioxetane residues was detected by TCL measurement. Washing with borate buffers of high molarities, however, resulted in the detection of all of the expected TCL. (The background luminescence of the borate buffer was negligible at all molarities). The increase of TCL in the presence of borate buffer can be explained by complex formation of borate salts and 1,2-dioxetane residues at 240°C. This complexing prevents the 1,2-dioxetane TCL from quenching by all kinds of compounds (e.g., metals) present on the surface and partially embedded in the Kapton 500H material. Kapton 500 H disks coated with bovine serum albumin labeled with <sup>125</sup>I showed that about 400 to 800 ng BSA/cm<sup>2</sup> was coated on Kapton 500H. This corresponds nicely with the amount of TCL found from the disks, coated with TCL labeled albumin and washed with the borate buffers of optimal molarity (Figure 7).

For the above mentioned reason, the Kapton 500H disks were washed with 100 mM borate buffer after the incubation of antigen and antibodies. Higher molarities of borate buffer were not used, because of practical reasons (The pick up mechanism of the automated TCL reader was contaminated upon use of borate buffers of high molarity).

We are tempted to believe that, especially at low concentrations of the antigen in a thermochemiluminescent immunoassay, such quenching, despite the presence of 100 mM borate buffer, occurs. Treatment of the Kapton disks in such a way that TCL quenching impurities on the surface of this solid-phase material are removed, is a goal for the near future.

### III. DISCUSSION

Sensitive TCL immunoassays are possible. Until now, neither the hCG TIA nor the hCG FTIA is competitive with a sensitive hCG enzyme immunoassay. Probably, this is caused by a decrease of TCL by quenching of the stable 1,2-dioxetane luminescence by impurities, present on the surface of Kapton 500H. It was proven that such quenching can be inhibited by complexation of the 1,2-dioxetane with  $\beta$ - and  $\gamma$ -cyclodextrin.<sup>9</sup> Presently, work is done to change the Kapton 500H surface to such an extent that luminescence quenching of a monomolecular layer of 1,2-dioxetane labeled proteins is reduced substantially.

Another possibility to improve the TCL immunoassay is the development of a label, containing a combination of a stable 1,2-dioxetane moiety and an efficiently fluorescent singlet energy acceptor. Thus, an extremely high specific activity of a conjugate can be obtained without the need of coupling many labels to an antibody. As a consequence, both the affinity and the immune kinetics of the conjugate are expected to be preserved more efficiently.

The advantages of using stable 1,2-dioxetanes in immunoassay are (i) the high specific activity of antibodies labeled with both (1) and (3), (ii) the large range of antigen concentration that can be measured by TCL immunoassay; the range of measurement of stable 1,2-dioxetanes is only restricted by the apparatus used (our apparatus has a measuring range of seven orders of magnitude), (iii) the inherently present signal (the signal is switched on simply by raising the temperature, without the need of a waiting time or additional chemicals), and (iv) the background luminescence in the immunoassay solely originates from the 1,2-dioxetanes. Proteins show very low background luminescence upon heating under an atmosphere of nitrogen.

A present disadvantage of the TCL technique is the need of a special heating apparatus. Although we were able to design a device, capable of heating a sample (or a series of samples) from room temperature up to 250°C within a few seconds, improvement is obviously needed. The precision of the TCL assay being inferior by  $\approx 5\%$  to that of the enzyme immunoassay can be partly explained by the heating system allowing substantial variation in the temperature program during TCL detection. Other possibilities to decompose 1,2-dioxetanes are laser excitation,<sup>16-18</sup> room temperature catalytic luminescent decomposition of stable 1,2-dioxetanes,<sup>19</sup> and chemical and enzymatic triggering of suitably substituted 1,2-dioxetanes.<sup>20-22</sup> It is clear that the ultimate use of stable 1,2-dioxetanes as labels (or enzyme substrates) for immunoassay has not yet been achieved. (See Chapter 17.)

### IV. EXPERIMENTAL PART

#### A. MATERIALS

Bovine serum albumin (Sigma, Fraction V), Sephadex<sup>®</sup> PD-10 (Pharmacia), boric acid (Merck), sodium tetraborate (Merck), and Tween<sup>®</sup> 20 (Sigma) were used without further purification. Kapton 500H was purchased from Dupont in sheet format. Monoclonal anti-hCG, hCG standards, and peroxidase labeled monoclonal anti-hCG were kindly provided by Dr. T. C. J. Gribnau and Mr. T. van Sommeren (Organon Int. BV/Organon Teknika).

#### B. CLEANING OF KAPTON 500H DISKS

Sheets of Kapton 500H were cut in disks ( $\phi 9$  mm). These disks were refluxed in EtOH during 4 h. The ethanol was replaced four times. Hereafter, the disks were refluxed during 1 h in distilled water. The distilled water was replaced once. Refluxing in H<sub>2</sub>O longer than 1 h resulted in swelling of the disks.

After refluxing and thoroughly washing the disks with excess cold distilled water, the disks were transferred into a glass petri dish and heated for 2 h in an oven at 300°C.

The background TCL of such cleaned disks was checked in the TCL reader. Optimally cleaned Kapton 500H disks showed a background of less than 150 counts per minute.

### C. ENZYME IMMUNOASSAY

#### 1. Coating of Kapton 500H Disks with Monoclonal Anti-hCG

About 500 disks were put in an Erlenmeyer flask (200 ml) containing 70 ml of a solution of 40  $\mu\text{g}$  monoclonal anti-hCG per milliliter in a coating buffer containing 0.14 M NaCl, 1.5% (w/v) polyethylene glycol 6000, 6% saccharose, and 4.8 g  $\text{NaH}_2\text{PO}_4$  per liter, pH 7.4 (Q).

Coating was performed at 4°C overnight, without shaking. Subsequently, the disks were incubated during 2 h in 1% BSA (70 ml) in the above mentioned buffer (Q) at room temperature. Hereafter, the disks were thoroughly washed with 200 ml buffer Q. Disks were stored in buffer Q, containing 0.05%  $\text{NaN}_3$ .

#### 2. Coating of Microtitration Plates with Anti-hCG

Microtitration plates (Polystyrene, Organon Int.) were coated in the manner described above. To each well, 135  $\mu\text{l}$  of a solution of 40  $\mu\text{g}$  antibody per milliliter buffer Q was added. Subsequently, the plates were coated overnight, preincubated with 1% BSA in buffer Q during 2 h, and thoroughly washed with buffer Q. The microtitration plates were dried over silica, sealed in aluminum bags, and stored at 4°C.

#### 3. hCG Enzyme Immunoassay on Kapton 500H

Kapton 500H disks, coated with monoclonal anti-hCG, were put in a home made microtitration plate, consisting of a PVC holder containing 60 tube plugs ( $\phi 12.5$  mm). The tube plugs were used as wells for the immunoassay. The disks were washed twice with 100  $\mu\text{l}$  borate buffer (100 mM; pH 8.5). Subsequently, the disks were incubated with hCG standards in human serum and peroxidase labeled anti-hCG (total volume 100  $\mu\text{l}$ ) on a Sarstedt TPM-2 shaking machine during 30 min, and washed 3 times with the borate buffer. Finally, 200  $\mu\text{l}$  of a solution containing 3,3', 5,5'-tetramethyl benzidine and urea hydrogen peroxide was added. The development of blue color was stopped with 200  $\mu\text{l}$  1 N  $\text{H}_2\text{SO}_4$  after 30 min. The resulting yellowish color was measured in a twin reader at 450 nm by putting 200  $\mu\text{l}$  of the colored solution in a clean microtitration plate.

For the incubation of Kapton 500H disks during the immunoassay, 200  $\mu\text{l}$  of solvent is needed in order to have the surface of the Kapton 500H disks embedded properly. Nevertheless, only 100  $\mu\text{l}$  was used during the incubation of hCG and peroxidase labeled antibody to allow optimal comparison between the different assay types. For practical reasons, twice (200  $\mu\text{l}$ ) the standard volume for color and stop-reagent solution had to be taken. Consequently, the signal found in Figure 2 for Kapton 500H must be multiplied by a factor 2 for a comparison with the hCG enzyme immunoassay on microtitration plate (Organon Teknika). This concession leads to suboptimal results for the Kapton 500H enzyme immunoassay.

#### 4. hCG Enzyme Immunoassay on Microtitration Plates

The immunoassay was performed as described above except for the volume of color and stop-reagents (100  $\mu\text{l}$ ).

### D. LABELING OF ANTI-hCG WITH 1,2-DIOXETANE LABEL (1)

Into 1 ml of a solution of 3.1 mg monoclonal anti-hCG per milliliter in 0.9% NaCl was pipetted 1 ml borate buffer (100 mM, pH 8.3) and 200  $\mu\text{l}$  of a solution of 1.25 mg (1) per milliliter in 1,4-dioxane (molar ratio antibody: (1) = 1:25). The solution remained clear. The reaction was performed at room temperature during 2 h. The product was purified by

dialysis against borate buffer (100 mM, pH 8.3; three times 1:1). The labeled antibody was stored in borate buffer (100 mM, pH 8.3; 0.05% NaN<sub>3</sub>) at 4°C. The heavily labeled conjugates (Table 3, entries 2, 3) were prepared in the same way, except that the volume of the freshly prepared solution of label added was changed to 100 µl 5 mg (1) per milliliter and 200 µl 5 mg (1) per milliliter, respectively.

#### **E. DUAL LABELING OF ANTI-hCG**

To 1 ml of a 1.47 mg/ml solution of 1,2-dioxetane labeled anti-hCG (entries 1, 2, and 3; Table 3) in borate buffer (100 mM, pH 8.5) was added 50 µl of a solution of 8.7 mg (3) per milliliter in 1,4-dioxane. The reaction mixtures immediately turned turbid upon the addition of (3). The reactions were allowed to remain for 5 h at room temperature, with gentle stirring (magnetic bar). Thereafter, insoluble material was removed by centrifugation (Eppendorf centrifuge). The supernatant was dialyzed against several changes of borate buffer (100 mM, pH 8.5).

The conjugate "1:25:89" (entry 4; Table 3) was further purified on a Sephadex<sup>®</sup> PD-10 column (Pharmacia) with borate buffer (100 mM, pH 8.5) as the eluent.

Conjugates of entries 7 and 8 (Table 3) were prepared by adding (1) and (3) simultaneously (antibody concentration 1 mg/ml during the labeling reaction). The reaction conditions were identical to those of the above-mentioned procedure. In both cases, the reaction turned turbid.

#### **F. DETERMINATION OF IMMUNE REACTIVITY OF LABELED ANTIBODY**

Wells of a microtitration plate, coated with anti-hCG, were incubated with 200 IU hCG per liter, 0.1 µg anti-hCG labeled with peroxidase, and different amounts of unlabeled monoclonal antibody during 30 min. The microtitration plate was washed three times with distilled water (200 µl), and the peroxidase activity was determined as described above. The immune reactivity of labeled antibodies was determined by means of a reference curve of the unlabeled antibody. The labeling procedure in which 5% 1,4-dioxane was used did not lead to a diminished immune reactivity.

#### **G. THERMOCHEMILUMINESCENCE IMMUNOASSAY**

Kapton 500H disks were coated with anti-hCG as described above. The disks were washed once with borate buffer (100 mM, pH 8.5) in a PVC petri dish (about 10 ml borate buffer per 100 disks). Thereafter, the disks were transferred to plug stops (12.5 mm; Thovadec) which were placed in home made microtitration holders. Each plug contained 200 µl borate buffer (100 mM, pH 8.5).

A vial from a frozen stock solution of hCG (10,000 IU/l) in 0.9% NaCl was diluted in a solution containing bovine serum in such a way that all standards contained 80% bovine serum and 0.9% NaCl. Finally, the conjugate was diluted in bovine serum. The incubation volume was 160 µl. The disks were shaken during incubation. After the incubation, the disks were washed three times with 300 µl borate buffer (100 mM, pH 8.3). Then, the disks were stored in 200 µl borate buffer until the TCL measurement was performed.

#### **H. FTIA FOR hCG**

This immunoassay was identical to that described for TIA, unless stated otherwise in the text. All immunoassays performed with dually labeled conjugates were done at room temperature during 1 h on a shaking plateau (Sarstedt TPM-2).

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