Multicenter Evaluation of New Enzyme-Linked Immunoassays of Follitropin and Lutropin in Serum or Plasma


Results from a multicenter evaluation of two new enzyme-linked immunosorbent assays [Enzymun-Test® for follitropin (FSH) and lutropin (LH)] are presented and compared with results from 11 other commercial immunoassays, radioactive as well as nonradioactive. Enzymun-Test FSH and LH assays are suitable for automated systems and manual applications. The tests were reproducible (CV <5%), highly specific, and sensitive enough (<0.5 int. unit/L) to measure the hormones directly in almost all patients’ samples, except for LH measurements in prepubertal children. We did not find interference by heterophilic antibodies or other factors. A comparison of assays for FSH found very good agreement among all modern two-site assays; competitive immunoassays almost invariably yielded systematically lower results for FSH, probably because of the heterogeneity of the International Reference Preparation (2nd IRP FSH, 78/549). For LH also we found good agreement, with no systematic differences among the various reagents. Guidelines for reference values with the new reagents are given.

Additional Keyphrases: interlaboratory performance, reference values, variation, source of

Measurements of peptide and steroid hormones in serum play an important role in both the investigation and treatment of male and female reproductive disorders. In general, follitropin (FSH, follicle-stimulating hormone)13 and lutropin (LH, luteinizing hormone) are among the key hormones measured in investigations of delayed or precocious puberty, hypogonadism, fertility problems, polycystic ovarian disease, menopause, and hypothalamic–pituitary disorders (1, 2). Because the pituitary gland secretes both hormones in a pulsatile fashion (3), frequent, sequential LH measurements are used to assess ovarian function [e.g., in sports medicine (4) and during stimulation of follicles by gonadoliberin (5)]. Assessment of pubertal disorders (6, 7) and of the inhibition of ovarian function during breast cancer therapy (8) requires reliable procedures capable of measuring very low concentrations of both hormones.

Since the first applications of immunoassays to LH (9–11) and FSH (12–14), the increasing quality of available antisera has permitted the specific determination of these hormones in small serum or plasma samples. The introduction of monoclonal antibodies has especially stimulated the development of two-site immunometric assays with superior specificity. Several commercial assay systems for FSH and LH are available (15, 16), usually based on the use of radioactive reagents.

Here, we present two new enzyme-linked immunosorbent assays (ELISAs). Because of the specificity of the combined monoclonal antibodies, both assays are highly specific and accurate. The assays permit assessment of samples with low and high concentrations of the analytes without interferences by antibody-binding substances, including heterophilic antibodies. Both assays are available for automated systems and manual applications. We present results from a multicenter evaluation and a comparative study.

Materials and Methods

Samples. The multicenter evaluation was performed in 10 laboratories. FSH and LH were measured in serum from ~1100 subjects. The samples (serum or plasma) were obtained from local patients and control subjects, subdivided into eight groups: children, men, nonpregnant women [follicular phase, midcycle (less than two days before ovulation), luteal phase, and postmenopausal], pregnant women, and women during stimulation in in-vitro fertilization programs. FSH and LH enzyme immunoassays. All determinations were done with Enzymun-Test FSH® and Enzymun-Test® LH (Boehringer Mannheim GmbH, Mannheim, F.R.G.). Each of the participating laboratories received the following reagents.

Bottle 1 (incubation buffer for both assays): 40 mmol/L phosphate buffer, pH 7.4, containing 9.0 g of sodium chloride, 6.0 g of Pluronic F68, 10.0 g of polyethylene glycol (40 kDa), 2.0 g of bovine serum albumin, and 1.0 g of bovine IgG per liter.

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13 Nonstandard abbreviations: FSH, follitropin; LH, lutropin; ELISA, enzyme-linked immunosorbent assay; IRP, International Reference Preparation; IS, International Standard; hCG, human choriongonadotropin; TSH, thyrotropin; and UK EQAS, U.K. External Quality-Assessment Schemes.
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Bottle 2: either anti-FSH-peroxidase conjugate (10.6 kU/L) or anti-LH-peroxidase conjugate (6.6 kU/L) lyophilized from a buffer, pH 7.4, containing, per liter, 8.6 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 2.9 g of Tris, plus 54 g of D(+)-mannitol, 9.0 g of saccharose, 4.0 g of bovine serum albumin, 3.7 g of EDTA, and 0.4 g of 8-anilino-1-naphthalenesulfonic acid, ammonium salt. Monoclonal mouse IgG (γ, κ), polymerized by proprietary procedures (European patent application no. 0-331-068), had been added to prevent interference by heterophilic antibodies in individual blood samples. Reagents were obtained from Boehringer Mannheim.

Bottle 3 (six different solutions of either FSH or LH): appropriate concentrations of highly purified human FSH (h-FSH) or human LH (h-LH) standard material in a bovine serum-based matrix.

Bottles 4 and 5: 100 mmol/L phosphate–citrate buffer, pH 4.4, plus sodium perborate, 3.2 mmol/L (substrate buffer; bottle 4), or 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt chromogen, 1.9 mmol/L (bottle 5). The contents of these two bottles must be combined before starting the determinations. All buffers were prepared with combinations of two preservatives to prevent microbial growth. Each set of reagents had 100 or 200 plastic tubes coated with monoclonal antibodies (IgG) to either FSH or LH.

Assay principle. Enzymun-Test FSH and Enzymun-Test LH are two-site sandwich immunoassays specific for intact FSH or LH holohormone only. Specificity is achieved by the combination of a monoclonal antibody that recognizes an epitope on the β-subunits of the gonadotropin with another monoclonal antibody, which recognizes the associated α/β-hormone only. In both assays, the antibody coated to the tube (anti-β for FSH and anti-α/β for LH) is IgG, whereas the antibody fragment (Fab) of the second antibody (anti-α/β for FSH and anti-β for LH) is conjugated with horseradish peroxidase (EC 1.11.1.7). The principle of both assays is illustrated in Figure 1.

Assay procedure. In this study, 10 participating laboratories performed the assays, after appropriate training, according to the instructions of the manufacturer, with use of either the ES22 analyzer (three laboratories) or the automated ES600 system (seven laboratories), both from Boehringer Mannheim. (Both assays can be performed manually as well.) The procedure consisted of the following steps:

1) incubating 100 μL of standard or sample in anti-FSH- or anti-LH-coated plastic tubes with 1000 μL of buffer containing anti-FSH-peroxidase or anti-LH-peroxidase conjugate, respectively, for 120 min at 25 °C;

2) aspirating the supernate and washing (three times) with a wash solution of, per liter, 4.2 mmol of NaCl and 4 μmol of CuSO4 (preservative);

3) incubating with 1 mL of the chromogen solution (bottle 5) for 30 min at 25 °C;

4) measuring the absorbance at 422 nm; and

5) calculating the unknown concentrations, based on a calibration with six calibrators (range, 0 to 135 int. units/L for both FSH and LH).

Standardization. Enzymun-Test FSH has been standardized against the Second International Reference Preparation (2nd IRP) pituitary FSH/LH (78/549), and Enzymun-Test LH against the 1st IRP pituitary LH (68/40). These Reference Materials are designated in international units per ampoule. One ampoule of each Reference Material preparation was dissolved in FSH- and LH-free serum, obtained by immunosorption of human serum, and was used to calibrate 10 master calibrators; these master calibrators were used to calibrate each individual lot of kit calibrators.

Hormone preparations. To test the specificity of the assays described, we added various amounts of commercially available (Boehringer Mannheim) preparations of pituitary hormones, their α- and β-subunits, and chorionic gonadotropin (hCG) to human sera having no detectable concentrations of FSH or LH. These preparations, which had been calibrated against the appropriate IRPs, were h-LH (contamination with FSH <0.6%); h-FSH (contamination with LH <1%); human thyrotropin (h-TSH; contamination with FSH <1%, with LH <4%); hCG (contamination with both FSH and LH <1%); h-LHβ (<1% h-LHα); hCGβ (<0.1% hCGα); h-TSHα (<1% h-TSHβ); and h-LHα (<2% h-LHβ). The highest quantities used in the tests are indicated later (see Table 2).

Evaluation procedure. Besides making a practical evaluation of the reagents sets of the manufacturer, all participating laboratories measured FSH and LH concentrations in all samples, using their standard inhouse techniques. Some of the participating laboratories used more than one set of reagents. These techniques included five competitive radioimmunoassays and six two-site sandwich assays, as described in Table 1. All participants performed these assays according to the manufacturer's instructions. All results were expressed in international units, based on reference preparations 78/549 for FSH and 68/40 for LH.
Table 1. Summary of Alternative Reagents Used by the Participating Laboratories to Measure FSH and LH

<table>
<thead>
<tr>
<th>RIA</th>
<th>1. Amerlex-125I-radioimmunoassay; Amersham (Amersham, Bucks., U.K.)</th>
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<tbody>
<tr>
<td></td>
<td>2. RIA-mat; Mallinckrodt Diagnostics (Dietzenbach, F.R.G.)</td>
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<td></td>
<td>3. Double-antibody; Diagnostic Products Corp. (Los Angeles, CA)</td>
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<td></td>
<td>4. Inhouse RIA (U.K.)</td>
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<td></td>
<td>5. ELISA CIS (Gif-sur-Yvette, France)</td>
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<tr>
<td>EIA</td>
<td>1. Sensibead; Terumo (Tokyo, Japan)</td>
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<td></td>
<td>2. IRMA Sensibead</td>
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<td></td>
<td>3. EIA</td>
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<td></td>
<td>4. Amerlite</td>
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<td>5. TERUMO</td>
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<td>6. CIS</td>
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Calculation of data. All participating laboratories supplied one of the authors (A.C.K.) with the raw data (absorbances, absorptivities) generated by the automated instruments. From these data, the results for standards and for the concentrations of gonadotropins in human sera were calculated by standard computerized programs developed according to described procedures (17).

Statistics. We compared the methods by the main-component procedure for linear regression described by Passing and Bablock (18), because this nonparametric method does not require special assumptions about the distribution of the results.

Results

Assay Performance

Reproducibility of standards. The standard curves for both determinations consist of a zero-calibrator plus five standard points at 2, 9, 22, 70, and 135 int. units/L. The coefficient of variation (CV) for the absorbances of these five standard points, obtained by measuring each point 20 times on different occasions, varied between 1.6% and 4.9%, with no significant differences between the two instruments used, the ES 600 for LH and the ES 22 and the ES 600.

Detection limit. Potential sensitivity was assessed by 20 within-assay replicates of the zero calibrators in each laboratory. The mean result + 3 SD, based on the results from all participating laboratories, determined the lower limit of detection: 0.42 int. unit/L for LH and 0.27 int. unit/L for FSH.

In addition, an unknown sample (horse serum) was distributed and analyzed by all laboratories. The median concentration of LH and FSH measured in this sample was 0.7 int. unit/L each. The results from the individual laboratories varied between 0.2 and 1.1 int. units/L for LH and between 0.4 and 1.2 int. units/L for FSH.

Precision. We obtained data on reproducibility from blood samples analyzed ≥20 times in one series of determinations (intra-assay), from three different control sera (PN-IM, PP-IM, and Lyphochek III) analyzed ≥10 times in different series (interassay), and from 11 control samples [samples from the U.K. External Quality-Assessment Schemes (UK EQAS)] analyzed in all laboratories (interlaboratory). Two of the control sera used in the assessment of the interassay variation contained very similar FSH concentrations.

Total results for intra- and interassay variation are shown as the precision profiles for LH and FSH in Figure 2A. Intra-assay CV for LH was <3% in 24 of the 27 samples tested in the concentration range of 5 to 85 int. units/L; at lower concentrations, the CV was higher. For FSH, the intra-assay CV was <4% in 23 of 26 samples with concentrations of 6 to 135 int. units/L.

The interassay CV for both assays was <6% at concentrations of ≥20 int. units/L. In the control serum with the lower concentration of LH and FSH, the interassay CV for both assays varied between 2% and 9% in the nine laboratories. The precision of the automated ES600 analyzer was in general better (the variation was less) than that of the ES22 analyzer or with use of manual pipetting (results not shown).

Eleven blood samples were distributed to all labora-

Fig. 2. Intra-assay (+) and interassay (Q) variation for Enzymun Test LH (top) and Enzymun Test FSH (bottom) in participating laboratories.
tories. The resulting interlaboratory CV at an FSH concentration of 8 int. units/L was 13% (n = 76); at 10 to 25 int. units/L, it was between 10% and 11% (n = 114). For LH of 3 to 5 int. units/L, the interlaboratory CV was between 10% and 15% (n = 72); at concentrations between 6 and 34 int. units/L, the CV was 9% (n = 126).

**Accuracy.** Accuracy was tested by analyzing samples to which known amounts of Reference Material (1st IRP for LH and 2nd IRP for FSH) had been added. Analytical recovery was always within 5% of the expected value. The calculated recovery from UK EQAS samples, consisting of pools of human serum supplemented with either FSH or LH, was 94 (SD 7)% (n = 18) for LH and 97 (SD 7)% for FSH (n = 19).

**Specificity.** To human serum samples with no detectable FSH or LH we added various amounts of available pituitary (LH, FSH, TSH, and human growth hormone) or placental hormones (hCG). Cross-reactivity was calculated as apparent FSH or LH, in int. units/L (Table 2). No interference resulted from the addition of 500 μg/L of the specific subunits h-LHα, h-LHβ, h-FSHβ, h-TSHα, or hCGα (data not shown).

Also, individual laboratories, using different FSH and LH preparations, detected no cross-reactivity of any significance (<1%). [Note that the available TSH reference preparation (68/38; National Institute for Biological Standards and Control, Hampstead, London, U.K.) is contaminated with LH.]

**Calibration.** In four of the participating laboratories, the LH and FSH IRP-based standards of the tests were compared with locally available material of the 2nd IRP pituitary FSH and of the 1st IRP pituitary LH. Results of these comparisons were within 2% of the stated values.

In addition, the master calibrators used to standardize the kit standard materials were calibrated against the 1st International Standard (IS) for FSH, 83/757, and the 2nd IS for LH, 80/552. The results of extensive testing (n = 299) revealed clear differences in the magnitude of the international unit of the previous and the new standard preparations. For FSH, 1 int. unit of the 1st IS is equivalent to 2.92 int. units of the 2nd IRP. For LH, 1 int. unit of the 2nd IS is equivalent to 0.90 int. unit of the 1st IRP. These differences will be important when the recommended reference preparations are changed.

**Linearity.** Untreated human sera with high concentrations of FSH, LH, or both, were serially diluted with zero calibrator. No deviations were >5% of the calculated concentrations for FSH or LH, showing a good linearity of the reagents.

All participating laboratories analyzed LH and FSH in five samples, obtained from the UK EQAS, consisting of mixtures of two different plasma pools. Linearity was excellent: the mean values of the measured FSH and LH were within 3% of the calculated values. These results, based on the mean measurements in all laboratories, are illustrated in Figure 3.

**High-dose "hook" effects.** Adding large quantities of purified FSH or LH to human sera showed that concentrations as great as 4000 int. units/L for FSH and 3000 int. units/L for LH caused no high-dose "hook" effects.

**Interferences.** Interference with the determinations was tested by diluting sera with high FSH and LH concentrations with hemolytic, lipemic, icteric, uremic, dysproteinemic, or rheumatoid samples. No evidence was found for interference by any of these factors.

The effects of heterophilic antibodies were studied by using blood samples obtained after screening 10 000 human serum samples for non-LH/non-FSH antibody-binding substances with a special ELISA designed to detect murine antibodies. Of 16 sera that were positive for these antibodies, four (13) caused falsely increased FSH (or LH) values when assayed with the original reagents. Addition of monoclonal mouse-IgG (γκ), polymerized according to a proprietary procedure (patent pending), prevented this interference. Because the reagents used in the study described here had been so modified, no interference from heterophilic antibodies was seen.

The final concentrations of the additives used during this evaluation did not interfere with the quantitative recovery of added FSH or LH.

**Multicenter Evaluation**

Each of the 10 participating laboratories analyzed between 35 and 194 blood samples by their usual method and compared the results, expressed in the same international units, with those obtained with Enzymun-Test FSH and LH. Statistical analysis by the standardized Passing-Bablock (18) method showed excellent correlations between the various methods.

For FSH (Table 3), the correlation coefficients were between 0.947 and 0.997, regardless of the principle of the inhouse assay used. The calculated slopes of the regression lines showed more variation; in particular, the differences between the two-site sandwich Enzymun-Test FSH and competitive immunoassays (RIAs 1 to 5) were remarkable. In all cases, the more-specific two-site Enzymun-Test FSH yielded greater plasma concentrations than did the competitive assays. The
differences between Enzymun-Test FSH and other modern two-site assays were in general less remarkable, and a good agreement between these assay systems was observed. However, agreement between comparative results of laboratories using identical reagents (fluoroimmunoassays 1 and 2) was not very good.

For LH (Table 3), the correlation coefficients varied between 0.928 and 0.994, with one exception—0.844, from laboratory 2, which tested a relatively large number of sera from postmenopausal women. These samples may represent a population with an atypical relation between the results of the two methods. In other laboratories, however, no discrepancies were found with samples from postmenopausal women. Contrary to the results for FSH, the LH comparisons showed no systematic differences between the results of competitive assays and two-site noncompetitive assays. Again, the lack of agreement between users of identical reagents can be seen (fluoroimmunoassay 1 and immunoradiometric assay 1).

Reference Values

Because the results in the participating laboratories were comparable, we tried to define reference values for FSH and LH concentrations in serum. A summary of the results for normal subjects is given in Table 4. The criteria used by the participating laboratories to decide whether to include the data from a particular individual were based on information they had on the absence of abnormalities. The midcycle samples were collected within 48 h of ovulation, which was detected by daily sonographic evaluation. These values must be considered as guidelines, and each individual laboratory must still compile its own reference values.

Discussion

In the first part of this paper, we described a multicenter evaluation of two new ELSAs for FSH and LH. The results indicate that these assays meet reliability requirements. The results for precision and accuracy
were well within the accepted limits of adequate performance for immunoassays. The linearity of the results after diluting sera with the zero calibrator or after mixing different serum samples was excellent. Furthermore, a problem of some immunometric assays—a decrease in the measured signal at high concentrations of the analyte (the so-called hook effect)—could not be demonstrated, even at very high concentrations that are never reached under physiological conditions, even after hormonal stimulation.

The high specificity for both gonadotropins is demonstrated by the very low cross-reactivity of the related glycoproteins. In addition, no other interferences could be found, and adding treated murine immunoglobulins prevented the problem of interference from heterophilic antibodies. The sensitivity is satisfactory and, because of the large working range of the reagents, almost all determinations can be done directly, without prior dilution, by using the reagents described. An exception may be the determination of the very low concentrations of gonadotropins, particularly LH, in prepubertal children, 90% of whom have LH concentrations <0.5 int. unit/L (19).

We did not try to separate the results according to the instruments used. The reagents can be used manually, but fully automated analyses can be performed on the ES600. In the evaluation, the reproducibility of the results from laboratories using this equipment was slightly better than from those working manually or semi-automatically with the ES22.

Comparing the results of the Enzymun-Test FSH and Enzymun-Test LH with other commercially available reagents requires special attention. In general, correlations between the two new assays and other kits were good; in only one series of LH determinations in one laboratory was the correlation coefficient <0.93. Because this systematic discrepancy was observed almost exclusively in the comparison between the Enzymun-Test LH and this set of reagents, the discrepancy may well be caused by this particular combination of reagents.

The other participating laboratories had occasional discrepancies in the results for LH, mainly those laboratories using competitive immunoassays. These differences could not be attributed to laboratory errors, as shown by repeating the assays with both sets of reagents. The most likely explanation for such differences in assay results is cross-reactivity with the hCG present in some of the samples and the heterogeneity of the glycoproteins analyzed. The circulating forms of these hormones are heterogeneous, and several reports have documented the physicochemical separation of subpopulations of the molecular forms of LH and FSH (20–22). At least one study showed that the subpopulations of LH are bioactive, as assessed by an in vitro bioassay (20).

Immunological determinations of glycoproteins such as FSH and LH are based on comparison of the substance(s) present in biological samples with those present in nonhomogeneous standard preparations. Therefore, differences between determinations performed with different antibodies are not unexpected and indeed have been observed previously. Discrepancies between bioassays and immunological assays have also been described (23). De Hertogh et al. (24) recently concluded that immunometric assays more accurately reflect the function of the gonadotropic cells, especially in children near puberty.

Researchers discussing true values for the concentration of gonadotropins in serum usually consider bioassay results the closest to the truth. Recent findings, however, indicate that substances can be found in human plasma that interfere with the commonly used in vitro bioassay for LH (25). Thus, which method gives results closest to reality for the determination of gonadotropins in small blood samples is still uncertain.

References

Table 4. Reference Ranges (Central 95%) for FSH and LH from All Laboratories

<table>
<thead>
<tr>
<th></th>
<th>FSH, int. units/L</th>
<th>LH, int. units/L</th>
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<tbody>
<tr>
<td></td>
<td>n*</td>
<td>n</td>
</tr>
<tr>
<td>Men</td>
<td>200</td>
<td>0.6–6.2</td>
</tr>
<tr>
<td>Women</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular phase</td>
<td>52</td>
<td>3.3–9.9</td>
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<tr>
<td>Midcycle</td>
<td>34</td>
<td>6.3–31.1</td>
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<td>Luteal phase</td>
<td>52</td>
<td>2.2–8.4</td>
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<tr>
<td>Postmenopausal</td>
<td>124</td>
<td>26.5–139</td>
</tr>
<tr>
<td>Prepubertal children</td>
<td>23</td>
<td>&lt;0.5–3.4</td>
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* No. of individual samples tested.
Detection of Human Apolipoprotein E3, E2, and E4 Genotypes by an Allele-Specific Oligonucleotide-Primed Polymerase Chain Reaction Assay: Development and Validation

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A polymerase chain reaction (PCR) assay has been developed and validated by using allele-specific oligonucleotide (ASO) primers to specifically amplify E3, E2, and E4 polymorphic sequences of the human apolipoprotein E (apo E) genes. Degenerate ASOs containing one or two additional 3' mismatches provided greater specificity than did ASOs containing a single mid-sequence or 3' allele-specific mismatch with plasmid pEB4 or genomic DNA as template. Optimal specificity and efficiency of amplification did not correlate with primer annealing conditions, whether determined theoretically or via oligo-melting experiments. Pre-cycling denaturation times and high cycling denaturation temperatures were also required for optimal amplification, presumably because of the high G:C content (75–85%) of apo E gene sequences. Conditions permissive for amplification and discrimination with plasmid DNA did not transpose favorably to amplification from human genomic DNA from peripheral blood leukocytes; the latter required nested primer reactions. These data may be valuable in predicting PCR assay conditions for other G:C-rich sequences containing polymorphic sequence differences. The assay described is both more accurate and rapid (24 h) than previously described methods for phenotyping or genotyping human apo E from blood specimens.

Additional Keyphrases: PCR efficiency of plasmid DNA and human leukocyte DNA compared • phenotyping

The human apolipoprotein (apo) E gene located on chromosome 19 is highly polymorphic, with three alleles (designated e2, e3, and e4) encoding the main isoforms apo E2, E3, and E4 (I, 2).6 These isoforms represent amino acid substitutions at residues 112 (E3 Arg → E4 Cys) and 158 (E3 Cys → E2 Arg), corresponding to single-base changes of T to C or vice versa at the relevant position in the gene coding sequence (3). Several other isoforms have also been reported (4–8), principally E2x (E3 Cys → E2x Arg at residue 145) and E2

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6 Nonstandard abbreviations: apo, apolipoprotein; LDL, low-density lipoprotein; ASO, allele-specific oligonucleotide; PCR, polymerase chain reaction; and Tm, melting temperature.