Short Technical Reports

Genotyping of Human Apolipoprotein E Alleles by the New Qualitative, Microplate-Based CASSI-Detection Assay

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ABSTRACT

A new qualitative PCR product detection assay called competitive amplified single mutation detection by selective probe hybridization immunoassay (CASSI) was developed for genotyping the most common apolipoprotein E (apoE) polymorphisms. Single target DNA strands immobilized using biotin on streptavidin-coated microplates were hybridized in separate wells with two distinct, 5¢-fluorescein isothiocyanate (FITC)-labeled oligonucleotides, complementary to either the 112Arg or 158Arg encoding site. With this assay, only correctly matched hybrids that form between probe and target DNA can be cleaved with the Hha*I restriction endonuclease, leading to loss of probe label in corresponding wells. However, allele-specific, probe-target mismatches due to G®T exchanges in the* Hha*I recognition sequences are not cleaved. After digestion, the remaining microplate-adsorbed signal is measured colorimetrically by using anti-FITC, Fab-horseradish peroxidase conjugates. Our results show maximum intensity was detected when the respective probe hybridized incompletely to the target (i.e., no cleavage), and minimum signal was obtained when the probe matched the target completely (complete cleavage); whereas, an intermediate signal was recorded at 50% complementarity (i.e., heterozygote alleles). With this assay, we could demonstrate a high prevalence of the apoE2 allele in patients suffering from coronary artery disease even though they displayed normal triglyceride and cholesterol levels. Corresponding results were obtained by CASSI compared with conventional restiction fragment-length polymorphism analysis.*

INTRODUCTION

Apolipoprotein (apoE) participates in cellular uptake of cholesterol and triglycerides because it interacts with distinct apoB/E and apoE receptors and is an essential component of chylomicrons, very-low-density lipoproteins and low-density lipoproteins. Three common variants expressed from multiple alleles at a single apoE locus are known, which give rise to three homozygous genotypes (E2/2, E3/3 and E4/4) and three heterozygous genotypes (E3/2, E4/2 and E4/3). These variants differ by amino acid substitutions at position 112 and 158 of the 299 residue protein resulting from single G→T base exchanges. ApoE3, the most common isoform, has cysteine (Cys) at position 112 and arginine (Arg) at position 158; apoE2 differs in having Cys at position 158; and apoE4 has Arg at position 112 (3,5,13). These differences can have important effects on the structural and physiological properties of the proteins. Particularly, apoE4 appears to play a causal role in Alzheimer's disease (AD) pathogenesis (13), while familial hyperlipoproteinemia type III is associated with the apoE genotype E2/2 (3). Therefore, apoE genotyping in clinical specimens helps to assess the individual's risk for developing AD or coronary artery disease (CAD).

Diagnosis usually involves analyzing short subsequences of exons 2 and 3 (containing both polymorphic sites) that are amplified by polymerase chain reaction (PCR). Genotyping of apoE variants by means of analyzing PCR products following amplification is performed by either *(i)* dot blot hybridization with allele-specific oligonucleotide probes (3), *(ii)* oligonucleotide ligation assay (2), *(iii)* single-strand conformational polymorphism (12) or *(iv)* restriction fragment-length polymorphism (RFLP) analysis immediately following PCR using the restriction endonuclease *Hha*I, which selectively cleaves the GCG↓C motifs affected by the depicted polymorphisms (5). The majority of published procedures require analysis of DNA digests by gel electrophoresis, which has several disadvantages, including: labor intensiveness, limited automation and occasional empirical evaluation, particularly when PCR by-products or contaminants interfere with visualization of DNA bands of interest. Screening of clinical samples for apoE isoforms (e.g., large risk patient groups) requires simpler, more precise methods that can be automated. Therefore, we developed the competitive amplified single mutation detection by selective probe hybridization immunoassay (CASSI)-PCR product detection assay, which is able to distinguish quantitatively between nucleic acid sequences that differ by just one base. Besides assessment of triglycerides and lipoprotein subfraction distributions, the assay was used for typing apoE alleles in a case-control study including normocholesterolemic and normotriglyceridemic CAD patients and healthy control subjects. Results obtained by CASSI were compared with those obtained by conventional RFLP analysis.

MATERIALS AND METHODS

Patients and DNA Sample Preparation

Thirty normocholesterolemic and normotriglyceridemic CAD patients (men, age: 61 ± 8 years; mean cholesterol: 5.20 ± 0.73 mM; mean triglyceride: 1.31 ± 0.31 mM) who had been scheduled to undergo coronary bypass surgery were analyzed. Leukocytes were prepared from 3 mL EDTA-treated blood, and genomic DNA was isolated by a previously described salting-out procedure (10).

PCR

One hundred-nanogram aliquots of vacuum-dried, redissolved DNA pellet were amplified in 50 µL standard PCR mixtures containing 2 µL of each 3′ and 5′ primer (10 pmol/µL), 5 µL 10× *Taq* DNA polymerase buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.01% [wt/vol] gelatin, pH 8.3), 1.5 U AmpliTaq® DNA Polymerase [Perkin-Elmer, Norwalk, CT, USA], 4 µL dimethyl sulfoxide and 8 µL dNTPs (0.2 mM each, [Promega, Madison, WI, USA]) using 2′-deoxyuridine 5′-triphosphate (dUTP) (Boehringer Mannheim, Mannheim, Germany) instead of dTTP. Primers used were the forward primer F6APOE (5′-TAAGCTTGGC ACGGCTGTCC AAGG-3′; nucleotides [nt] 3868–3884), 5′-labeled with

biotin, and the reverse primer F4APOE (5′-ACAGAATTCGCCCCGGCCTG-GTACAC-3′; nt 4079–4095, GenBank® Accession No. M10065 yielding a 244-bp PCR product, essentially as previously described (3,5). For CASSI assay, primer F4APOE was replaced by the newly developed primer F4NAPOE (5′-ACAGAATTCGCCAGGGAGCC-ACAGT-3′; nt 4178–4194) yielding a 343-bp PCR product, flanked by a 5′-*Hin*dIII and 3′-*Eco*RI cloning site. Carryover contamination was prevented by addition of 0.2 U uracil-DNA glycosylase (UDG; Boehringer Mannheim) to each reaction tube (7). "Hot start" amplifications were run with an initial 15-min incubation step at 37°C to ensure destruction of contaminating PCR products, then a subsequent 10-min denaturation at 94°C was performed. PCR conditions included 6 starting cycles of 94°C for 45 s, 62°C for 45 s and 72°C for 1 min followed by an additional 35 cycles of 94°C for 45 s, 52°C for 45 s and 72°C for 1 min using a GeneAmp® 9600 Thermal Cycler (Perkin-Elmer).

RFLP Analysis Using Polyacrylamide Gel Electrophoresis (PAGE)

Aliquots (10-µL) of PCR sample were digested with 5 U *Hha*I overnight at 37°C. After 10 min of enzyme inactivation at 68°C, 5 µL of digested sample were electrophoresed through a 10% (wt/vol) polyacrylamide gel for 90 min at 30 mA using a Joey™ Gel Casting System (AGS, Heidelberg, Germany). The ethidium bromide or silver-stained gels were subjected to a GelPrint Video Documentation Workstation (MWG-Biotech, Ebersberg, Germany). ONE-Dscan™ Software (Scanalytics, Billerica, MA, USA) was used to determine the size of digested fragments.

PCR Product Analysis by CASSI and Genotyping of ApoE Alleles

PCR samples were diluted according to amplification yields as assessed by agarose gel electrophoresis (ca. 1:100–1:1000) with phosphate-buffered saline (PBS) sample dilution buffer (1 mM NaH_2PO_4, 14 mM Na_2HPO_4, 140 mM NaCl; pH 7.4, 0.1% [vol/vol] Tween® 20). Eight times 100-µL aliquots of each dilution were immobilized on a 8-well strip coated with streptavidin (NEN Life Science Products, Boston, MA, USA) for 1 h at 37°C under continuous shaking (30 rpm) using a Vortemp 56 EVC Mixer-Incubator (National Labnet, Woolbridge, NJ, USA). Following 3× washing with 300 µL wash buffer (PBS, pH 7.4; 1% [vol/vol] Tween 20), immobilized DNA was denatured with 100 µL denaturation solution (0.1 mM NaOH, 0.3 mM NaCl) for 10 min at room temperature. After repeated washing, the wells were blocked with 200 µL blocking solution (PBS, pH 7.4; 2% [vol/vol] Tween 20) at room temperature for 10 min. Hybridization was performed for 1 h at 37°C with 1× sodium chloride sodium phosphate EDTA (SSPE) (8.5 mM Na_2HPO_4, pH 7.4; 150 mM NaCl, 2 mM EDTA, 0.25% [wt/vol] dextran sulfate, 25% [vol/vol] formamide) supplemented with the FITC-labeled probes S112ARG (5′-FITC-CCAGGCGGC-CGCGCACGTCCTCCA; nt 3921–3944, 1 pmol per well, lanes A–D) or S158ARG (5′-FITC-CCTGGTACAC-TGCCAGGCGCTTCT; nt 4065– 4088, 0.25 pmol per well, lanes E–H), respectively. All probes were purified by NAP™-5 columns (Amersham Pharmacia Biotech, Uppsala, Sweden) and arbitrarily designed to be complementary to the CGC-motif encoding Arg at amino acid positions 112 and 158. After washing, restriction digestion of hybrid strands were performed directly in the wells. For this, lanes A, B, E and F were incubated with 100 µL restriction enzyme buffer (REB; 10 mM Tris acetate, pH 7.5; 10 mM magnesium acetate, 50 mM potassium acetate) alone or supplemented (lanes C, D, G and H) with 4 U *Hha*I endonuclease (Amersham Pharmacia Biotech) for 1 h at 37°C with continuous shaking. After washing, plate-adsorbed FITC haptens were detected with anti-FITC, Fab-horseradish peroxidase (POD) conjugates (150 U/mL; Boehringer Mannheim), diluted 1:4000 with PBS sample dilution buffer. Incubation was performed for 15 min at 37°C. POD activity was measured colorimetrically as described (8). The reaction was terminated with stop solution (0.25 mol/L H_2SO_4, 8.5 mol/L acetic acid). Absorbances were usually read at 450 nm, using an automated Neutral

Table 1. ApoE Genotypes and Theoretically Remaining Plate-Adsorbed Signal Intensity Following CASSI

ApoE Genotype (affected amino acids)	% Frequency in European Population (6)	Hybridized Probe	% *Hha*I Cleavage (idealized)	% Remaining FITC Signal after Cleavage (idealized)
2/2 (^{112}Cys/^{158}Cys)	1	S112ARG S158ARG	no no	100 100
3/3 (^{112}Cys/^{158}Arg)	61	S112ARG S158ARG	no 100	100 0
4/4 (^{112}Arg/^{158}Arg)	3	S112ARG S158ARG	100 100	0 0
4/2 (^{112}Cys,^{112}Arg/^{158}Cys,^{158}Arg)	2	S112ARG S158ARG	50 50	50 50
4/3 (^{112}Cys,^{112}Arg/^{158}Arg)	21	S112ARG S158ARG	50 100	50 0
3/2 (^{112}Cys/^{158}Cys,^{158}Arg)	12	S112ARG S158ARG	no 50	100 50

The intensity of microplate-immobilized signal following *Hha*I cleavage is dependent on the composition of sample apoE variants: *(i)* maximum FITC signal (ca. 100%) was measured when the respective probe hybridized incompletely to the target (i.e., no cleavage), *(ii)* minimum signal was obtained when the probe matched the target completely (i.e., complete cleavage), whereas *(iii)* an intermediate signal (ca. 50%) was recorded at 50% complementarity (i.e., heterozygous alleles).

Model 8001 ELISA Reader (Anthos Labtec Instruments, Salzburg, Austria), or, if required, at 405 nm, since this provides a three-fold enhancement of the measuring range (9). All other chemicals used were of analytical grade and provided by Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) and Boehringer Mannheim.

RESULTS AND DISCUSSION

Experimental Design

The newly developed CASSI assay was conceived as a quantitative adaptation of the PCR-enzyme-linked immunosorbent assay (ELISA) hybridization technique (4,8). Based on published protocols, apoE-derived PCR products were modified during amplification using a 5′-biotin-labeled primer oligonucleotide that immobilized them to the streptavidin-coated matrix. Following capture on the microplate, complementary DNA strands were removed by alkaline denaturation.

Figure 1 shows the new strategy of apoE allele isotyping by CASSI. Two distinct, 5′-FITC-labeled DNA oligonucleotide probes complementary to DNA sections encoding either the 112Arg or 158Arg polymorphic site were hybridized to immobilized single target strands in separate wells. Following *Hha*I digestion, the blank-corrected control signals (corresponding to the total amount of hybridized probes) and signals obtained after enzymatic cleavage (corresponding to the amount of incompletely hybridized probe) were measured in duplicate. The percentages of remaining plate-adsorbed signal were calculated and used for classification of the apoE variant (Table 1). Since the typical FITC signal was too weak to be measured directly with conventional fluorescence readers (3 models were tested), the measurement was performed with anti-FITC POD conju-

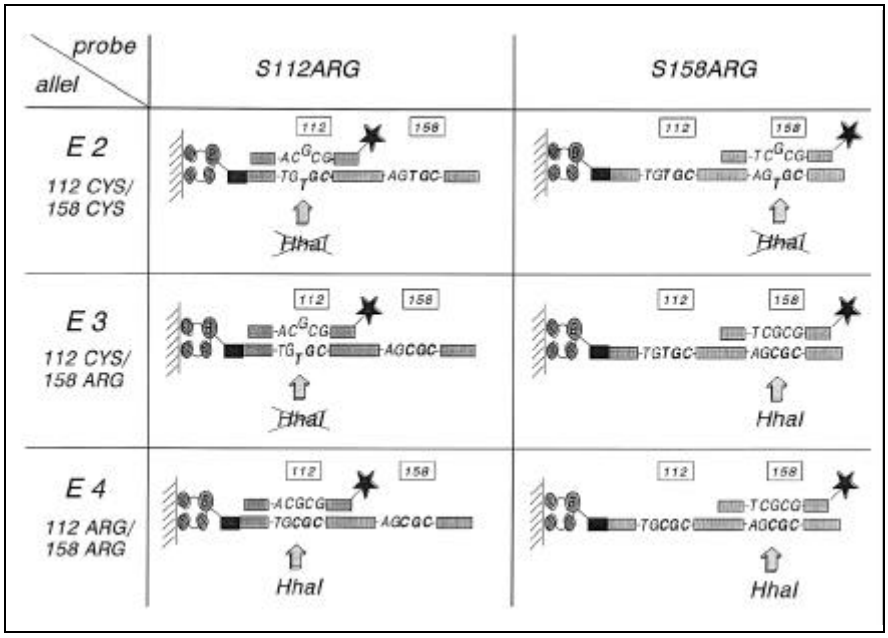

Figure 1. Schematic representation of CASSI assay for genotyping human apoE alleles on solid supports. Microplate immobilized single-stranded apoE PCR products were hybridized with FITC-labeled (indicated by star) probes S112ARG or S158ARG in different wells. Whereas only correctly matched ds-DNA segments were cleavable by *Hha*I-restricted digestion in the wells, mismatched hybrids were not cut. Thus, highly specific PCR product detection was combined with selective restricted digestion.

gates and subsequent colorimetric detection. This simple technique may be exploited even for quantitative differentiation between highly similar PCR products, which is usually necessary when heterozygously apoE-allele-expressing patients were analyzed.

Whereas both synthesis and immobilization of biotinylated PCR products to the microplates was uncomplicated, some critical difficulties of the assay had to be circumvented. At first, the minimum length of a suitable probe was unknown, since this should be as short and, consequently, as cheap as possible. Preliminary experiments revealed that probe-target hybrids can then be effectively cleaved by *Hha*I when the probe consists of at least 9 complementary nucleotides flanking both ends of the central GCG↓C recognition sequence. Probes merely consisting of 2 or 6 flanking residues were less effective, because they permitted less than 75% cleavage and nonspecifically detached (data not shown). Consequently, probes used for CASSI should be at least approximately 24–30 bases in length. High stringency hybridization was achieved with 1× SSPE buffer, which, supplemented with 25% formamide, allows a decrease of the hybridization temperature from 50° to 37°C. Since probe S158ARG unfortunately spanned a part of the F4APOE primer sequence in the 244-bp PCR products, amplification was performed with the corresponding new primer F4NAPOE, which annealed to the target further downstream.

The second and perhaps most critical point was determining reliable conditions for enzymatic cleavage. Towards this end, four recommended digestion buffers and several *Hha*I concentrations (0.5–5 U/well) were tested. The buffers tested were as follows: *(i)* acetate buffer (AB) [20 mM Tris acetate, pH 7.9, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol (DTT), 100 μg/mL bovine serum albumin (BSA)]; *(ii)* restriction buffer M (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl); *(iii)* KGB buffer (50 mM Tris acetate, pH 7.5, 200 mM potassium glutamate, 20 mM magnesium acetate, 100 μg/mL BSA, 1 mM β-mercaptoethanol [11]) and *(iv)* REB [see Materials and Methods]. Whereas restriction digestion with buffer AB was accompanied with nonspecific cleavage, buffers M and KGB yielded only poor digestion. Moreover, a significant nonspecific probe removal (up to 31%) even from undigested hybrid strands was detected in all cases. On the other hand, satisfactory results were obtained

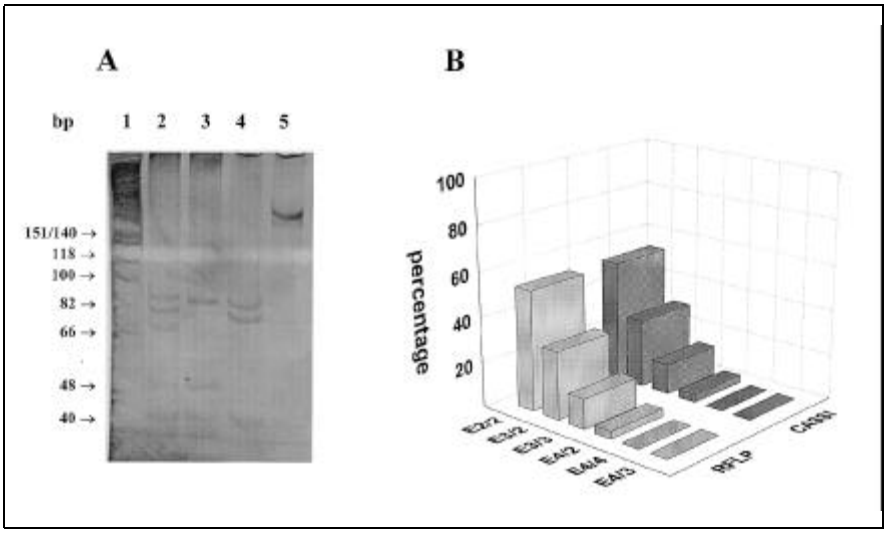

Figure 2. ApoE genotyping by CASSI compared with RFLP analysis. (A) Representative RFLP analysis of *Hha*I-digested, 244-bp products by PAGE (silver staining). Lane 1: ϕX174 DNA/*Hin*fI marker (Promega), lane 2: E4/2, lane 3: E3/3, lane 4: E2/2 and lane 5: uncut 244-bp fragment. Visible fragments corresponding to allele 2: 38, 81 and 91 bp; allele 3: 33, 38, 48 and 91 bp; and allele 4: 33, 38, 48 and 72 bp. (B) ApoE genotypes of 30 normolipemic CAD patients determined by CASSI and RFLP analysis.

with REB and cleavage with 4 U *Hha*I per well (data not shown). Since unsuitable buffer systems fundamentally differ from REB by addition of reducing agents, such as DTT and β-mercaptoethanol, these compounds appear to be responsible for nonspecific probe detachment.

After cleavage, the remaining immobilized signals corresponded well with the theoretically expected values summarized in Table 1. Mismatched probe-target hybrids yielded an average immobilized signal of 94% ± 16% and 87% ± 10% for probe S112ARG (n = 42) and S158ARG (n = 23), respectively. Amplified heterozygous alleles lead to intermediate cleavage corresponding to 47% ± 12% (n = 7) and 43% ± 13% (n = 16) of entire immobilized probe signals, whereas completely matched target-probe hybrids (exclusively assessed for probe S158ARG since no E4/4 variation was available) were cleaved to 11% ± 6% (n = 10) of the initial signal, which corresponds to an average cutting efficiency of 89%. Since sometimes slight detachment of probes or decreased enzymatic cleavage was observed, the simultaneous analysis of reference samples (e.g., variants E2/2 and E4/3 indicating none, complete and intermediate cleavage of probe-target hybrids, respectively) is required.

Furthermore, note that under the optimized conditions, the restriction enzyme *Hha*I apparently neither cleaved single-strand DNA nor displayed star activity (although nonspecific cleavage was observed at suboptimal conditions). Since carryover contamination was prevented by strictly replacing all dT nucleotides contained in the PCR mixture with dU nucleotides, it might be advantageous to select restriction enzymes that exclusively recognize DNA cleavage sites encoded by G and C residues.

ApoE Genotyping by CASSI and RFLP Analysis in Clinical Samples

In Figure 2A, some representative apoE RFLP restriction patterns are shown. Identical results were obtained when all individual samples were analyzed by either the CASSI or RFLP method (Figure 2B).

As shown by both methods, the selected group of normolipemic CAD patients was characterized by a high prevalence of the apoE2 allele: 53% of all patients expressed this variation homozygously and 33% heterozygously (30% E3/2, 3% E4/2). This result strongly diverged from the average allele frequency observed in the European population (Table 1). The remaining patients were apportioned to the most common allelic variation E3/3. The frequent apoE2 genotype in this CAD group seems to be in accordance with a reduced chylomicron remnant catabolism and the subsequent atherogenic potential of delayed postprandial clearance of triglyceride-rich lipoproteins (unpublished observation).

We conclude that the CASSI assay originally developed for quantitation of competitively amplified PCR products, which differ in sequence by only one nucleotide, is a reliable qualitative detection method for the most common apoE allele polymorphisms. Compared to recent alternative procedures (2,3, 5,11), this assay has a multitude of advantages: *(i)* it is readily automated [e.g., tested with a DIAS Ultra™ ELISA Processor (Dynex Technologies, Chantilly, VA, USA)]; mainly because no further electrophoretical separation and assessment is necessary, *(ii)* the method involves a specific hybridization step that is required by German law as specified by DIN-Norm 58967-60 for diagnostic applications; enzymatic cleavage of probe-target hybrids is a further proof for amplification specificity, *(iii)* the pitfall of heteroduplex formation, which may strongly influence RFLP analysis of highly similar PCR products (1), is avoided (since exclusively single target DNA strands are detected), *(iv)* the dUTP/UDG-strategy may be applied, which allows a high degree of contamination safety and *(v)* the assay takes only about 4 h, is relatively inexpensive (about $4.00 per patient assuming one 8-well strip), is non-isotopic and use of standard laboratory equipment suffices. To eliminate the necessity of concentration-dependent sample dilution, further experiments are now in progress that combine amplification directly performed in microplates coated with defined amounts of the upstream primer, followed immediately by detection in the same wells.

REFERENCES

1. **Becker-Andre, M. and K. Hahlbrock.** 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). Nucleic Acids Res. *17*:9437-9446.
2. **Baron, H., S. Fung, A. Aydin, S. Bahring, E. Jeschke, F.C. Luft and H. Schuster.** 1997. Oligonucleotide ligation assay for detection of apolipoprotein E polymorphisms. Clin. Chem. *43*:1984-1986.
3. **Emi, M., L.L. Wu, M.A. Robertson, R.L. Myers, R.A. Hegele, R.R. Williams, R. White and J.-M. Lalouel.** 1988. Genotyping and sequence analysis of apolipoprotein E isoforms. Genomics *3*:373-379.
4. **Hahn, M., V. Dörsam, P. Friedhoff, A. Fritz and A. Pingoud.** 1995. Quantitative polymerase chain reaction with enzyme-linked immunosorbent assay detection of selectively digested amplified sample and control DNA. Anal. Biochem. *229*:236-248.
5. **Hixson, J.E. and D.T. Vernier.** 1990. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *Hha*I. J. Lipid Res. *31*:545-548.
6. **Jaross, W. and J. Hänisch.** 1990. Apolipoprotein E, ein lebensnotwendiges Protein. Med. aktuell *16*:460-462.
7. **Köhler, T., A.-K. Rost and H. Remke.** 1997. Calibration and storage of DNA competitors used for contamination-protected competitive PCR. BioTechniques *23*:722-726.
8. **Laßner, D.** 1995. Quantitation of mRNA by the ELOSA technique using external standards, p. 117-123. *In* T. Köhler, D. Laßner, A.-K. Rost, B. Thamm, B. Pustowoit and H. Remke (Eds.), Quantitation of mRNA by Polymerase Chain Reaction—Nonradioactive PCR Methods. Springer-Verlag, Heidelberg.
9. **Madersbacher, S. and P. Berger.** 1991. Double wavelength measurement of 3,3′,5,5′-tetramethylbenzidine (TMB) provides a threefold enhancement of the ELISA measuring range. J. Immunol. Methods *138*:121-124.
10. **Miller, S.A., D.D. Dykes and H.F. Polesky.** 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. *16*:1215-1218.
11. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. CSH Laboratory Press, Cold Spring Harbor, NY.
12. **Tsai, M.Y., P. Suess, K. Schwichtenberg, J.H. Eckfeldt, J. Yuan, M. Tuchman and D. Hunninghake.** 1993. Determination of apolipoprotein E genotypes by single-strand conformational polymorphism. Clin. Chem. *39*:2121-2124.
13. **Utermann, G.** 1994. The apolipoprotein E connection. One particular variant of the polymorphic protein apolipoprotein E appears to be a risk factor for Alzheimer's disease, possibly because it directly promotes amyloid formation. Curr. Biol. *4*:362-365.

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Development of a Yeast Trihybrid Screen Using Stable Yeast Strains and Regulated Protein Expression

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ABSTRACT

We describe a yeast trihybrid system that facilitates rapid screening of cDNA libraries. Novel yeast vectors were developed that direct integration of cDNA encoding the bait and third protein component into the yeast chromosome. A recombinant yeast strain is thus generated (screening strain) and is available for library transformation. Transformation with the library DNA is a single, efficient transformation event, allowing the cDNA library to be represented in one step. Recovery of the library plasmid from the yeast is also simplified, since it is the only episomal plasmid. Assay of trihybrid interaction and identification of positive clones is faciliated by regulating expression of the third protein component using the yeast MET3 *promoter, which is repressed in the presence of exogenous methionine. Trihybrid interactions are detected only on media lacking methionine. This trihybrid system uses the standard* E. coli LacZ *and yeast* HIS3 *reporter genes and is compatible with most available Gal4 activation domain cDNA libraries. We describe the successful application of this yeast trihybrid system to the study of phosphoprotein interactions involved in T-cell signaling.*

INTRODUCTION

In recent years, the yeast two-hybrid assay for direct protein-protein interactions has been adapted to allow the investigation of complex molecular interactions mediated by several components or between post-translationally modified proteins. Several such assays have been described, including co-expression of a protein tyrosine kinase (PTK) as a modifying enzyme to assay the interaction between phosphoproteins (8), introduction of adapter or ligand bridges to assay complex ternary interactions (7,17) and assay of RNA-protein interactions (9,13). These assays invariably involve the presentation of three heterologous components in the yeast cell and have been described using a variety of terms incuding tribrid, three-hybrid and trihybrid assays. The system we describe involves the expression of three hybrid fusion-proteins in the yeast cell, and we use the definition trihybrid.

Introduction of three components into the yeast cell is generally achieved by serial transformation. To facilitate the use of the trihybrid assay in screening cDNA libraries, we have chosen to generate stable yeast strains (designated screening strains), expressing both the bait protein and the third protein component from constructs stably integrated into the yeast genome. The library plasmid DNA can then be introduced in a single efficient transformation event, and because the library plasmid is the only episomal plasmid, recovery from the yeast is simplified. The screening strains are also readily available for repeated screens. To facilitate rapid identification of positive trihybrid clones, we have chosen to regulate expression of the third protein component independently of the other fusion proteins using the repressible yeast *MET3* promoter (1,3). A similar strategy was recently de-