Quantitation of apolipoprotein A-IV in human plasma using a competitive enzyme-linked immunosorbent assay

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Summary A method for measuring human apolipoprotein A-IV has been developed using the competitive enzyme-linked immunosorbent assay (ELISA) system. The assay described is relatively easy, rapid, and inexpensive to perform, uses convenient dilutions of plasma (1/8–1/32) but is sensitive enough to quantitate the apoA-IV content of lipoproteins following gel filtration of small (0.3–0.5 ml) volumes of plasma. The working range is 100–600 ng of apoA-IV per 50-μl sample and the intra- and interassay coefficients of variations are 7.5 and 10.2% (means), respectively. The mean apoA-IV concentration of 100 subjects was found to be 16.4 ± 5.4 mg/dl. The assay can be performed on untreated plasma samples which may be stored frozen (−20°C) for up to 2 months.—**Kondo, K., C. Allan, and N. Fidge.** Quantitation of apolipoprotein A-IV in human plasma using a competitive enzyme-linked immunosorbent assay. *J. Lipid Res.* 1989. **30:** 939–944.

Supplementary key words ELISA • HDL • apoA-I • apoA-II

Whereas most of the apolipoproteins that have been purified and sequenced have been assigned physiological functions, a few exceptions remain and apoA-IV is a notable example. Recently, several laboratories have reported observations that are consistent with a role for apoA-IV in cholesterol transport (1–4) and structural studies have revealed interesting repeated sequence homologies between A-IV, A-I, and E apolipoproteins (5, 6).

The levels of apoA-IV in plasma may affect or reflect metabolic events related to lipid transport by as yet undefined mechanisms. Some earlier studies found that the concentration of apoA-IV was influenced by absorption of dietary fat (7, 8) although other studies found that, in the rat, A-IV apolipoprotein synthesis was not regulated by the rate of intestinal triglyceride transport (9). More recent studies, which were designed to examine factors regulating apoA-IV synthesis, showed that apoA-IV mRNA levels in the liver were significantly decreased in hypothyroid rats but not in cholesterol-fed animals (10) whereas intestinal apoA-IV mRNA was not altered.

To aid our continuing interest in elucidating a functional role for apoA-IV (11), we required an assay system that would enable us to accurately measure variations in the plasma concentration of this apolipoprotein that occur as a result of dietary or drug manipulations. This report describes the development of a competitive ELISA that is relatively inexpensive, rapid, and sensitive and that could readily be adopted by most laboratories interested in lipoprotein metabolism.

EXPERIMENTAL PROCEDURE

Isolation and purification of apoA-IV

ApoA-IV was isolated from lymph triglyceride-rich lipoproteins as described previously (12). Chylomicrons were delipidated with chloroform–methanol–ether (12) and applied to Sephacryl-300 columns (2.5 × 100 cm) equilibrated with 3 M guanidine-HCl in 0.05 M Tris-HCl, pH 8.0. ApoA-IV peaks were pooled and after rechromatography were shown to be homogeneous on SDS-polyacrylamide gels and by two-dimensional gel immunoblotting as described previously (12). The purity of the apoA-IV preparation was further confirmed by the sequence of the first 10 amino acid residues (6), determined on the Applied Biosystems 470A sequencer with an on-line 120A PTH analyzer and by the absence of immunoprecipitin lines against any other apolipoprotein except apoA-IV.

Preparation of antisera

Antisera to human apoA-IV were produced by injecting New Zealand white rabbits as described previously (12). The monospecificity of the antisera used in the immunoassay system (see below) was further established following Western blots of mixtures of apolipoproteins, whole serum, or HDL and the lipoprotein-free fraction obtained after gel permeation chromatography as described below. After electrophoretic transfer from 10% polyacrylamide slab gels to nitrocellulose (13), the sheets were blocked and incubated with rabbit anti-A-IV serum, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) to visualize bound antibody.

ApoA-IV quantitation by competitive ELISA

Coating procedure. ApoA-IV was dissolved in coating buffer (0.035 M $NaHCO_3$, 0.014 M Na_2CO_3, pH 9.6) at a concentration of 50 μg/dl and 100 μl was added to wells of 96-well microtitre plates (Immulon 2, Dynatech) which were covered and incubated overnight at 37°C. Significant variation in coating capacity of apoA-IV was observed for different plates and plastic surfaces; the above plates were most suitable using our assay conditions. The solution containing the unbound apoA-IV was removed and blocking buffer (0.01 M phosphate-buffered saline (PBS) containing 0.5% BSA and 0.05% Tween 20) (PBS-BSA-Tween 20) was added to the wells for 1 h at room temperature. Then the wells were washed three times with PBS-Tween 20.

Plasma samples were diluted 1:16 in PBS and 50 μl was added to the plates. Standards of apoA-IV, ranging from 1 to 20 μg per ml in PBS were included in each plate. The concentration of apoA-IV present in stock solutions was

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

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calculated from the amino acyl mass following acid hydrolysis and amino acid composition analysis on the Beckman 6300 analyzer. This was followed by the addition of 50 μl of apoA-IV-antiserum diluted 1:50,000 in PBS-BSA-Tween 20 and the samples were incubated for 2 h at 37°C. After washing three times with PBS-Tween 20, 100 μl of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Bio-Rad), diluted (1:2000) in PBS-BSA-Tween 20 was added to each well and incubated at room temperature for 1 h and washed as before to remove excess enzyme-labeled antibody. Quantitation of the bound peroxidase was determined using H_2O_2 as substrate and *o*-phenylenediamine (OPD) as hydrogen donor. Ten μl of 30% H_2O_2 and 1 ml of 1% OPD in methanol (freshly prepared before each assay) were added to 49 ml of distilled water and mixed. One hundred fifty μl of the chromogen-substrate solution was pipetted into each well, and the color was developed at room temperature in the dark for 30 min. The enzymatic reaction was stopped by the addition of 50 μl of 8 M H_2SO_4 to each well. Then the plates were read in a Titertek multiscan MC ELISA plate reader at 492 nm. The results were plotted as absorbance or B/Bo against log apoA-IV (standard curve).

Quantitation of plasma apoA-IV concentrations

Blood was obtained from individuals attending a hospital coronary risk-reduction clinic. Samples for apoA-IV analysis were immediately placed on ice and spun at 4°C. Plasma was used fresh or after storage at −20°C for 2 months. In order to demonstrate that apoA-IV in the lipoprotein-free form showed the same immunoreactivity as apoA-IV in plasma lipoproteins, particularly HDL which transports most of the lipoprotein bound apoA-IV (8), we isolated HDL and the lipoprotein-free fraction by gel permeation chromatography (as described below) of 0.5 ml plasma. The fractions were concentrated by ultrafiltration to allow the preparation of a range of dilutions selected to fit within the linear range of the apoA-IV standard curve.

Lipid and apolipoprotein A-I, A-II assays

Each plasma sample was further analyzed for total cholesterol and triglyceride using the enzymatic colorimetric method (Boehringer Mannheim GmbH) in the Cobas Bio centrifugal analyzer (Roche). Apolipoprotein A-I and A-II concentrations were determined by immunoturbidometric assays (Boehringer Mannheim Biochemica) in the Cobas Bio. The assay mixture consisted of 5 μl sample, 40 μl diluent, and 250 μl of a solution containing 6% (w/v) polyethylene glycol 6000 and 2.5% (v/v) buffer concentrate (OVEC 40/41, Behringwerke, Marburg, West Germany). After incubation for 2 min at 37°C, 20 μl sheep anti-human apoA-I or A-II antiserum (Boehringer-Mannheim, West Germany) was added and the incubation was continued for a further 20 min. Absorbances were read at 340 nm and compared with standard calibration serum supplied by Boehringer-Mannheim. HDL cholesterol was estimated after precipitation of VLDL and LDL with phosphotungstic acid and Mg^{2+}. After centrifugation at 4,000 rpm for 10 min, the clear supernatant was recovered and aliquots were removed to determine the cholesterol content by the CHOP-PAP method (Boehringer Mannheim GmbH) in the Cobas.

Determination of apoA-IV distribution in plasma

Plasma samples (maintained at 4°C with 2 mM *p*-chloromercuriphenyl sulphonate (PCMPS)) were fractionated on Superose 12 to separate lipoproteins from plasma proteins. Plasma (0.5 ml) was loaded onto a column (0.5 × 20 cm) which was attached to the Pharmacia FPLC system. The fractions were eluted with 0.15 M NaCl in 0.05 M Tris-HCl, pH 7.4, and monitored at 280 nm. Columns were calibrated with VLDL, LDL, HDL_2, HDL_3, and albumin to determine elution volumes of lipoprotein fractions.

Treatment and storage of plasma samples

To determine conditions for exposing optimal antigenicity of apoA-IV in the ELISA system, plasma was treated with detergents (0.25% Tween 20 in PBS, or 0.1% SDS in PBS) heat-treated at 56°C for 10 min, or delipidated with 10 vol alcohol–ether 3:1 (v/v), and then reconstituted in PBS. Because apoA-IV occurs in oligomeric as well as monomeric forms in plasma, the effects of adding dissociating agents to plasma at concentrations of 4 M urea or 3 M guanidine-HCl (before dilution) were compared with untreated samples.

RESULTS

The purity of the apoA-IV used to produce rabbit antiserum is shown in **Fig. 1a**. Fig 1b shows the monospecificity of the antiserum following Western blotting. The antisera reacted against pure apoA-IV and only against apoA-IV whether present in whole plasma, or HDL and lipoprotein-free fractions separated by size exclusion chromatography on Superose 12 columns. **Fig. 2** demonstrates the standard curve obtained using isolated apoA-IV and the results describe the mean data ± SD from five experiments. The absorbances at 492 nm ranged from approximately 0.2 to 1.5, and although changes in absorbance and slopes of the standard curve could be manipulated by altering first and second antibody concentrations, the conditions described in the Methods section produced the most reproducible results. Using the linear part of the curve, ranging from approximately 100 to 500 ng/50 μl (0.16–1.2 mg/dl) of apoA-IV, convenient dilutions of plasma from 1/8 to 1/32 could be used to fit within this working range. Purified A-I, A-II, or E apolipoproteins at concentrations of approximately 2 mg/ml did not produce displacement as shown in Fig. 2. The intra-assay coefficient of variation ranged between 5.9

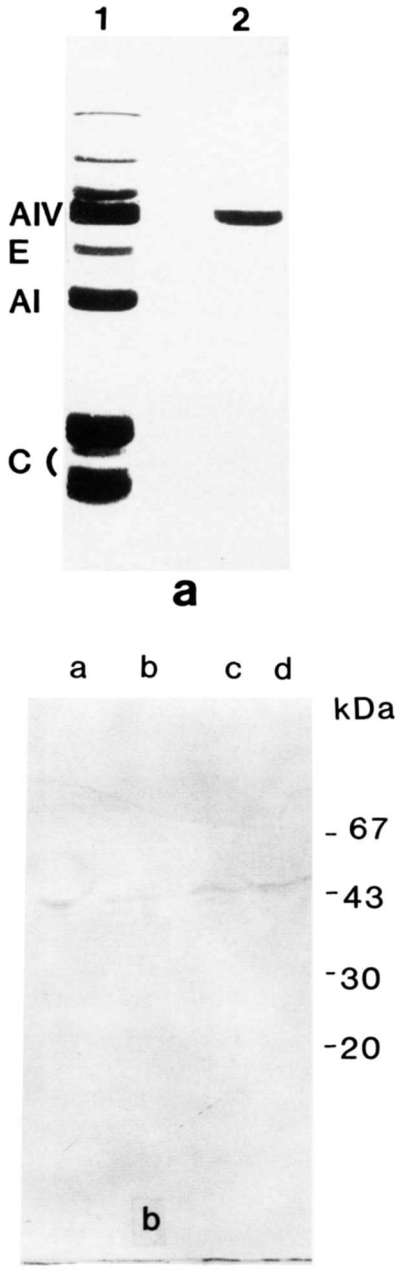

Fig. 1. a: Separation of apolipoproteins on SDS-polyacrylamide (15%) gels of 1) 65 μg of chylomicron apolipoproteins; and 2) 5 μg of apoA-IV purified as described in Methods. Gels were stained with Coomassie blue. b: Western blotting to test monospecificity of apoA-IV. SDS-gels were loaded with a) whole plasma, b) HDL from a Superose 12 column, c) lipoprotein-free fraction from Superose column, or d) pure apoA-IV. After electroblotting, the nitrocellulose sheet was incubated with apoA-IV antisera and second antibody, as described in the text.

and 9.1% and the inter-assay C.V. was 8.9–11.5%. ApoA-IV present in either the HDL or lipoprotein-free fractions of plasma (which together comprise the bulk of plasma apoA-IV) showed parallel displacement curves as shown in **Fig. 3**.

Treatment of plasma by several methods did not significantly alter the values obtained for plasma apoA-IV concentrations as shown in **Table 1**. Samples could be stored frozen (−20°C) for 6–8 weeks with no significant change in values although storage at 4°C for several weeks gave slightly lower values (range 5–15%) than fresh samples (data not shown). Most of the treatments gave inconsistent results, and the intra- or inter-assay C.V. values were also increased, rather than reduced by these procedures. Even delipidation, or treatment with detergents such as Tween 20 and SDS, produced inconsistent results and did not expose further antigenicity of plasma apoA-IV. Heat treatment at 56°C for 1 h produced some increase but showed a wider variation than assays of untreated samples.

Table 2 shows the plasma levels of apoA-IV measured in 100 individuals and compares values between normolipemic subjects and those with varying combinations of mild hyperlipoproteinemia. Although not statistically significant (data analyzed by paired *t*-test) there was a suggestion of increased levels in the group with mild hypertriglyceridemia (19.7 vs. 16.4 mg/dl). This group was also associated with lower HDL cholesterol levels (0.97 vs. 1.26 mmol/l). The ratio of apoA-IV to apoA-I remained relatively constant from 0.14 to 0.17 (w/w). Values for males and females are also shown in Table 2, and in the normal group, mean values of 14.8 and 17.7 mg/dl for men and women were not significantly different, nor were the values different between men and women for the apoA-IV concentrations in the various lipemic groups.

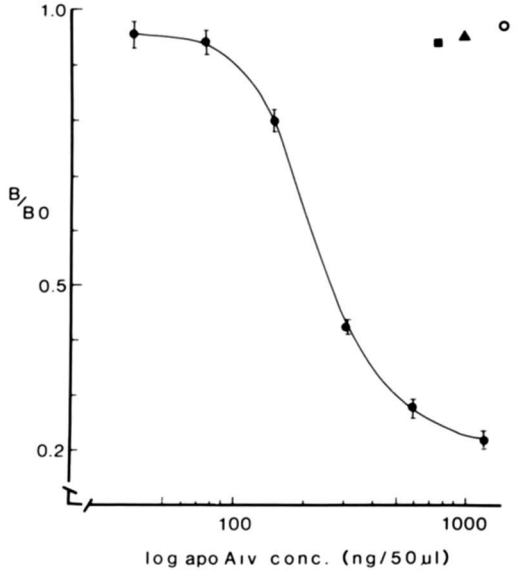

Fig. 2. Standard curve obtained with human apoA-IV in the competitive ELISA. Results are mean ± SD of five different displacement assays performed on different microwell plates; B, absorbance, at 492 nm, in the presence of apoA-IV standard; and B_0, absorbance in the absence of standard. Lack of competition of other apolipoproteins is demonstrated by the high B/B_0 values after addition of high concentrations of apoA-I (▲), apoE (■), and apoA-II (○).

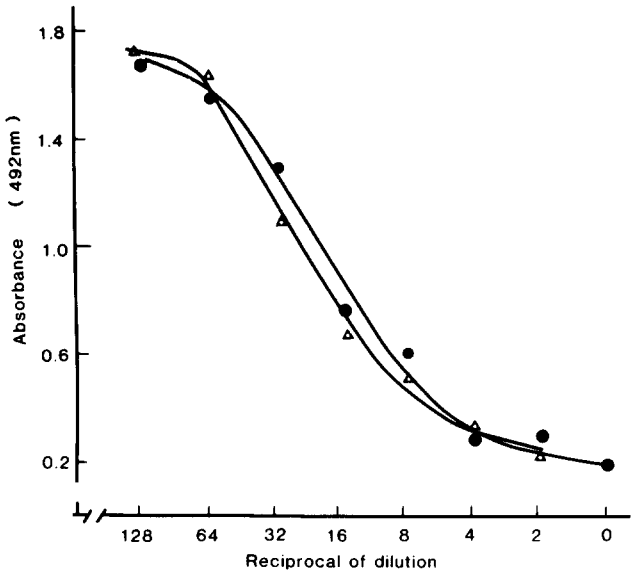

Fig. 3. Comparison of the immunoreactivity of apoA-IV when present in either HDL (●) or lipoprotein-free fraction (LFF) (△) of plasma. After separating HDL or LFF by size exclusion chromatography, fractions were pooled and concentrated to enable serial dilutions to be tested within the apoA-IV standard curve range. Displacement assays were performed as described in the text, and color development was measured by absorbance at 492 nm.

The competitive ELISA method was also compatible with the detection requirements for estimating the distribution of apoA-IV in small quantities of plasma. **Fig. 4** shows the distribution in two subjects following application of 0.5 ml fresh plasma, without additions or incubation, to a Superose column as described in Methods. The distribution in four other plasma samples was also determined (not shown). Most of the apoA-IV was associated with HDL (mean, 39%) and the nonlipoprotein fraction (mean, 51%) but small quantities were found in the VLDL/LDL elution region (mean, 10%).

TABLE 1. Effect of various treatments on plasma apoA-IV immunoassay

Treatment	Percent of Value Obtained on Untreated Plasma[a]
Delipidation[b]	101 ± 12
Guanidine HCl, 3 M[c]	90 ± 8
Urea, 4 M[c]	107 ± 13
Tween-20, 0.25%[c]	67 ± 36
SDS, 0.1%[c]	90 ± 56
Heating 56°C, 1 h	114 ± 22
Heating 56°C, 4 h	82 ± 14

[a]Eleven plasma samples treated.
[b]Delipidation performed with alcohol-ether 3:1 (v/v).
[c]Refers to concentration in plasma before dilution.

DISCUSSION

The objective of the experiments described in this report was to establish a simple, sensitive, moderately inexpensive, and rapid assay for measuring apoA-IV levels in human plasma and lipoprotein fractions. Various immunoassay systems have been reported previously and they have provided adequate quantitation for their respective applications. Electroimmunoassays were used to measure apoA-IV concentrations in human plasma and in lipoproteins separated by ultracentrifugation (7, 14–16). Radioimmunoassays (RIA) have also been used to provide the sensitivity and accuracy required to enable quantitation of apoA-IV in plasma and in lipoproteins fractionated by size exclusion chromatography (3, 8, 17). Most values reported for apoA-IV

TABLE 2. Mean values for lipid and apoA-I, A-II, A-IV in human plasma

	N	Age	Total Cholesterol	Triglyceride	HDL Cholesterol	Apoprotein A-I	Apoprotein A-II	Apoprotein A-IV	A-IV:A ratio
			mmol/l				*mg/dl*		*w/w*
Normal[a]	54	38 ± 10[b]	4.64 ± 0.81	0.81 ± 0.26	1.26 ± 0.40	118 ± 24	33.9 ± 7.0	16.4 ± 5.4 14.8 ± 4.9[c] 17.7 ± 5.6[d]	0.14
TC ≥ 5.5, TG < 1.5	30	52 ± 11	6.36 ± 0.55	0.97 ± 0.29	1.45 ± 0.41	134 ± 22	37.7 ± 6.3	18.5 ± 5.0 19.7 ± 4.4[c] 17.8 ± 5.3[d]	0.14
TC ≥ 5.5, TG ≥1.5	11	43 ± 9	7.04 ± 1.12	2.45 ± 1.46	1.06 ± 0.25	123 ± 24	41.1 ± 8.6	18.4 ± 4.6 17.3 ± 4.5[c] 21.3 ± 4.0[d]	0.15
TC < 5.5, TG ≥ 1.5	5	28 ± 9	4.99 ± 0.45	1.96 ± 0.33	0.97 ± 0.08	114 ± 32	36.2 ± 9.7	19.7 ± 9.3 18.6 ± 4.9[c] 20.5 ± 12.6[d]	0.17

[a]Normal normolipemic individuals with cholesterol < 5.5 mmol/l, TG < 1.5 mmol/l.
[b]Mean ± SD.
[c,d]Values for male and female plasma, respectively.

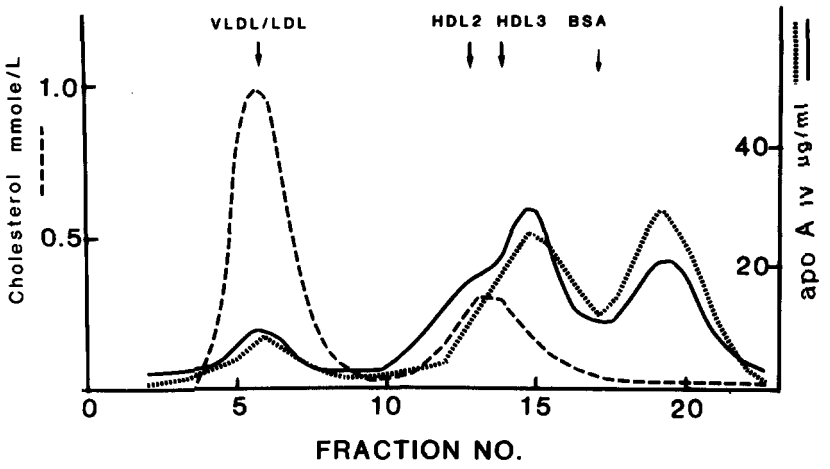

Fig. 4. Determination of apoA-IV in two samples of fresh unincubated human plasma following size exclusion chromatography on Superose 12 (Pharmacia/FPLC). Plasma samples (0.5 ml) were loaded and after elution, fractions were assayed for apoA-IV (····· , ——— : subjects 1 and 2, respectively) or for cholesterol (— — —: subject 1 only).

have ranged from 13.1 to 17.2 mg/dl, but in one study (8) a value of 37.4 mg/dl was found for normolipemic human serum. The values reported in this study (16 mg/dl) fall within these ranges and are provided by a method that may offer advantages over other assays. The ELISA method is rapid and, using pre-coated plates, provides answers within a few hours compared with the 3–4 days required for RIA. The competitive ELISA uses no radiolabel and provides a greater sensitivity than electroimmunoassays which require larger quantities of antibody than the present method. Immunoturbidometric methods are easy and rapid but require expensive equipment, larger quantities of antibody, and costly reagents.

The antibody used in our system, produced by immunizing rabbits with apoA-IV originating from lymph chylomicrons, reacted only against apoA-IV whether it was present in plasma, HDL, or the lipid-poor fraction of plasma. We detected no differences in displacement curves between these two fractions (which comprise the major pools of circulating apoA-IV) suggesting that, under conditions of our ELISA immunoassay, these apoA-IV-containing fractions are immunochemically equivalent.

Most of the problems that arise in quantitating apoA-IV appear common to all immunoassay systems and probably reside in the nature of this apolipoprotein which has some unusual properties. It has a relatively high affinity for self-association (18) which may have a profound influence on its biological behavior, including its capacity to interact with antibodies. If the dimeric, or other oligomeric forms of apoA-IV, have lower affinities for antibody than monomeric apoA-IV, considerable variation may be expected if the ratios of dimer–monomer differ appreciably between individuals and/or lipoprotein particles. It is interesting to note that some investigators have used detergents to expose maximum antigenicity of apoA-IV (8, 13, 17) and the highest value (37.4 mg/dl) was obtained using high concentrations of SDS (1%) in the assay system (8). On the other hand, values of 15.7 mg/dl have been reported even when 8 M urea was included in reagent buffers (7). In our studies, addition of either urea or guanidine-HCl did not improve immunochemical detection of apoA-IV and most treatments were inconsistent as shown in Table 1. We have therefore chosen to omit denaturing agents from the apoA-IV ELISA until further information is obtained regarding the immunochemistry of oligomeric and monomeric forms of this apolipoprotein.

Using the competitive apoA-IV ELISA we have found values for apoA-IV in plasma ranging from 14.8 to 21.3 mg/dl with a mean of 16.4 ± 5.4 mg/dl. This assay, which also enabled the distribution of plasma apoA-IV to be determined following size exclusion chromatography of 0.5 ml plasma, may also be useful as a rapid, sensitive, and moderately inexpensive clinical routine assay.

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