Sensitive, Rapid Procedure for Time-Resolved Immunofluorometry of Lutropin

Mohammad J. Khosravi,[1] Robert C. Morton,[1] and Eleftherios P. Diamandis[1,2,3]

In this new immunofluorometric method for quantification of lutropin in serum, the "sandwich" principle is combined with time-resolved fluorescence measurements, with the europium chelate 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) used as label. A monoclonal antibody to the alpha-subunit of lutropin is adsorbed onto the walls of white-opaque microtiter wells to form the solid-phase capture antibody, and a biotin-labeled soluble monoclonal antibody is used for antigen quantification. The detection system is completed with streptavidin, which has been linked to a protein bulking agent labeled with multiple BCPDA residues. In the presence of excess europium, the fluorescence of the final complex attached to captured lutropin molecules is measured on the dried solid phase with an automated time-resolved fluorometer. The assay can be performed as a rapid (<60 min incubation) or regular (150 min incubation) procedure. The rapid assay is well-suited for routine daily monitoring of increasing or ovulatory lutropin concentrations; the regular assay, with its greater sensitivity (0.5 int. unit/L), is a practical procedure for lutropin measurements in hyposecretory states. The assay measures up to 240 int. units/L, and results compare well with those by a commercially available radioimmunoassay, an immunoradiometric assay, and another time-resolved immunofluorometric procedure.

Additional Keyphrases: *RIA, immunoradiometric assay compared · hypersecretory states · fertility studies*

Measurement of lutropin (luteinizing hormone, LH) in human serum is useful in many areas of reproductive endocrinology (*1–7*). The radioimmunoassays (RIAs) used routinely for many years (*8–11*) are now being replaced with the more sensitive immunoradiometric procedures (*12–13*). A number of non-isotopic detection systems have recently been introduced in which enzymes (*14*), erythrocytes (*15*), or fluorescence probes (*16*) are used as labels.

The fluorescent Eu^{3+} complexes exhibit attractive properties as immunological tracers (*17–19*). Because the fluorescence emitted from these complexes is long lived, the tracer can be detected after a pulsed excitation with a gated fluorometer working in a time-resolved mode. Other favorable features of the Eu^{3+} complexes have been reviewed (*19–21*). We have recently described a time-resolved immunofluorometric assay for choriogonadotropin with a new europium chelate [4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-decarboxylic acid, BCPDA] as label (*20*). Using this same chelate, we have developed an immunofluorometric assay of lutropin. It is a two-site "sandwich"-type assay in which a monoclonal antibody to lutropin-alpha subunit, immobilized in microtiter wells, forms the solid phase, while a biotin-labeled monoclonal antibody to lutropin-beta subunit forms the detection antibody. The detection system is completed by interfacing the biotinylated antibody to streptavidin, which has been covalently linked to a bulking protein agent carrying multiple BCPDA residues. In the presence of excess europium, the final fluorescent complex on the dried solid phase is then measured in an automated time-resolved fluorometer.

Contamination with europium constitutes the principal drawback of the currently available time-resolved fluorometric immunoassays described by a number of investigators for lutropin (*16*) and other analytes (*21*). In the present procedure, this major disadvantage has been eliminated by using BCPDA as label and performing the assay in the presence of a saturating concentration of europium.

Materials and Methods

Instrumentation

Time-resolved fluorescence measurements at the bottom of dried white microtitration wells were performed with the Model 615 Immunoanalyzer (CyberFluor Inc.). Data reduction was done automatically by the machine (*19*). Time-resolved fluorescence measurements of liquids (for the "Delfia" kit) were performed with the Arcus fluorometer (LKB Wallac, Turku, Finland). Radioactivity counting was performed with the LKB 1275 Minigamma counter.

Materials

Chemicals. Human follitropin (follicle-stimulating hormone) was from Scripps Labs., San Diego, CA 92103. Human choriogonadotropin was from Calbiochem–Behring Diagnostics, La Jolla, CA 92037. Human thyrotropin (thyroid-stimulating hormone) was from Sigma Chemical Co., St. Louis, MO 63178. Bovine serum albumin, bovine globulin, bovine thyroglobulin, and streptavidin were also from Sigma. Sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-Biotin) was from Pierce Chemical Co., Rockford, IL 61105. Europium(III) chloride hexahydrate was from Aldrich Chemical Co., Milwaukee, WI 53233. White opaque microtiter strips (12-well), "Microfluor," are products of

[1] CyberFluor Inc., 179 John Street, Toronto, Ontario, M5T 1X4, Canada.

[2] Department of Clinical Biochemistry, University of Toronto, 100 College Street, Toronto, Ontario, M5G 1L5, Canada.

[3] Address correspondence to this author at CyberFluor Inc.

Received March 25, 1988; accepted April 28, 1988.

Dynatech Laboratories Inc., Alexandria, VA 22314.

Buffers. The coating buffer is 10 mmol/L Tris, pH 8.25, containing 5.8 g of NaCl per liter. The blocking buffer is 0.1 mol/L carbonate, pH 8.3, containing 10 g of bovine serum albumin and 0.5 g of sodium azide per liter. The assay buffer is 50 mmol/L Tris, pH 7.8, containing 9 g of NaCl, 0.5 g of sodium azide, 5 g of bovine serum albumin, and 0.5 g of bovine globulin per liter. The streptavidin–europium buffer is 50 mmol/L Tris, pH 7.8, containing 9 g of NaCl, 10 g of bovine serum albumin, 10 μmol of $EuCl_3$, and 0.5 g of sodium azide per liter. The wash solution is a 9 g/L NaCl solution containing 0.5 mL of Tween 20 [polyoxyethylene (20) sorbitan monolaurate] per liter.

LH standards. Purified LH (specific activity, 6000 kilo-int. units/g in terms of the first IRP, 68/40, for immunoassay) was obtained from Scripps Labs. The preparation was reconstituted according to the manufacturer's recommendation and diluted in LH-free human serum (Scantibodies Laboratory, Santee, CA 92071) to give the desired standard concentrations.

Monoclonal antibodies. We used two mouse monoclonal antibodies, one directed against the LH alpha subunit and the other against the LH beta-subunit. These antibodies, immunoglobulin fractions partly purified from ascites fluid by column chromatography on diethylaminoethyl Sephadex, can be obtained from CyberFluor Inc.

Specimens. Human serum samples were provided by Dr. M. D'Costa (Mount Sinai Hospital, Toronto, Canada, M5G 1X5). The samples, obtained from individuals who were under investigation for gonadal or hypothalamic/pituitary disorders, had been stored at −20 °C. To exclude any possible effect of storage on LH concentrations, the samples were concurrently tested with the present method and the comparison kits. Lyphochek immunoassay control serum (human) levels I, II, and III were purchased from Bio-Rad Labs., Clinical Division, Richmond, CA 94801.

Preparation of biotin–anti-LH conjugate. The biotinylation procedure has been described previously (*20, 22*). After dialysis, the biotin–anti-LH beta subunit conjugate was tested at various dilutions to determine the optimal concentration for the assay, and it was stored at 4 °C. Before use, the stock preparation was diluted in the assay buffer to prepare a 5 mg/L solution of biotinylated antibody solution.

Preparation of the tracer (streptavidin–thyroglobulin–BCPDA–Eu^{3+}). The preparation of BCPDA-labeled streptavidin–protein conjugates is detailed elsewhere (*23*). The concentration of streptavidin in the working solution of BCPDA-labeled streptavidin–thyroglobulin conjugate was 0.15 mg/L; that of $EuCl_3$ was 10 μmol/L.

Coating microtiter wells with anti-LH antibody. The coating antibody solution was prepared by diluting the monoclonal anti-LH-alpha subunit antibody in the coating buffer to a concentration of 5 mg/L. We then added 100 μL of the antibody solution per well into wells of microtiter strips and allowed the antibody to adsorb at 4 °C. After overnight incubation, the wells were washed twice with the wash solution, 200 μL of the blocking buffer was added per well and incubated for 1 h at room temperature to block the remaining active sites. Before use, the wells were washed twice with the wash solution.

Comparison Methods

We used an RIA and an immunoradiometric assay (IRMA) as comparison methods (Diagnostic Products Corp., Los Angeles, CA 90045). The RIA is a conventional competitive RIA, based on a double-antibody separation technique. In the IRMA, ligand-coated tubes, two monoclonal antibodies labeled with radioactive iodine, and a third monoclonal linked to the ligand are used. The ligand-coated tube/anti-ligand bridge method is used for the separation.

A third comparison method was the "Delfia" hLH assay (LKB-Wallac), also a time-resolved immunofluorometric procedure, in which the solid phase is a monoclonal antibody to the LH beta-subunit, immobilized onto wells of microtiter strips. The tracer is a monoclonal anti-LH alpha-subunit labeled with europium. All kits are calibrated against the First International Reference Preparation (IRP) 68/40 for human pituitary LH. Procedures recommended by the manufacturer were followed for duplicate measurements of the specimens.

Assay Protocol

The assay was performed according to a one-step procedure previously described for choriogonadotropin (*20*). Pipet 50 μL of standards, controls, or patients' samples, followed by 50 μL of the biotinylated antibody working solution (5 mg/L, in the assay buffer), into the antibody-coated wells in duplicate. Shake the wells for 2 min in an automatic shaking device and incubate at 37 °C (air-oven) for 90 min. Aspirate the contents of each well and wash the wells three times with the wash solution, using a 12-well aspirating–washing system. With a semi-automatic dispenser, pipet 100 μL of the tracer working solution into each well. After a 30-min incubation at 37 °C, wash the wells twice with the wash solution and dry for 5 min under cool air. Then measure the fluorescence on the dried solid phase with the CyberFluor 615 Immunoanalyzer. Data reduction is automatic.

The assay may be speeded by shortening the two incubation periods to 30 and 25 min, respectively. Otherwise, the procedure is identical to the one described above. Because the calibration curve of the rapid assay is linear, one can use only the 0.0, 5.0, and 240 int. units/L standards for the calibration curve. However, in this assay it is important to limit the number of test specimens so that standards, controls, and samples are pipetted within 5 min. This will help minimize variation in incubation time and ensure better reproducibility.

Results

Analytical Variables

Calibration curve and detection limit. Figure 1 shows typical standard curves (log-log plot) for lutropin obtained with the two assay procedures. In both cases, the response (mean fluorescence intensity of duplicate measurements after subtraction of the zero-standard signal) is nearly linear over the working concentration range (1.8 to 240 int. units of LH per liter).

The limit of detection, defined as the concentration corresponding to the mean plus three standard deviations of the zero-standard signal for 12 replicates, was 0.5 and 1.0 int. units/L for the normal and speeded assay, respectively. In both assays, the zero standard fluorescence was about 500 to 800 arbitrary fluorescence units.

Figure 1 also shows the precision profiles of the assays, deduced from 12 replicate measurements of each standard point. The CV is $<$10% over a concentration range of 1.8 to 240 int. units of lutropin per liter.

For 50 clinical samples assayed by the normal and the

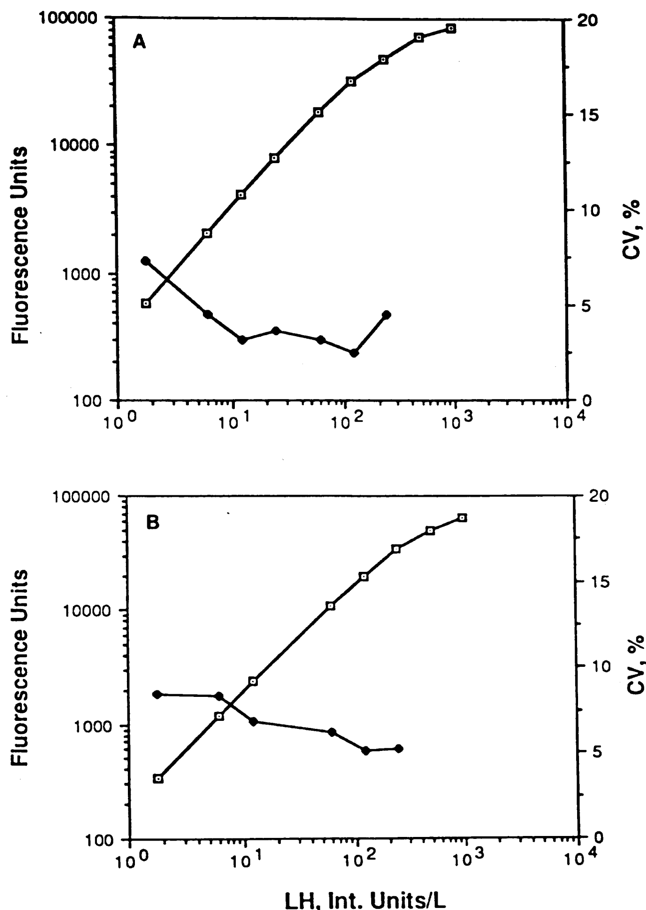

Fig. 1. Dose–response curve, and precision profile (within-run) of the normal (*A*) and the rapid (*B*) assays

Each point represents the mean fluorescence of duplicates from which the mean fluorescence of the zero standard has been subtracted

speeded procedures there was close agreement between values. The following regression equation summarizes these results:

$$y \text{ (rapid assay)} = 0.6143 + 0.9156x \text{ (normal assay)}, r = 0.99$$

Precision. Within-run precision was studied by assaying human control sera in three concentrations 15 times each. We assessed between-run precision by analyzing control samples in five successive runs, day-to-day precision by testing the control samples on 15 consecutive days. In all cases, the CVs obtained by the regular (Table 1) and the speeded assay (data not shown) were found to be well below 10%.

Dilution test. We evaluated the dilution linearity of the procedure by assaying samples serially diluted with LH-free human serum (Table 2). The expected values were derived from the initial concentrations of LH in the undiluted samples. Correlating the results obtained by the regular (samples 1 to 3) and the speeded assay procedure (samples 4 and 5) with the expected concentrations, the dilution curves were linear over the whole range of concentrations. Expected and measured values correlated well.

Analytical recovery of lutropin. LH-supplemented serum samples were prepared by adding known concentrations of exogenous lutropin (13 to 100 int. units/L) to aliquots of pooled serum samples. Analytical recovery was assessed by analyzing the samples before and after the additions and subtracting the estimated endogenous LH concentrations.

Table 1. Precision of the Proposed Lutropin Assay[a]

LH, int. units/L			
Mean	SD	CV, %	n
Within-run			
5.9	0.4	6.8	22
20.5	1.4	6.8	22
92.4	5.7	6.2	22
Between-run			
5.6	0.5	8.9	10
20.6	1.0	4.8	10
88.6	7.0	7.9	10
Day-to-day			
5.6	0.5	8.9	15
23.2	2.0	8.8	15
96.5	7.1	7.3	15

[a] Regular procedure.

Of the added lutropin, 88 to 103% (mean 95%) could be accounted for by the regular assay and between 87% and 103% (mean 94%) by the rapid assay procedure.

Specificity. The proposed procedure was tested for cross-reactivity by the related glycoprotein hormones. LH-free human serum was supplemented with various amounts of follitropin (preparation 78/549), thyrotropin (preparation 68/38), and choriogonadotropin (preparation 75/537) and assayed. The equivalent concentration of LH (in int. units/L) was then calculated from the response obtained.

Concentrations of follitropin and thyrotropin up to 200 int. units/L and 200 milli-int. units/L, respectively, did not interfere (cross-reactivity $<1\%$). Only choriogonadotropin cross-reacted to a significant degree (5% cross-reaction at choriogonadotropin concentrations of 100–500 int. units/L).

Correlation with Other Methods

Table 3 summarizes the accuracy of the proposed procedure as assessed by analyzing serum samples with the three comparison methods.

Table 2. Dilution Linearity of Samples with High Lutropin Concentration

Samples[a,b]	Undiluted	2X	4X	8X	16X	32X
1						
Expected	—	50	25	12.5	6.2	3.1
Observed	100	52.2	26	13.6	7.8	4.1
2						
Expected	—	47.1	23.6	11.8	5.8	3.0
Observed	94.2	48.8	25.8	13.0	7.1	3.8
3						
Expected	—	37.9	18.9	9.5	4.7	2.4
Observed	75.9	32.8	16.0	8.2	4.0	2.2
4						
Expected	—	47.0	23.5	11.7	5.8	2.9
Observed	94.0	43.6	24.8	11.0	5.7	2.7
5						
Expected	—	49.0	24.5	12.2	6.1	3.0
Observed	98.0	41.5	23.6	13.9	8.6	4.1

[a] Samples 1, 2, and 3 were tested by the normal assay; samples 4 and 5 by rapid assay.

[b] Regression analysis of the expected (x) vs measured (y) values (int. units/L):

Sample no.	*Slope*	*Intercept*	*r*
1	1.019	1.005	0.9998
2	1.019	1.092	0.9998
3	0.863	−2.536	0.9990
4	0.936	0.541	0.9971
5	0.792	3.331	0.9973

Table 3. Correlation of Results Obtained for Lutropin with the Proposed and Other Methods

Comparison method[a]	Slope	Intercept	r	n
DPC, RIA	0.736	−0.15	0.99	100
DPC, IRMA	1.05	0.53	0.97	81
Delfia	0.943	0.24	0.95	105

[a] Linear regression with the comparative method on the x-axis.

Discussion

Many reports deal with the use of Eu^{3+}-labeled antibodies for the quantification of LH (*16*) and several other analytes in serum (*21*). The use of Eu^{3+} as a label has a number of advantages (*17–19*) but suffers from the drawback of the assays being vulnerable to contamination with exogenous Eu^{3+}. The assay proposed here is based on a different principle and a new detection system (*19*) that is completely insensitive to Eu^{3+} contamination. In the new system BCPDA-labeled reagents are used, and it operates under conditions of excess Eu^{3+}.

The detection principle is based on the biotin–streptavidin system, modified to achieve high amplification. This was made possible by creating a multi-labeled streptavidin derivative (streptavidin–thyroglobulin conjugate). In this conjugate, thyroglobulin carries the fluor at a load of about 160 residues of BCPDA per thyroglobulin molecule. The creation of labeled streptavidin–protein derivatives and their use in time-resolved fluoroimmunoassays are described in detail elsewhere (*23*).

The performance characteristics of the assays are in many respects equivalent to or better than those reported for radioimmunoassays (*8–11*) and enzyme immunoassays (*14*). The detection limit is about 0.5 int. unit of LH per liter, compared with about 1 to 5 int. units/L for currently available RIAs. Several factors contribute to the high sensitivity of the assay, notable among them are the amplification achieved by the use of multiple labeling, the rejection of optical background by measurement in the time-resolved mode, and the use of high concentrations of monospecific antibodies. High sensitivity may be useful for the investigation of individuals suffering from LH hyposecretory states such as in prepubertal children, in subjects with hypopituitarism, and during the luteal phase of the menstrual cycle.

The working range of the assay is between 0 and 240 int. units/L. Because the fluorescence intensity continues to increase in response to LH concentrations of at least 1000 int. units/L, the assay allows LH measurements through the entire pathophysiological range. The wide measuring range in comparison with that of RIAs (*10, 11*), and the observed insensitivity to the "high dose" hook effect, characteristic of some "sandwich"-type assays, would ensure accuracy of results and eliminate the need for any sample dilution. Only samples with lutropin values >240 int. units/L may require result validation by assay of diluted samples.

In one-step sandwich-type immunoassays, cross-reactivity may have a positive or a negative effect, depending on the extent of the antibodies' specificity and the concentration of the cross-reacting analyte. The falsely positive response may occur when such analytes compete for binding to both the solid phase and the soluble antibody. A falsely negative response may result when the cross-reactant binds only to the soluble antibody. Testing the response of the closely related hormones in the presence or absence of LH, we found the present assay to be insensitive to any interference from follitropin. The cross-reactivity from thyrotropin was minimal (<1%). A relatively higher cross-reactivity (5%) was observed with choriogonadotropin. Because very high concentrations of choriogonadotropin can be attained, this assay is not recommended for use during pregnancy or immediately postpartum.

In the speeded procedure for LH the detection limit is somewhat higher than the regular procedure, but the assay requires less than 1 h. The linearity of the calibration curve permits the use of only three standard points. Because the rapid surge in lutropin concentration is a highly sensitive index of ovulation required for artificial insemination and in vitro fertilization programs, the rapid assay may be of value to such programs for monitoring lutropin daily. With this procedure the pre-ovulatory rise in lutropin can be detected shortly after blood sampling, thus allowing the time interval between the occurrence of ovulation and the performance of in vitro fertilization or artificial insemination to be greatly shortened.

References

1. Kletzky OA, Davajan V, Nakamura RM, et al. Classification of secondary amenorrhea based on distinct hormone patterns. J Clin Endocrinol Metab 1975;41:660–8.

2. Odell WD, Swerdloff RS. Abnormalities of gonadal function in men. J Clin Endocrinol 1978;8:149–80.

3. McDonough PB. Amenorrhea—ectopic approach to diagnosis. Fertil Steril 1978;30:1–15.

4. Benson RC. Diseases of ovaries. In: Benson RC, ed. Handbook of obstetrics and gynecology. Los Altos, CA: Lange Med. Pubns., 1980:650–65.

5. Franchimont P. Pituitary gonadotropins. J Clin Endocrinol Metab 1977;6:101–16.

6. Croxatto HB, Ortiz ME, Diaz S, et al. Studies on the duration of egg transport by human oviduct. II. Ovum location at various intervals following luteinizing hormone peak. Am J Obstet Gynecol 1978;132:629–34.

7. Yussmann HA, Taylor ML. Serum follicle stimulating hormone, luteinizing hormone and plasma progesterone levels related to ovulation by corpus luteum biopsy. J Clin Endocrinol Metab 1970;30:396–9.

8. Midgley Jr AR. Radioimmunoassay: a method for human chorionic gonadotropin (hCG) and human luteinizing hormone (LH). Endocrinology 1966;79:10–18.

9. Odell WD, Ross GT, Rayford PL. Radioimmunoassay for human luteinizing hormone. Metabolism 1966;15:287–9.

10. Beinlich CJ, Piper JA, O'Neal JC, White OD. Evaluation of dual-label simultaneous assays for lutropin and follitropin in serum. Clin Chem 1985;31:2014–8.

11. Wains Jr FH, Dev J, Powell MM, Heald JI. Evaluation of simultaneous measurement of lutropin and follitropin with the Simultron™ radioimmunoassay kit. Clin Chem 1986;32:887–90.

12. Hunter WM, Bennie JG, Kellett HA, et al. A monoclonal antibody-based immunoradiometric assay for hLH. Am Clin Biochem 1984;21:275–83.

13. Odell WD, Griffin J. Two-monoclonal-antibody "sandwich" type assay of human lutropin, with no cross reaction with choriogonadotropin. Clin Chem 1987;33:1603–7.

14. Wada HG, Danisch RJ, Baxter SR, et al. Enzyme immunoassay of the glycoprotein tropic hormones—choriogonadotropin, lutropin, thyrotropin—with solid-phase monoclonal antibody for the α-subunit and enzyme-coupled monoclonal antibody specific for the β-subunit. Clin Chem 1982;28:1862–6.

15. Siddle K, Gard T, Thomas D, et al. Red cell-labeled monoclonal antibodies for assay of human chorionic gonadotropin and luteinizing hormone by reverse-passive haemagglutination. J Immunol Methods 1984;73:169–76.

16. Stenman U-H, Alfthan H, Koskimies A, et al. Monitoring the LH surge by ultrarapid and highly sensitive immunofluorometric

assay. Ann NY Acad Sci 1984;442:544–50.

17. Soini E, Kojola H. Time-resolved fluorometer for lanthanide chelates—a new generation of non-isotopic immunoassays. Clin Chem 1983;29:65–8.

18. Hemmila I, Dakubu S, Mukkala V-M, et al. Europium as label in time-resolved immunofluorometric assays. Anal Biochem 1984;137:335–43.

19. Diamandis EP. Immunoassays with time-resolved fluorescence spectroscopy. Principles and applications [Review]. Clin Biochem 1988;21:139–50.

20. Khosravi MJ, Diamandis EP. Immunofluorometry of choriogonadotropin by time-resolved fluorescence spectroscopy, with a new europium chelate as label. Clin Chem 1987;33:1994–9.

21. Soini E, Lovgren T. Time-resolved fluorescence of lanthanide probes and applications in biotechnology [Review]. Crit Rev Anal Chem 1987;18:105–54.

22. Chan MA, Bellem AC, Diamandis EP. Time-resolved immunofluorometric assay of alpha-fetoprotein in serum and amniotic fluid with a novel detection system. Clin Chem 1987;33:2000–3.

23. Diamandis EP, Morton RC, Reichestein E, Khosravi MJ. Multiple fluorescence labeling for highly sensitive time-resolved immunoassays. Submitted for publication.