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A time-resolved fluorescence immunoassay for insulin in rodent plasma

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Abstract

We describe a time-resolved fluoroimmunoassay (TR-FIA) for quantification of insulin in rodent serum and plasma in the picomolar levels typical of these samples. The method is a solid-phase, sequential saturation assay based on competition of unlabeled insulin and biotinamidocaproyl-labeled insulin for anti-insulin antibody. Europium-labeled streptavidin allows the DELFIA system (Wallac) to be used for detection. The assay is sensitive (0.1 fmol detection limit, $EC_{50} = 58 \pm 3$ pM), accurate (> 95% recovery of 88–880 pM insulin added to the samples), and simple enough to be automated in a 96-well microtiter plate format. Blood samples of 5 μl can be quickly processed and analyzed within a working concentration range of 40–200 pM, allowing direct measurement of insulin levels in rodents from a tail bleed. We used the TR-FIA to assess insulin levels in mouse and rat samples. In studies of streptozotocin-induced diabetes, as well as glucose load experiments, the assay gave results consistent with known literature. The measured insulin levels correlated significantly with values obtained by radioimmunoassay ($R^2 = 0.996$). The intra-assay and inter-assay coefficients of variation were 2.3% and 15%, respectively. We compared results of this assay with an enzyme-linked immunosorbent assay (ELISA) method. The TR-FIA method was comparable to the ELISA but had higher sensitivity and required only one-tenth as much sample. The assay can be performed using commercially available reagents that allow for high sensitivity and practicability.

Keywords: Insulin; Rat; Mouse; Rodent; Time-resolved fluorescence; Competitive immunoassay; ELISA; Radioimmunoassay; Non-isotopic; Delfia

1. Introduction

Quantification of circulating insulin levels is an essential element of many investigations in endocrinology. Following the landmark work by Yalow and Berson in 1960 [1], radioimmunoassay (RIA) has been widely used for this purpose. Improvements have been made since then [2–7]. However, RIA methods have a number of disadvantages. Among the more prominent are the costs, inconvenience and potential hazards involved with using radioiodinated labels, and the stability problems of these reagents.

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One alternative to RIAs for insulin is the enzyme immunoassay (EIA) that uses enzyme-labeled specific antigen or antibodies [8–10]. A competitive ELISA using peroxidase-labeled insulin demonstrated potential advantages over the RIA, giving adequate sensitivity and reproducibility [10,11]. However, the method does not seem to have gained widespread acceptance, possibly due to the relatively large amount of sample required for analysis (≥ 100 μl), and potential for interference in assays of serum or plasma. Another approach is the use of a two-site EIA [8,12,13]. This technique has shown acceptable results when assessing insulin levels from human serum samples. However, this method is not suitable for studies involving rodents, because significant sample volume is still required, and two antibodies simultaneously reactive with rodent insulin are needed. Such 2-site assays for rodent insulin have not appeared in the literature as of this writing.

As researchers continue to rely on rodent models for diabetes and endocrine research, the need for a convenient and practicable method to determine rodent insulin levels remains. We undertook to develop a non-isotopic assay for determination of insulin levels in the microliter amounts available from multiple tail bleeds of mice. It was necessary for the method to be convenient and capable of automation.

We describe a time-resolved fluoroimmunoassay (TR-FIA) for determination of insulin levels in serum or plasma in the picomolar range. The assay takes advantage of the high sensitivity of the dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) technique, utilizing lanthanide chelates and time-resolved fluorometry [14–16]. Using commercially available reagents and materials, we demonstrate that the assay produces results comparable with those in the literature for various normal and diabetic rat and mouse models [17–19]. The assay uses whole blood, requires minimal volumes, and is simple yet robust. The method appears to be more reproducible and sensitive than an EIA using otherwise comparable reagents.

2. Materials and methods

2.1. Materials

Rat insulin was a generous gift of Dr. Ron Chance of Lilly Research Laboratories (Indianapolis). The lyophilized rat insulin was reconstituted as directed to a working concentration of 0.5 μg ml^{-1}, aliquoted, and stored at -20°C until ready for use. Rat plasma samples were graciously provided by Judith Heisserman of Lilly Research Laboratories (Indianapolis). Lyophilized guinea pig anti-rat insulin serum was obtained from Linco Research (St. Charles MO). Normal rat serum (lyophilized) was obtained from ICN Biomedicals. The ICN normal rat serum was reconstituted in 10.0 ml of water according to manufacturer's instructions, aliquoted and stored at -20°C. Coat-A-Count® Insulin radioimmunoassay was purchased from Diagnostic Products (Los Angeles, CA). Rabbit anti-guinea pig IgG (whole molecule), peroxidase labeled insulin (from bovine pancreas), normal mouse serum, streptozotocin (STZ), bovine gamma globulin (BGG), bovine serum albumin (BSA), Triton X-100, and biotinamidocaproyl-labeled insulin (lyophilized powder containing approximately 80% insulin), were obtained from Sigma. TMB Microwell Peroxidase Substrate System was purchased from Kirkegaard and Perry (Gaithersburg, MD). A stock solution of biotinamidocaproyl-labeled insulin (IBC) was prepared by resuspending the powder in water to a final concentration of 2 mg ml^{-1} and storing at 4°C. Nunc plates (96-well Nunc-Immuno MaxiSorp plates) were purchased from Nunc Brand Products. Europium-labeled Streptavidin (Eu-SA), DELFIA® Enhancement Solution, DELFIA® Assay Buffer, and DELFIA® Wash Solution (25× concentrate) were purchased from Wallac (Turku, Finland). All other reagents were of analytical grade and purchased from either Gibco or Sigma.

2.1.1. Insulin standards

Purified rat insulin standards were prepared at concentrations of 2, 9, 18, 36, 91, 180, 360, 910, and 4600 pM (0.01–25 ng ml^{-1}) using PNEBN

(0.04 M phosphate buffer, 0.1 M NaCl, 6% BSA, 0.05% NaN_3, pH 7.5) or PBS-BSA (50 mM NaPi, 150 mM NaCl, 5 mM EDTA, 0.1% BGG, 0.05% NaN_3, pH 7.5) for the TR-FIA or ELISA procedures, respectively. The standards were assayed in triplicates.

2.1.2. Assay controls and pooled test samples

The samples assayed in the fluoroimmunoassay and ELISA consisted of (a) three pools of rat plasma controls (48A, B, and C), (b) normal rat serum control (obtained from ICN Biomedicals), (c) normal mouse sera (from individual animals numbered 1–4, as well as a pool of these) prepared by retro-orbital bleeding of four fasted C57BL/6J mice (Jackson Laboratories), and (d) two pools of sera from diabetic mice. The first was from obese (*ob*/*ob*) mice; the second was from diabetic (*db*/*db*) mice. These two strains of animals are commonly used in studies of diabetes because their characteristic hyperinsulinemia and insulin resistance are traits found in human type 2 (NIDDM) diabetes [17,20,21]. Each of these controls (rat plasma 48A-C, ICN normal rat serum) or test samples (normal mouse sera, *db*/*db* and *ob*/*ob* mouse sera) was used neat and/or diluted appropriately using PNEBN assay buffer. To evaluate recovery we spiked some of the samples to the level of 88, 180, or 880 pM rat insulin.

2.1.3. Anti-insulin serum

Guinea pig anti-rat insulin serum (Linco Research, St. Charles, MO) was reconstituted from lyophilized powder with 10 ml PNEBN assay buffer and further diluted 1:24 with assay buffer prior to addition to TR-FIA Nunc assay plate. We determined this titer of antibody to be optimal, based on displacement of binding afforded by 360 pM rat insulin.

2.2. Methods

2.2.1. Protocol for TR-FIA

Nunc plates were coated with 100 μl of rabbit anti-guinea pig IgG (1:200 dilution) in bicarbonate buffer ($NaHCO_3$, 50 mM, pH 9.0) and stored overnight at 4°C. The plates were washed three times with 300 μl of TBST buffer (20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.4). Then we added to each well 60 μl of pretitered guinea pig anti-rat insulin together with 20 μl of sample, rat insulin standard, or control. Insulin standards, controls, and samples were assayed in duplicate or triplicate as convenient. Following overnight incubation at 4°C, 20 μl of purified biotinamidocaproyl labeled insulin (1:1500 dilution, about 40 pg) was added so that the total volume in the wells was 100 μl. The plates were incubated overnight at 4°C. After washing the plates three times with 300 μl TBST buffer, we used the DELFIA wash solution to wash the plates two more times (200 μl each). We then added 100 μl of 0.1 μg ml^{-1} Eu-SA in DELFIA assay buffer and incubated the plates for 30 min at room temperature. Following washes with TBST buffer and DELFIA wash solution as above, 100 μl of DELFIA enhancement solution was added. After 10 min, we determined fluorescence using a Wallac 1234 DELFIA Research Fluorometer with standard settings for Eu^{3+} (excitation wavelength of 340 nm and an emission wavelength of 613 nm). The calibration curve and EC_{50} values were determined by fitting data to a sigmoidal curve with variable slope using GraphPad Prism 2.0a for Power PC Macintosh (GraphPad Software, San Diego, CA). The equation used for the curve fitting is

$$Y = \mathrm{MIN} + (\mathrm{MAX} - \mathrm{MIN})/[1 + 10^{(\mathrm{Log\ EC_{50}} - X) \times H}]$$

where X is the logarithm of concentration, Y is the response, MIN is the minimum Y value (i.e. at high concentrations of insulin), MAX is the maximum Y value (i.e. as concentration of insulin approaches zero), and H is the Hill slope. The minimum detectable concentration (MDC) was calculated as the interpolated dose at a response two standard deviations below the upper asymptote of the calibration curve (i.e. the MAX value). Sample potencies were determined by interpolation routines included in Prism 2.0a.

2.2.2. Protocol for rat insulin ELISA

We followed the method of Kekow et al. [10], except that we used the Linco guinea pig anti-rat insulin to compare the two methods exactly.

Briefly, microtiter plates were coated with anti-guinea pig IgG as above with $NaHCO_3$ buffer, and incubated overnight at 4°C. The plates were washed with TBST buffer. Then 20 μl of a pretitered dilution of guinea pig anti-rat insulin in PBS-BSA buffer and 80 μl of samples, controls, or standards were added to the plate. The pretitered dilution of guinea pig anti-rat insulin was determined as above for the TR-FIA. After overnight incubation at 4°C, plates were washed with TBST buffer and 20 μl of a pretitered dilution of peroxidase-labeled insulin was added such that the final volume in all the wells was 120 μl. Following a 4-h incubation at 4°C, the plates were washed five times with TBST buffer. Peroxidase activity was assayed using the TMB Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories) that uses 0.04% 3,3′,5,5′-tetramethylbenzidine and 0.02% H_2O_2 as substrate. A 5-min kinetic read of the plate was performed at a wavelength of 650 nm.

2.2.3. Radioimmunoassay

The RIA was performed at Lilly Research Laboratories using a commercially available kit (Coat-A-Count® Insulin) from Diagnostic Products Corporation (Los Angeles, CA). The assay was performed as described in the product literature provided with the kit, except that rat insulin standard from Lilly Research Laboratories was substituted for assay calibration. Samples to be assayed were diluted in Diagnostic Products' zero calibrator. A volume of 0.2 ml was required. The rat plasma controls (48A-C) had been previously assayed on 29 separate occasions.

2.2.4. Glucose load experiments

Normal C57BL/6J mice (Jackson Laboratories) were fasted overnight and subsequently given PBS (control) or 2.0 g kg^{-1} oral glucose load the following day (seven mice/treatment group). We took blood samples from tail bleeds at time points of 0 (baseline), 30 min, 1, 2, and 4 h. Approximately 5 μl was taken for glucose determination by Glucometer Elite (Bayer, Elkhart, IN); at the same time we took another 5 μl sample for insulin determination. Each 5 μl sample for insulin was immediately diluted into 45 μl of PBE Buffer (0.04 M NaPi, 0.1 m NaCl, 5 mM EDTA, 0.5% BSA, pH 7.4). Following centrifugation at $12000 \times g$ for 10 min at 4°C, supernatants were removed and stored at −20°C until ready for analysis.

2.2.5. Collection of streptozotocin (STZ) plasma

Sixteen Sprague–Dawley male rats (Charles River Laboratories) were given doses i.p. of STZ ranging from 0 to 100 mg kg^{-1} (four rats/group). Control rats were given 50 μM citrate buffer (pH 4.5). Four days later blood samples were collected and processed as described above.

All experiments and procedures involving animals were approved for use by the Animal Experimentation Advisory Committee.

2.2.6. Assessment of assay parallelism

Assay parallelism was evaluated by diluting *ob/ob* serum samples (collected as described above) at 1:100, 1:200, 1:400, and 1:800 dilutions with PNEBN assay buffer and evaluating as described for the TR-FIA. Lower dilutions (e.g. 1:10) of serum were not assessed because the high levels of insulin found in *ob/ob* serum at such dilutions would be beyond the linear range of the standard curve.

3. Results

3.1. Standard curve and range of detection for TR-FIA, ELISA, and RIA

A typical standard curve for the TR-FIA is shown in Fig. 1. The EC_{50} was 58 ± 3 pM (S.D., $n = 3$). The minimum detectable concentration (MDC) was 5.2 ± 0.6. In comparison, the standard curve we obtained by the ELISA method of Kekow et al. [10] had an EC_{50} value of 34 ± 10 pM with an MDC of 2.2 ± 0.5 pM. These results were similar to those reported in their work, but we observed a discontinuity in the form of a plateau in the lower half of the calibration curve (at insulin concentrations around 350 pM). At higher insulin doses, the curve resumed its expected slope. This behavior was consistently seen in three separate experiments. For this reason there was greater variance associated with the

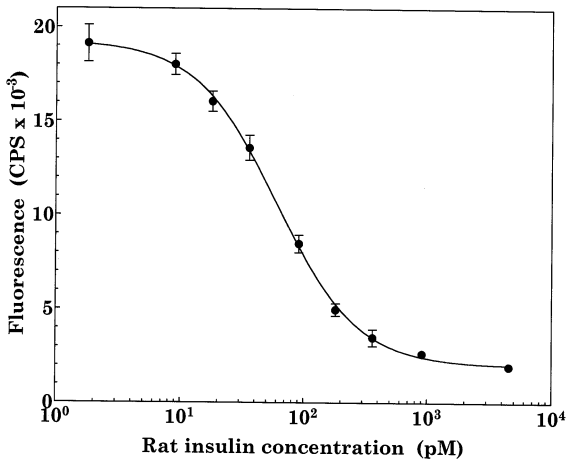

Fig. 1. Standard curve for rat insulin by time-resolved fluoroimmunoassay. Error bars represent one standard deviation. Non-specific binding, assessed as amount of tracer bound in absence of guinea pig anti-rat insulin antibody, was 1600 cps.

ELISA's EC_{50} value. For the RIA the EC_{50} was 440 ± 50 pM and the MDC was 3.9 ± 2 pM.

3.2. Correlation of TR-FIA results with RIA and ELISA

We compared the accuracy and precision of the TR-FIA with that of the ELISA and RIA by assaying samples of rat plasma and serum. We found good correlation between the results of the TR-FIA and RIA, but some deviations with the ELISA (Table 1). For instance, in the analyses of the rat plasma samples 48A and 48B, we saw close agreement in the results when using the TR-FIA and RIA (where the reported values were within two standard deviations of each other). In contrast, we were unable to determine the concentrations of the samples by ELISA because the dose values were greater than the upper limit of the standard curve. For the 48C rat plasma sample the concentration and experimental error determined by ELISA were significantly higher than that determined by either the TR-FIA or RIA. The TR-FIA and RIA values for the ICN normal rat serum diverged significantly. This is not uncommon for samples whose concentrations fall near the lower limit of detection for immunoassays, particularly when it is necessary to run them undiluted. The correlation between the ELISA and TR-FIA for this sample could not be discerned because of high error in the ELISA value. For all samples analyzed by TR-FIA and RIA, the correlation coefficient was 0.996 (a correlation coefficient was not determined for the ELISA because of inconsistency in results). These studies also afforded sufficient data to assess as-

Table 1
Comparison of TR-FIA with RIA and ELISA

Sample	Potency (pM)[a]		
		Coat-a-count®	
	TR-FIA	RIA	ELISA
48A rat plasma	54 ± 12 ($n = 3$)	93 ± 22 ($n = 29$)	>6000[b] ($n = 4$)
48B rat plasma	210 ± 36 ($n = 4$)	330 ± 38 ($n = 29$)	>6000[b] ($n = 4$)
48C rat plasma	1200 ± 187 ($n = 13$)	1000 ± 92 ($n = 29$)	2600 ± 1400 ($n = 4$)
ICN normal rat serum	25 ± 6 ($n = 9$)	130 ± 10 ($n = 2$)	24 ± 22 ($n = 4$)
ob/ob mouse serum	$1.4 \pm 0.06 \times 10^4$ ($n = 4$)	$0.91 \pm 0.06 \times 10^4$ ($n = 2$)	$1.3 \pm 0.2 \times 10^4$ ($n = 4$)

[a]Values reported are the mean ± S.D.
[b]Data greater than upper limit of standard curve.

Table 2
Analytical recovery of 88, 180, and 880 pM insulin determined by TR-FIA

Sample	Analytical recovery (pM)		% Recovery
	Observed[a]	Expected	(observed/expected)
48C rat plasma	1200	880	140
	140	180	78
ICN normal rat serum	960	880	110
	100	88	110
Normal mouse serum pool	960	880	110
	180	180	100
db/*db* mouse serum pool	820	880	93
	110	88	130
Mouse serum 1	1000	880	110
Mouse serum 2	1100	880	120
Mouse serum 3	980	880	110
Mouse serum 4	780	880	89
Mean ± S.E.M.			110 ± 5

[a] Results shown have had unspiked value subtracted.

say variability. The TR-FIA gave good intra-assay reproducibility with a typical coefficient of variation less than 5% ($n = 97$). To examine inter-assay variability, we performed the assay on separate days using the same stock of rat sera and plasma samples. The inter-assay coefficient of variation was $\leq 15\%$ ($n = 3$).

3.3. Analytical recovery

To assess analytical recovery we assayed serum samples from normal, *db*/*db* and *ob*/*ob* mice, and normal rats in the presence and absence of 88, 180, or 880 pM (0.5, 1, or 5 ng ml^{-1}) spike (Table 2). The recovery ranged from 78 to 140%. The average recovery was 110 ± 5%.

3.4. Assay parallelism

Samples from *ob*/*ob* mice, which normally contain high concentrations of insulin [17,21], were diluted in assay buffer and assayed for insulin levels by TR-FIA (Table 3). Parallel results were observed among dilutions that fell within the sensitive range of the assay. Thus there appeared to be no significant dilution bias for these samples.

3.5. Glucose load experiments

We used the TR-FIA to evaluate the insulin levels in normal mice following a 2.0 g kg^{-1} oral glucose load. Fig. 2 shows the change in plasma insulin and glucose levels over a 4-h time course. We observed a typical rise and fall in insulin and blood glucose levels within 1 h of glucose administration, with no notable change for untreated animals [22,23].

4. Discussion

The complexity and cost of radioimmunoassay for insulin in large numbers of rodent serum

Table 3
Parallelism of *ob*/*ob* mouse serum samples

ob/*ob* serum dilution	Potency (pM)	Corrected for dilution (pM)
1:100	140	1.4×10^4
1:200	72	1.4×10^4
1:400	39	1.6×10^4
1:800	21	1.7×10^4

Each of the dilutions was assayed in quadruplicate.

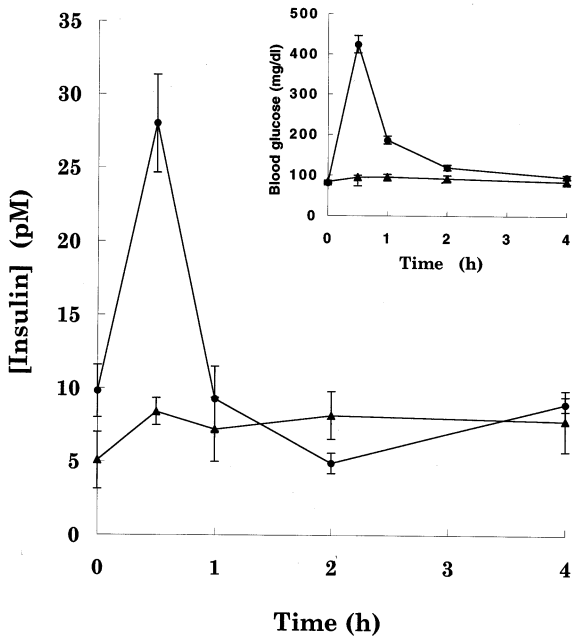

Fig. 2. Results of glucose load experiment. C57BL/6J mice (seven per group) were given 2.0 g kg^{-1} oral glucose load at time 0. Samples for glucose and insulin were taken simultaneously as described. Insulin values by TR-FIA. Control (PBS) —▲— ; Glucose —●—.

samples impelled us to develop a non-isotopic method. The DELFIA technique has been widely employed in clinical and research labs, serving primarily as a platform for 2-site fluorometric "sandwich" assays [8,12,13,24]. The competitive fluoroimmunoassay we developed shows that the high sensitivity of competitive RIA is also achievable in this new format. We also evaluated the ELISA method of Kekow, because it was the only alternative non-isotopic method that could be practically implemented in our laboratory. Although all three assay formats showed similar values for minimum detectable concentration, the TR-FIA required 4–5 fold less sample and therefore had the lowest mass detection limit, 0.1 fmol, vs. 0.2 and 0.4 fmol for the ELISA and RIA, respectively. Comparison of EC_{50} values for the TR-FIA with those of the RIA and ELISA showed a marked reduction in EC_{50} value from that of the RIA, and increased reproducibility from that of the ELISA. This high sensitivity allowed us to quantify insulin in serum samples from normal fasting mice at a 1:10 dilution, using only 5 μl of whole blood.

Our correlation studies showed that values determined by TR-FIA were comparable to those measured by RIA, but agreement with ELISA was less satisfactory, as the ELISA gave extremely high values for some of the rat plasma controls. This may be due to inhibition of the peroxidase on the labeled insulin by interfering substances present in some samples. Further investigation may be required to characterize the source of the discrepancies.

To assess the experimental utility of the assay, we performed a glucose load experiment in normal mice. The expected rise and fall in insulin and glucose levels that we observed showed that the TR-FIA produces results consistent with the literature [22,23]. Significantly, we were able to use single animals and obtain multiple time points for studies that normally require more animals to perform.

We also assessed assay performance by ability to monitor insulin levels from serum samples of STZ-treated rats. STZ has been used extensively to produce diabetes in animal models [19,25–27]. STZ has been shown to be cytotoxic to islet B-cells, resulting in acute hyperglycemia, and substantial or complete loss in insulin secretion within 48 h of administration [19,28–30]. In agreement with these reports, we found that plasma insulin levels were substantially reduced from ~ 300 pM to levels below the range of the TR-FIA when normal rats were treated with ≥ 50 mg kg^{-1} STZ (data not shown).

To assess analytical recovery we assayed several samples of rat and mouse serum or plasma spiked with rat insulin. The good recovery data reported in Table 2 indicate that problems due to serum effects did not occur. The analytical recovery was robust with respect to animal species (rat vs. mouse) and type of processing of sample (serum vs. plasma, or lyophilization).

An advantage of the TR-FIA over the other methods is the commercial availability of the reagents. A possible concern with biotinamidocaproyl-labeled insulin is that biotination of insulin can produce at least three different products. By analogy with methods for preparation of mono-iodinated insulin [31] or fluorescein-labeled insulins of defined composition [32], one could undertake to separate these products by HPLC and

characterize their performance individually. We found that the IBC could indeed be resolved by HPLC into four immunoreactive fractions of different specific activity. However, we did not observe a significant advantage in using the best of these fractions over the unpurified IBC. Although we have optimized conditions of the assay (e.g. antiserum-dilution, biotinyl-insulin tracer concentrations, incubation times) for maximal sensitivity, one may be able to reduce assay time at the possible expense of sensitivity by adjusting these or other assay conditions.

In conclusion, we have shown the TR-FIA to be a clear alternative to both conventional RIA and a previously reported ELISA. The assay is simple and relatively inexpensive because it uses commercially available reagents. The TR-FIA needs only 5 μl of whole blood, a fraction of the volume required to perform the ELISA or RIA. This also allows multiple analyses to be completed in a convenient 96-well plate format suitable for automation. In addition, the TR-FIA avoids the hazards and high disposal costs of radionuclides. Overall, these features make the assay ideal for routine monitoring of insulin levels in animal studies where only limited amounts of sample can be obtained.

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