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Background: Cell-free fetal DNA circulating in maternal blood has potential as a safer alternative to invasive methods of prenatal testing for paternally inherited genetic alterations, such as cystic fibrosis (CF) mutations. Methods: We used allele-specific PCR to detect mutated CF D1152H DNA in the presence of an excess of the corresponding wild-type sequence. Pfx buffer (Invitrogen) containing replication accessory proteins and Taq polymerase with no proofreading activity was combined with TaqMaster PCR Enhancer (Eppendorf) to suppress nonspecific amplification of the wild-type allele. The procedure was tested on DNA isolated from plasma drawn from 11 pregnant women (gestational age, 11-19.2 weeks), with mutation confirmation by chorionic villus sampling. Results: The method detected 5 copies of the CF D1152H mutant allele in the presence of up to [~]100 000 copies of wild-type allele without interference from the wild-type sequence. The D1152H mutation was correctly identified in one positive sample; the only false-positive result was seen in a mishandled sample. Conclusions: This procedure allows for reliable detection of the paternally inherited D1152H mutation and has potential application for detection of other mutations, which may help reduce the need for invasive testing.

http://www.clinchem.org/cgi/content/abstract/50/4/702
Background: The orthopox viruses that are pathogenic for humans include variola major virus (VAR), monkeypox virus (MPV), cowpox virus (CPV), and to a lesser extent, camelpox virus (CML) and vaccinia virus (VAC). PCR is a powerful tool to detect and differentiate orthopox viruses, and real-time PCR has the further advantages of rapid turnaround time, low risk of contamination, capability of strain differentiation, and use of multiplexed probes. Methods: We used real-time PCR with fluorescence resonance energy transfer technology to simultaneously detect and differentiate VAR, MPV, CPV/VAC, and CML. An internal control generated by cloning and mutating the PCR target gene facilitated monitoring of PCR inhibition in each individual test reaction. Results: Strain differentiation results showed little interassay variability (CV, 0.4-0.6%), and the test was 100-fold more sensitive than virus culture on Vero cells. Low copy numbers of DNA could be detected with 95% probability (235-849 genome copies/mL of plasma). Conclusions: The real-time PCR assay can detect and differentiate human pathogenic orthopox viruses. The use of an internal control qualifies the assay for high sample throughput, as is likely to be needed in situations of suspected acts of biological terrorism, e.g., use of VAR.


Measurement of plasma butyrylcholinesterase (BChE) activity and inhibitor-based phenotyping are standard methods for identifying patients who experience post-succinylcholine (SC) apnea attributable to inherited variants of the BChE enzyme. Our aim was to develop PCR-based assays for BChE mutation detection and implement them for routine diagnostic use at a university teaching hospital.

Methods: Between 1999 and 2002, we genotyped 65 patients referred after prolonged post-SC apnea. Five BChE gene mutations were analyzed. Competitive oligo-priming (COP)-PCR was used for flu-1, flu-2, and K-variant and direct DNA sequencing analysis for dibucaine and sil-1 mutations. Additional DNA sequencing of BChE coding regions was provided when the five-mutation screen was negative or mutation findings were inconsistent with enzyme activity.

Results: Genotyping identified 52 patients with primary hypocholinesterasemia attributable to BChE mutations, and in 44 individuals the abnormalities were detected by the five-mutation screen (detection rate, 85%). Additional sequencing studies revealed mutations in eight other patients, including five with novel mutations. The most common genotype abnormality was compound homozygous dibucaine and homozygous K-variant mutations. No simple homozygotes were found. Of the remaining 13 patients, 3 had normal BChE activity and gene, and 10 were diagnosed with hypocholinesterasemia unrelated to BChE gene abnormalities.

Conclusion: A five-mutation screen for investigation of post-SC apnea identified BChE gene abnormalities for 80% of a referral population. Six new BChE mutations were identified by sequencing studies of 16 additional patients.

Background: The aim of the present study was to investigate the interactions between the circulating concentrations of 1,25-dihydroxyvitamin D3 \([1,25(\text{OH})_2\text{D}3]\) and the mRNA concentration of its specific nuclear receptor in human leukocytes. Methods: We measured vitamin D receptor (VDR) mRNA extracted from leukocytes by use of TaqMan fluorescence analysis applied to the reverse transcription-PCR (RT-PCR) technique in 16 volunteers before and after calcitriol administration. VDR mRNA was also measured in leukocytes from calcium-stone-formers (37 hypercalciuric and 34 normocalciuric patients). The relationship between VDR mRNA concentrations and genetic VDR polymorphisms was analyzed in these patients. Results: Imprecision (CV) of RT-PCR was 1.3% within assay \((n = 10)\) and 1.7% between assays \((n = 4)\). Oral 1,25(OH)2D3 increased mean \((SE)\) serum 1,25(OH)2D3 1.6 (0.3)-fold and VDR mRNA 1.6 (0.1)-fold 8 h after administration. The maximum VDR mRNA was reached 3.6 (1.3) h after 1,25(OH)2D3 ingestion. No differences in leukocyte VDR mRNA concentrations were found between normocalciuric and hypercalciuric stone-formers in the absence of stimulation. Finally, no association was found between VDR mRNA concentrations and genetic VDR polymorphisms in stone-formers. Conclusions: The TaqMan RT-PCR assay is a rapid and accurate method to measure VDR mRNA, and leukocytes are a useful model to study VDR and 1,25(OH)2D3 interactions. In humans, VDR mRNA is increased by agonist 1,25(OH)2D3, a finding resembling previously reported results obtained in cellular and animal models.

Background: Mutations in the retina-specific ABC transporter (ABCA4) gene have been associated with several forms of macular degenerations. Because the high complexity of the
molecular genotype makes scanning of the ABCA4 gene cumbersome, we describe here the first use of denaturing HPLC (DHPLC) to screen for ABCA4 mutations. Methods: Temperature conditions were designed for all 50 exons based on effective separation of 83 samples carrying 86 sequence variations and 19 mutagenized controls. For validation, samples from 23 previously characterized Stargardt patients were subjected to DHPLC profiling. Subsequently, samples from a cohort of 30 patients affected by various forms of macular degeneration were subjected to DHPLC scanning under the same conditions. Results: DHPLC profiling not only identified all 132 sequence alterations previously detected by double-gradient denaturing gradient gel electrophoresis but also identified 5 sequence alterations that this approach had missed. Moreover, DHPLC scanning of an additional panel of 30 previously untested patients led to the identification of 28 different mutations and 29 polymorphisms, accounting for 203 sequence variations on 29 of the 30 patients screened. In total, the DHPLC approach allowed us to identify 16 mutations that had never been reported before. Conclusions: These results provide strong support for the use of DHPLC for molecular characterization of the ABCA4 gene.


http://www.clinchem.org/cgi/content/abstract/48/12/2147

Background: The genotype of hepatitis C virus (HCV) is a predictor of antiviral therapeutic response. We describe an approach for HCV genotype determination by real-time PCR and melting curve analysis. Methods: After automated nucleic acid extraction, we used reverse transcription-PCR in a block cycler to amplify nucleotides 6-329 of the 5'-untranslated region of HCV. The product was further amplified by single-tube real-time seminested PCR in a LightCycler™ instrument (Roche). The final product was analyzed by melting curves with the use of fluorescence resonance energy transfer (FRET) probes. The FRET sensor probe was directed at nucleotides 151-170 of type 1 HCV and was designed to distinguish types 1a/b, 2a/c, 2b, 3a, and 4, with melting temperatures (Tms) predicted to differ by 1 {degrees}C. Genotypes were compared in a blinded fashion with those of the INNO-LiPATM test (Bayer Diagnostics) on 111 serum samples. Results: In preliminary experiments, the Mg2+ concentration was found to be critical in allowing clear separation of melting points, with the best separation at a Mg2+ concentration of 2 mmol/L. The results for 111 samples clustered at expected Tms for genotypes 1a/b (n = 78), 2a/c (n = 2), 2b (n = 11), 3a (n = 14), and 4 (n = 2). Of the 111 samples, results for 110 were concordant with the comparison method at the level of type 1, 2, 3, or 4. Subtyping results were discordant for two samples, both of type 2. For 108 samples concordant with INNO-LiPA at the genotype and subtype levels, the mean Tms were 64.1, 59.5, 54.2, 52.6, and 50.1 {degrees}C for types 1a/b, 2a/c, 4, 2b, and 3a, respectively, with SDs of 0.2, 0.3, 0.3, 0.2, and 0.3 {degrees}C. All 78 samples identified as type 1 were concordant with results of the comparison method. Conclusions: Melting analysis with a single pair of FRET probes can rapidly provide information about HCV genotypes and identifies type 1 samples with high specificity.


http://www.clinchem.org/cgi/content/abstract/48/12/2155

Background: Most pathogenic human mitochondrial DNA (mtDNA) mutations are heteroplasmic (i.e., mutant and wild-type mtDNA coexist in the same individual) and are difficult to detect when
their concentration is a small proportion of that of wild-type mtDNA molecules. We describe a simple methodology to detect low proportions of the single base pair heteroplasmic mutation, A3243G, that has been associated with the disease mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) in total DNA extracted from blood. Methods: Three peptide nucleic acids (PNAs) were designed to bind to the wild-type mtDNA in the region of nucleotide position 3243, thus blocking PCR amplification of the wild-type mtDNA while permitting the mutant DNA to become the dominant product and readily discernable. DNA was obtained from both apparently healthy and MELAS individuals. Optimum PCR temperatures were based on the measured ultraviolet thermal stability of the DNA/PNA duplexes. The presence or absence of the mutation was determined by sequencing. Results: In the absence of PNAs, the heteroplasmic mutation was either difficult to detect or undetectable by PCR and sequencing. Only PNA 3 successfully inhibited amplification of the wild-type mtDNA while allowing the mutant mtDNA to amplify. In the presence of PNA 3, we were able to detect the heteroplasmic mutation when its concentration was as low as 0.1% of the concentration of the wild-type sequence. Conclusion: This methodology permits easy detection of low concentrations of the MELAS A3243G mutation in blood by standard PCR and sequencing methods.


Background: Because cyclosporin A (CsA) and glucocorticoids inhibit the production of interleukin-2 (IL-2) and other cytokines, quantitative analysis of cytokine mRNA might constitute a pharmacodynamic measure for immunosuppressive drug effects. We investigated whether immunosuppressive drugs influence cytokine mRNA expression kinetics during T-cell costimulation. Methods: We used a human whole blood assay to determine basal (unstimulated) IL-2, IL-4, and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) mRNA concentrations and expression kinetics after anti-CD3/anti-CD28 monoclonal antibody costimulation in kidney transplant recipients undergoing CsA-based immunosuppressive triple therapy and in healthy controls (ex vivo study I). The effect of CsA on IL-2 mRNA expression kinetics was also determined ex vivo in patients undergoing CsA monotherapy (ex vivo study II) and after in vitro addition of CsA. Results: In ex vivo study I, basal TNF-\(\alpha\) mRNA but not IL-2 and IL-4 mRNA was decreased in kidney transplant patients. We observed shifts in peak IL-2 and IL-4 (from 8 to 24 h) and TNF-\(\alpha\) (from 4 to 8 h of costimulation) mRNA expression in kidney transplant patients after T-cell costimulation. In patients undergoing CsA monotherapy (ex vivo study II), the inhibitory effect of CsA was detectable as an individually delayed increase in IL-2 mRNA during costimulation. In vitro addition of CsA also induced a dose-independent displacement of IL-2 mRNA expression kinetics (i.e., a delay). Conclusions: A delayed increase in cytokine mRNA expression during T-cell costimulation may represent a sensitive effect of immunosuppression. The single analysis of one absolute or peak mRNA value could be misleading. For prospective studies involving measurement of cytokine mRNA, we therefore suggest the parameter "area of cytokine mRNA expression over time", which should include absolute cytokine mRNA values at two different time points of mRNA kinetics.


Background: Microelectronic DNA chip devices represent an emerging technology for genotyping.
We developed methods for detection of single-nucleotide polymorphisms (SNPs) in clinically relevant genes. Methods: Primer pairs, with one containing a 5'-biotin group, were used to PCR-amplify the region encompassing the SNP to be interrogated. After denaturation, the biotinylated strand was electronically targeted to discrete sites on streptavidin-coated gel pads surfaces by use of a Nanogen Molecular Workstation. Allele-specific dye-labeled oligonucleotide reporters were used for detection of wild-type and variant sequences. Methods were developed for SNPs in genes, including factor VII, \((\beta)\)-globin, and the RET protooncogene. We genotyped 331 samples for five DNA variations in the factor VII gene, >600 samples from patients with \((\beta)\)-thalassemia, and 15 samples for mutations within the RET protooncogene. All samples were previously typed by various methods, including DNA sequence analysis, allele-specific PCR, and/or restriction enzyme digestion of PCR products. Results: Analysis of amplified DNA required 4-6 h. After mismatched DNA was removed, signal-to-noise ratios were >5. More than 940 samples were typed with the microelectronic array platform, and results were totally concordant with results obtained previously by other genotyping methods. Conclusions: The described protocols detect SNPs of clinical interest with results comparable to those of other genotyping methods.


http://www.clinchem.org/cgi/content/abstract/48/12/2164

Background: Mutations in codons 12, 13, and 61 of the N-ras gene are common alterations in cutaneous malignant melanoma. We evaluated pyrosequencing, a simple and rapid method used mainly for single-nucleotide polymorphism analysis, as a possible alternative to single-strand conformation polymorphism (SSCP) analysis and sequencing of N-ras. Methods: We evaluated the sensitivity and accuracy of the pyrosequencing method for identification of mutations in N-ras codons 12, 13, and 61. Nucleotide dispensation orders were created to produce distinct pyrogram peak profiles for the most frequent mutations in codon 61 and codons 12 and 13, respectively. Results: The detection limits for the two most common codon 61 mutations found in malignant melanoma, which code for Arg and Lys, were 30% and 15%, respectively. To evaluate the pyrosequencing method on clinical samples, we performed a parallel analysis of 82 melanoma metastases using SSCP analysis and pyrosequencing. All mutations detected by SSCP analysis and confirmed by sequencing were also correctly identified by pyrosequencing. Codon 61 mutations were identified in 26 of the 82 samples (32%), whereas no mutations were found in codons 12 and 13. Four types of codon 61 mutations, Arg (17%), Lys (10%), Leu (4%), and His (1%), were identified. Conclusion: Pyrosequencing is an attractive alternative to SSCP analysis for N-ras mutation detection in malignant melanoma tumor samples because it displays the same sensitivity and accuracy as SSCP analysis and is simple and rapid.


http://www.clinchem.org/cgi/content/abstract/49/12/1981

Background: Hereditary hemochromatosis is a recessive disorder characterized by iron accumulation in parenchymal cells, followed by organ damage and failure. The disorder is mainly attributable to the C282Y and H63D mutations in the HFE gene, but additional mutations in the HFE, transferrin receptor 2 (TfR2), and hepcidin genes have been reported. The copresence of mutations in different genes may explain the phenotypic heterogeneity of the disorder and its
variable penetrance. Methods: We used denaturing HPLC (DHPLC) for rapid DNA scanning of the HFE (exons 2, 3, and 4), hepcidin, and TfR2 (exons 2, 4 and 6) genes in a cohort of 657 individuals with altered indicators of iron status. Results: DHPLC identification of C282Y and H63D HFE alleles was in perfect agreement with the restriction endonuclease assay. Fourteen DNA samples were heterozygous for the HFE S65C mutation. In addition, we found novel mutations: two in HFE (R66C in exon 2 and R224G in exon 4), one in the hepcidin gene (G71D), and one in TfR2 (V22I), plus several intronic or silent substitutions. Six of the seven individuals with hepcidin or TfR2 coding mutations carried also HFE C282Y or S65C mutations. Conclusion: DHPLC is an efficient method for mutational screening for the genes involved in hereditary hemochromatosis and for the study of their copresence.

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http://www.clinchem.org/cgi/content/abstract/49/2/209

Background: \(\\beta\)-Thalassemia is endemic to many regions in Southeast Asia and India, and <20 \(\\beta\)-globin gene mutations account for \([\geq]\)90% of \(\\beta\)-thalassemia alleles in these places. We describe a multiplex minisequencing assay to detect these common mutations.

Methods: Gap-PCR was used to simultaneously amplify the \(\\beta\)-globin gene from genomic DNA and to detect the \(\Delta619\)bp deletion mutation. Multiplex minisequencing was then performed on the amplified \(\\beta\)-globin fragment to detect an additional 15 common Southeast Asian and Indian \(\\beta\)-thalassemia mutations. Site-specific primers of different lengths were subjected to multiple rounds of annealing and single-nucleotide extension in the presence of thermostable DNA polymerase and the four dideoxynucleotides, each labeled with a different fluorophore. Minisequencing products were separated and detected by capillary electrophoresis, followed by automated genotyping. The optimized assay was subjected to a double-blind validation analysis of 89 \(\\beta\)-thalassemia and wild-type DNA samples of known genotype.

Results: Homozygous wild-type or mutant DNA samples produced electropherograms containing only a single colored peak for each mutation site, whereas samples heterozygous for a specific mutation displayed two different-colored peaks for that mutation site. Samples were automatically genotyped based on color and position of primer peaks in the electropherogram. In the double-blind validation analysis, all 89 DNA samples were genotyped correctly (100% assay specificity).

Conclusions: The described semiautomated multiplex minisequencing assay can detect the most common Southeast Asian and Indian \(\\beta\)-thalassemia mutations, is amenable to high-throughput scale up, and may bring population-based screening of \(\\beta\)-thalassemia in endemic regions a step closer to implementation.

http://www.clinchem.org/cgi/content/abstract/50/2/313

Background: Scanning for mutations in BRCA1 and BRCA2 in a large number of samples is hampered by the large sizes of these genes and the scattering of mutations throughout their coding sequences. Automated capillary electrophoresis has been shown to be a powerful system to detect mutations by either single-strand conformation polymorphism or heteroduplex analysis (HA). Methods: We investigated the adaptation of gel-based HA of BRCA1 and BRCA2 to a fluorescent multipillar platform to increase the throughput of this technique. We combined multiplex PCR, three different fluorescent labels, and HA in a 16-capillary DNA sequencer and tested 57 DNA sequence variants (11 insertions/deletions and 46 single-nucleotide changes) of BRCA1 and BRCA2. Results: We detected all 57 DNA changes in a blinded assay, and 2 additional single-nucleotide substitutions (1186 A>G of BRCA1 and 3624 A>G of BRCA2), previously unresolved by conformation-sensitive gel electrophoresis. Furthermore, different DNA changes in the same PCR fragment could be distinguished by their peak patterns. Conclusions: Capillary-based HA is a fast, efficient, and sensitive method that considerably reduces the amount of "hands-on" time for each sample. By this approach, the entire coding regions of BRCA1 and BRCA2 from two breast cancer patients can be scanned in a single run of 90 min.

http://www.clinchem.org/cgi/content/abstract/48/1/35

Background: Fetal DNA has been detected in maternal plasma by the use of genetic differences between mother and fetus. We explore the possibility of using epigenetic markers for the specific detection of fetal DNA in maternal plasma. Methods: A differentially methylated region in the human IGF2-H19 locus and a single-nucleotide polymorphism in this region were chosen for the study. The methylation status in this region is maintained in such a way that the paternal allele is methylated and the maternal allele is unmethylated. The single-nucleotide polymorphism was typed by direct sequencing of PCR products. The methylation status of this region was ascertained by bisulfite conversion and methylation-specific PCR. Differentially methylated fetal alleles were detected in maternal plasma by direct sequencing and a primer-extension assay. Results: Women in the second (n = 21; 17-21 weeks) and third (n = 18; 37-42 weeks) trimesters of pregnancy were recruited. Among these 39 volunteers, the 16 who were heterozygous for the single-nucleotide polymorphism were chosen for further analysis. In 11 of these 16 cases, paternally inherited methylated fetal alleles were different from the methylated alleles of the respective mothers. Using direct sequencing, we detected paternally inherited methylated fetal DNA in 6 of 11 (55%) cases. In 8 of the 16 heterozygous cases, the fetuses possessed an unmethylated maternally inherited allele that was different from the unmethylated allele of the mother. Using a primer-extension assay, we detected fetal-derived maternally inherited alleles in maternal plasma of four of eight (50%) cases. Conclusions: These results represent the first use of fetal epigenetic markers in noninvasive prenatal analysis. These data may also have implications for the investigation of other types of chimerism.


http://www.clinchem.org/cgi/content/abstract/49/1/104

Background: Sensitive monitoring of minimal residual disease may improve the treatment of neuroblastoma in children. To detect and monitor neuroblastoma cells in blood and bone marrow, we developed a quantitative method for the analysis of tyrosine hydroxylase mRNA. Methods: We used real-time reverse transcription-PCR. The calibrator was constructed from a segment of tyrosine hydroxylase mRNA that included the target. Blood and bone marrow samples from 24 children with neuroblastoma and 1 child with ganglioneuroma were analyzed. Controls were blood samples from the cords of 40 babies, from 58 children 6 months to 15 years of age, and from 34 healthy adults, as well as from 12 children with other diseases. Results: The detection limit was [~]70 transcripts/mL. All 144 blood controls were below this limit. At diagnosis, blood tyrosine hydroxylase mRNA was higher in children with widespread disease (stage 4/4S; n = 6; range, 203-46 000 transcripts/mL) than in patients with localized disease (stages 1-3; n = 6; [\&lt;]=83 transcripts/mL; P = 0.002). Bone marrow from all five children with localized disease had concentrations <72 transcripts/mL, whereas five of six stage 4 patients had increased concentrations (6000-8 000 000 transcripts/mL; P <0.05). In nine children in whom tyrosine hydroxylase mRNA was measured repeatedly, the results corresponded to the clinical course. Conclusion: Quantitative analysis of tyrosine hydroxylase mRNA in blood and bone marrow is reliable and easy to perform and may be used for upfront staging, prognostic assessment, and treatment monitoring of neuroblastoma.

Background: The discovery of fetal DNA in maternal plasma has opened up an approach for noninvasive prenatal diagnosis. Despite the rapid expansion in clinical applications, the molecular characteristics of plasma DNA in pregnant women remain unclear. Methods: We investigated the size distribution of plasma DNA in 34 nonpregnant women and 31 pregnant women, using a panel of quantitative PCR assays with different amplicon sizes targeting the leptin gene. We also determined the size distribution of fetal DNA in maternal plasma by targeting the SRY gene.

Results: The median percentages of plasma DNA with size >201 bp were 57% and 14% for pregnant and nonpregnant women, respectively (P <0.001, Mann-Whitney test). The median percentages of fetal-derived DNA with sizes >193 bp and >313 bp were 20% and 0%, respectively, in maternal plasma. Conclusion: Plasma DNA molecules are mainly short DNA fragments. The DNA fragments in the plasma of pregnant women are significantly longer than those in the plasma of nonpregnant women, and the maternal-derived DNA molecules are longer than the fetal-derived ones.


Background: Interleukin 6 (IL-6) is a pleiotropic cytokine that plays an essential role in the pathogenesis of acute and chronic infections. As the role of the IL-6 G(-174)C polymorphism in determining serum concentrations of IL-6 is controversial, we studied the genotype-specific IL-6 response in a well-standardized model of systemic inflammation. Methods: A total of 76 healthy young males (age range, 19-35 years) received a single bolus of 2 ng/kg endotoxin [lipopolysaccharide (LPS)] intravenously. Plasma IL-6 was measured by enzyme immunoassay at 0, 2, 6, and 24 h after LPS infusion, and the IL-6 promoter genotype was analyzed by a mutagenic separated PCR assay. Results: IL-6 increased 300-fold 2 h after LPS challenge and returned almost to normal within 24 h. Neither basal IL-6 nor the IL-6 response to LPS was significantly affected by the IL-6 promoter genotype. Conclusions: The IL-6 G(-174)C promoter polymorphism does not significantly influence basal concentrations of IL-6 or peak IL-6 in human endotoxemia.

DNA samples previously genotyped by other methods, with complete concordance of results. Alternative multiplexed formats were explored: the combination of multiplex PCR with multiple addressing and/or hybridization allowed analysis of all nine mutations in the same sample on one test site of the chip. Conclusions: The open flexible platform can be designed by the user according to the local prevalence of mutations in each geographic area and can be rapidly extended to include the remaining mutations causing {beta}-thalassemia in other regions of the world.


http://www.clinchem.org/cgi/content/abstract/50/1/141

Background: To optimize immunosuppressive treatment in individual transplant patients, functional measurements of the effects of tacrolimus (FK 506) are of clinical importance. Previous investigations have demonstrated the occurrence of tacrolimus-resistant production of interleukin-2 (IL-2) in vitro, which may explain in part why rejection episodes are still a frequent problem despite attainment of therapeutic blood concentrations and HLA matching. However, an adequate surrogate marker to define the tacrolimus response in individual patients has not been established. Methods: We investigated the immunosuppressive effects of tacrolimus on anti-CD3/anti-CD28 T-cell costimulation in a human whole-blood assay, analyzing T-cell proliferation, activation marker expression (CD25, CD69), IL-2 protein expression, and cytokine mRNA expression in vitro (n = 11 healthy individuals). We also quantified IL-2 mRNA expression in patients undergoing tacrolimus (n = 4) or cyclosporin A (CsA; n = 4) monotherapy before ex vivo living-donor kidney transplantation. Results: T-cell proliferation; CD25, CD69, and IL-2 concentrations; and IL-4 mRNA were significantly decreased in vitro. In contrast, cytokine mRNA profiles revealed variable tacrolimus sensitivity. Whole-blood samples from 3 of 11 healthy individuals demonstrated marked suppression of IL-2 mRNA expression (>50%) when tacrolimus was administered in vitro. When CsA was added to whole-blood cultures, the influence on IL-2 mRNA expression was comparable to that of tacrolimus in 9 of 11 individuals. Two individuals responded conversely, indicating that differences in the in vitro response to tacrolimus and CsA among individuals may be attributable to potential heterogeneity in the involvement of the CD28 pathway. Kinetic profiles of IL-2 mRNA expression also revealed individually distinct degrees of calcineurin inhibitor sensitivity in patients undergoing tacrolimus or CsA monotherapy before living-donor kidney transplantation. Conclusions: Our results suggest an individual degree of calcineurin inhibitor sensitivity of activated whole-blood lymphocytes based on IL-2 mRNA expression. Our approach is potentially valuable for identifying transplant patients in whom IL-2 mRNA expression is unaffected or even enhanced after initiation of immunosuppressive therapy. Such individuals may be less sensitive to the immunosuppressive agent and therefore at increased risk of transplant rejection. Prospective studies are necessary to determine the correlation of IL-2 mRNA expression with the clinical risk of transplant rejection.


http://www.clinchem.org/cgi/content/abstract/50/1/67

Background: A novel coronavirus (CoV) was recently identified as the agent for severe acute respiratory syndrome (SARS). We compared the abilities of conventional and real-time reverse transcription-PCR (RT-PCR) assays to detect SARS CoV in clinical specimens. Methods: RNA
samples isolated from nasopharyngeal aspirate (NPA; n = 170) and stool (n = 44) were reverse-
transcribed and tested by our in-house conventional RT-PCR assay. We selected 98 NPA and 37
stool samples collected at different times after the onset of disease and tested them in a real-time
quantitative RT-PCR specific for the open reading frame (ORF) 1b region of SARS CoV.
Detection rates for the conventional and real-time quantitative RT-PCR assays were compared.
To investigate the nature of viral RNA molecules in these clinical samples, we determined copy
numbers of ORF 1b and nucleocapsid (N) gene sequences of SARS CoV. Results: The
quantitative real-time RT-PCR assay was more sensitive than the conventional RT-PCR assay for
detecting SARS CoV in samples collected early in the course of the disease. Real-time assays
targeted at the ORF 1b region and the N gene revealed that copy numbers of ORF 1b and N
gene sequences in clinical samples were similar. Conclusions: NPA and stool samples can be
used for early diagnosis of SARS. The real-time quantitative RT-PCR assay for SARS CoV is
potentially useful for early detection of SARS CoV. Our results suggest that genomic RNA is the
predominant viral RNA species in clinical samples.

Simultaneously Detect Eleven BRCA1 Mutations In Breast Cancer Families." Clin. Chem. 50(1):
203-206.

http://www.clinchem.org

Vaginal Bleeding (Threatened Abortion <20 Weeks) and a Surviving Fetus." Clin. Chem. 51(1):
224-227.

http://www.clinchem.org


http://www.clinchem.org/cgi/content/abstract/51/1/113

Background: Increased plasma DNA has been found in cancer patients and may have potential
as a tumor marker. The objectives of this study were to develop a controlled, quantitative PCR
(QPCR) assay to measure plasma DNA and then evaluate plasma DNA concentrations as a
tumor marker in patients with thoracic malignancies. Methods: We developed a QPCR assay for
DNA, using the human (beta)-actin gene. Plasma samples were analyzed from 58 patients with
esophageal cancer (EC; 20 banked samples and 38 prospectively collected samples) and 25
patients with lung cancer (LC; all prospectively collected). Control groups consisting of 51
patients with gastroesophageal reflux disease (GERD; 23 banked samples and 28 prospectively
collected) and 11 healthy volunteers were also analyzed. Results: The assay had an
experimental variability <4%. In our banked samples, the mean concentration of plasma DNA in
EC was 819.0 {micro}g/L (range, 46.2-4738.0 {micro}g/L) vs 432.0 {micro}g/L (6.0-2888.0
{micro}g/L) in GERD (P = 0.02). However, the prospectively collected samples had lower DNA
concentrations, and there was no difference between cancer patients and controls. The mean
DNA concentration was 10.6 {micro}g/L (range, 7.0-14.0 {micro}g/L) in healthy volunteers and
10.5 {micro}g/L (range, 4.0-23.5 {micro}g/L) in GERD controls vs 13.0 {micro}g/L (range, 4.5-46.5
{micro}g/L) in EC and 14.6 {micro}g/L (range, 3.0-30.0 {micro}g/L) in LC. Conclusions: Our data
indicate that plasma DNA concentrations are of limited diagnostic value when samples are
prospectively collected and uniformly handled. This is in contrast to previously published results. Qualitative analysis of DNA may be needed if plasma nucleic acids are to be used as a diagnostic tool in cancer screening.


http://www.clinchem.org/cgi/content/abstract/51/1/35

Background: Despite considerable advances, DNA sequencing has remained somewhat time-consuming and expensive, requiring three separate steps to generate sequencing products from a template: amplification of the target sequence; purification of the amplified product; and a sequencing reaction. Our aim was to develop a method to routinely combine PCR amplification and cycle sequencing into one single reaction, enabling direct sequencing of genomic DNA. Methods: Combined amplification and sequencing reactions were set up with Big DyeTM sequencing reagents (Applied Biosystems) supplemented with variable amounts of forward and reverse primers, deoxynucleotide triphosphates (dNTPs), and input DNA. Reactions were thermal-cycled for 35 or 45 cycles. Products were analyzed by capillary electrophoresis to detect sequencing products. Results: Reactions using two oligonucleotide primers at a ratio of 5:1 (500 nM primer 1 and 100 nM primer 2), 125 (micro)M supplemental dNTPs, and 35-45 thermal cycles optimally supported combined amplification and cycle sequencing reactions. Our results suggest that these reactions are dominated by PCR during early cycles and convert to cycle sequencing in later cycles. We used this technique for a variety of sequencing applications, including the identification of germline mutations/polymorphisms in the Factor V and BRCA2 genes, sequencing of tumor DNA to identify somatic mutations in the DPC4/SMADH4 and FLT3 genes, and sequencing of 16S ribosomal DNA for bacterial speciation. Conclusions: PCR amplification and cycle sequencing can be combined into a single reaction using the conditions described. This technique allows direct sequencing of genomic DNA, decreasing the cost and labor involved in gene sequencing.


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http://www.clinchem.org/cgi/content/abstract/48/7/989

Background: NO synthesized from L-arginine by the constitutive endothelial NO synthase (eNOS) plays a key role in the atherosclerotic process. We investigated whether common variants in the NOS3 gene (a T786C mutation in the 5' flanking region and the polymorphism on exon 7 that produced the Glu298Arg polymorphism in the protein) are associated with an increased risk of moderate to severe internal carotid artery (ICA) stenosis. Methods: We studied 88 patients consecutively operated for ICA stenosis and 133 healthy controls. A T786C mutation in the 5' flanking region and the polymorphism in exon 7 that produces the Glu298Asp polymorphism in the protein were explored by PCR and fluorescent probe analysis. Results: Genotype distribution
was significantly different between patients and controls only for T786C, the CC genotype frequency being 26% and 13%, respectively [odds ratio (OR), 2.26; 95% confidence interval (CI), 1.14-4.46; P = 0.018]. Moreover, the CC genotype was significantly more frequent in a subgroup of patients with ulcerative plaques compared with patients with nonulcerative lesions (44% vs 17%; OR, 3.82; 95% CI, 1.79-8.14; P = 0.003). Multiple logistic regression analysis using the most frequent risk factors and the eNOS gene variant showed that the CC genotype is an independent risk factor for ICA stenosis (P = 0.023). Conclusion: C allele homozygosity in position 786 of the eNOS promoter seems to be an independent risk factor for the development of moderate to severe ICA stenosis, especially ulcerative lesions.


http://www.clinchem.org/cgi/content/abstract/48/7/983

Background: Cytochrome P450-dependent monooxygenase 2D6 (CYP2D6) activity can be estimated by investigating the metabolism of model drugs or by genotyping the most common CYP2D6 alleles. For Caucasians, the CYP2D6 allele frequencies are well investigated, and single-step assays are available for genotyping, whereas allele analysis in mainland Chinese is limited. Methods: Two tetra-primer assays and one allele-specific amplification assay were developed to easily genotype the CYP2D6 alleles *8, *10, and *14 previously detected in Asians. Applying these assays in combination with established single-tube assays, we analyzed 223 DNA samples from Chinese volunteers for the CYP2D6 alleles *3, *4, *5, *6, *8, *10, and *14 and for duplication of CYP2D6. Results: Six different alleles were detected in mainland Chinese. The most frequent mutant allele was the intermediate metabolizer allele, CYP2D6*10, with a prevalence of 51.3%, followed by the poor metabolizer alleles CYP2D6*5 (7.2%) and a novel variant of CYP2D6*14. This novel *14B allele (2.0%) differs from the *14 allele by the absence of the C188T substitution and by the additional G1749C substitution. Furthermore, six duplication alleles of CYP2D6 were detected, including one duplication of the *10 allele (*10X2). Conclusions: The CYP2D6 allele frequencies in mainland Chinese shows some genetic diversity compared with Chinese from other regions: a novel *14B allele, a slightly higher frequency of the *5 allele, and a slightly lower frequency of the *10 allele than in most other Chinese populations.


http://www.clinchem.org/cgi/content/abstract/49/7/1066

Background: Characterization of fusion gene transcripts in leukemia that result from chromosome translocations provides valuable information regarding appropriate treatment and prognosis. However, screening for multiple fusion gene transcripts is difficult with conventional PCR and state-of-the-art real-time PCR and high-density microarrays. Methods: We developed a multiplex reverse transcription-PCR (RT-PCR) assay for screening and quantification of fusion gene transcripts in human leukemia cells. Chimeric primers were used that contained gene-specific and universal sequences. PCR amplification of fusion and control gene transcripts was achieved with use of an excess of universal primers to allow the ratio of abundance of fusion gene to endogenous or exogenous controls to be maintained throughout PCR. Multiplex RT-PCR products analyzed by an ABI 310 Genetic Analyzer were consistent with those of duplex RT-PCR (single analytical sample plus control). In addition, multiplex RT-PCR results were analyzed by an assay using an oligonucleotide microarray that contained probes for the splice-junction sequences of various fusion transcripts. Results: The multiplex RT-PCR assay enabled screening of >10 different fusion gene transcripts in a single reaction. RT-PCR followed by analysis with the
ABI Prism 310 Genetic Analyzer consistently detected 1 fusion-transcript-carrying leukemia cell in 100-10,000 cells. The assay covered a 1000-fold range. Preliminary results indicate that multiplex RT-PCR products can also be analyzed by hybridization-based microarray assay. Conclusions: The multiplex RT-PCR analyzed by either ABI Prism 310 Genetic Analyzer or microarray provides a sensitive and specific assay for screening of multiple fusion transcripts in leukemia, with the latter an assay that is adaptable to a high-throughput system for clinical screening.

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http://www.clinchem.org/cgi/content/abstract/48/6/818

Background: Congenital adrenal hyperplasia (CAH) is a frequent autosomal recessive disease, with a wide range of clinical manifestations, most commonly attributable to mutations in the 21-hydroxylase gene (CYP21). Large gene deletions, large gene conversions, a small 8-basepair deletion, and eight point mutations in CYP21 account for ~95% of all enzyme deficiencies. We developed a new strategy for a rapid CYP21 analysis. Methods: DNA samples from 40 CAH patients previously genotyped by direct DNA sequencing were reanalyzed by allele-specific amplification of the functional CYP21 gene followed by a multiplex minisequencing reaction using 13 primers. In addition, a second PCR that amplified a part of exon 3 was used to demonstrate the presence or absence of at least one functional gene. Results: The assay detected the P453S mutation and nine of the most common mutations (P30L, intron 2 splice, (Delta)8bp, I172N, exon 6 cluster, V281L, F306+t, Q318X, and R356W) caused by microconversions from the CYP21P pseudogene. The concordance was 100% for detecting these mutations, including gene deletions and large gene conversions. The 40 patient DNA samples were analyzed in 1.5 working days by one technician (actual hands-on time, 3.5 h). The material cost for analyzing one sample was approximately 10.00 (US $9.00). Conclusions: This novel mutation screening strategy rapidly detects 90-95% of all mutations associated with CAH and appears applicable as a tool for confirmation of increased 17-hydroxyprogesterone found in neonatal CAH screening.

http://www.clinchem.org/cgi/content/abstract/50/6/1002

Background: Analysis of fetal DNA in maternal plasma has recently been introduced as a new method for noninvasive prenatal diagnosis, particularly for the analysis of fetal genetic traits,
which are absent from the maternal genome, e.g., RHD or Y-chromosome-specific sequences. To date, the analysis of other fetal genetic traits has been more problematic because of the overwhelming presence of maternal DNA sequences in the circulation. We examined whether different biochemical properties can be discerned between fetal and maternal circulatory DNA. Methods: Plasma DNA was examined by agarose gel electrophoresis. The fractions of fetal and maternal DNA in size-fractionated fragments were assayed by real-time PCR. The determination of paternally and maternally inherited fetal genetic traits was examined by use of highly polymorphic chromosome-21-specific microsatellite markers. Results: Size fractionation of circulatory DNA indicated that the major portion of cell-free fetal DNA had an approximate molecular size of <0.3 kb, whereas maternally derived sequences were, on average, considerably larger than 1 kb. Analysis of size-fractionated DNA ([<=]0.3 kb) from maternal plasma samples facilitated the ready detection of paternally and maternally inherited microsatellite markers. Conclusions: Circulatory fetal DNA can be enriched by size selection of fragment sizes less than [-]0.3kb. Such selection permits easier analysis of both paternally and maternally inherited DNA polymorphisms.


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http://www.clinchem.org/cgi/content/abstract/49/3/415

Background: Optical trapping has traditionally been used to visually select and isolate nonadherent cells grown in suspension because cells grown in monolayers will rapidly reattach to surfaces if suspended in solution. We explored methods to slow cell reattachment that are also compatible with high-fidelity PCR. Methods: Using HeLa cells grown on plates and suspended after trypsinization, we measured the efficiency of capture by retention and movement of the cell by the laser. Success for removing a captured cell by pipette was determined by PCR amplification of the 5S rRNA gene. After optimizing PCR amplification of a 2049-bp region of the p53 gene, we determined PCR fidelity by DNA sequencing. Results: Addition of bovine serum albumin to suspended cells slowed reattachment from seconds to minutes and allowed efficient trapping. The success rate of removing a cell from the trap by pipette to a PCR tube was 91.5%. The 5S PCR assay also revealed that DNA and RNA that copurify with polymerases could give false-positive results. Sequence analysis of four clones derived from a single cell showed only three polymerase errors in 7200 bp of sequence read and revealed difficulties in reading the correct number in a run of 16 A:T. Comparison of the HeLa and wild-type human sequences revealed several previously unreported base differences and an (A:T)n length polymorphism in p53 introns. Conclusions: These results represent the first use of optical trapping on adherent
cells and demonstrate the high accuracy of DNA sequencing that can be achieved from a single cell.


http://www.clinchem.org/cgi/content/abstract/49/3/407

Background: With the invention of the DNA chip, genome-wide analysis is now a reality. Unfortunately, solid-phase detection systems such as the DNA chip suffer from a narrow range in quantification and sensitivity. Today the best methodology for sensitive, wide dynamic range quantification and genotyping of nucleic acids is real-time PCR. However, multiplexed real-time PCR technologies require complicated and costly design and manufacturing of separate detection probes for each new target. Methods: We developed a novel real-time PCR technology that uses universal energy transfer probes constructed from An Expanded Genetic Information System (AEGIS) for both quantification and genotyping analyses. Results: RNA quantification by reverse transcription-PCR was linear over four orders of magnitude for the simultaneous analysis of (beta)-actin messenger RNA and 18S ribosomal RNA. A single trial validation study of 176 previously genotyped clinical specimens was performed by endpoint analysis for factor V Leiden and prothrombin 20210A mutation detection. There was concordance for 173 samples between the genotyping results from Invader(R) tests and the AEGIS universal energy transfer probe system for both factor V Leiden and prothrombin G20210A. Two prothrombin and one factor V sample gave indeterminate results (no calls). Conclusion: The AEGIS universal probe system allows for rapid development of PCR assays for nucleic acid quantification and genotyping.


http://www.clinchem.org/cgi/content/abstract/50/3/500

Background: Routine tissue processing has generated banks of paraffin-embedded tissue that could be used in retrospective cohort studies to study the molecular changes that occur during cancer development. The purpose of this study was to determine whether a p53 microarray could be used to sequence the p53 gene in DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissues. Methods: DNA was extracted from 70 FFPE breast cancer tissue specimens. p53 was sequenced with an oligonucleotide microarray (p53 GeneChip(R); Affymetrix), and the results were compared with the results obtained from direct sequencing. Results: DNA was extracted from 62 of 70 cases. We identified 26 mutations in 24 of the 62 cases by the p53 GeneChip. No polymorphisms were detected, and exon 4 could not be evaluated in 20 cases. There were 43 genetic alterations detected by direct sequencing in 35 of the 62 cases. These consisted of 26 polymorphisms and 17 mutations in exons or splice sites. Fifteen mutations were identified by both methods. Direct sequencing detected significantly more gene alterations (43 of 54) in DNA extracted from FFPE tissue than the p53 GeneChip (26 of 54; P = 0.018). However, if the changes in exon 4 were eliminated from this comparison, the p53 GeneChip detected 26 of 27 mutations compared with direct sequencing, which identified 16 of 27 mutations. (P = 0.016). Conclusions: A combination of oligonucleotide microarray and direct sequencing may be necessary to accurately identify p53 gene alterations in FFPE breast cancer. The p53 GeneChip cannot be used to detect exon 4 polymorphisms (codon 72) in FFPE breast cancer tissue.
Background: Several recent reports have described the detection of circulating, cancer-related RNA molecules in serum or plasma from cancer patients, but little is known about the biology of this extracellular RNA. We aimed to determine how RNA is protected against degradation in serum, to optimize RNA isolation from large volumes of serum, and to test our optimized assays for serum-based cancer detection. Methods: We used quantitative reverse transcription-PCR (QRT-PCR) analysis to investigate the isolation and biology of extracellular plasma RNA. We then examined the presence of amplifiable RNA transcripts in plasma and serum from controls and from patients with esophageal cancer and malignant melanoma. Results: We found that extracellular RNA in plasma is highly degraded and can be isolated most efficiently by guanidinium-phenol extraction followed by precipitation. Extracellular RNA is stable in serum for up to 3 h but is destroyed immediately by addition of detergents. Extracellular RNA can be captured on 0.2 {micro}m filters, allowing concentration of RNA from several milliliters of plasma. When we concentrated RNA from up to 4 mL of serum, detection of cancer-related transcripts in serum from cancer patients and controls was infrequent and inconsistent. Conclusions: Extracellular RNA is most likely protected within protein or lipid vesicles, possibly apoptotic bodies, which can be disrupted by detergents. Despite optimizing many aspects of plasma RNA detection, we were unable to reproducibly detect cancer-related transcripts. Our data suggest that measurement of circulating RNA may not be a good approach to early cancer diagnosis.

Background: Multiple endocrine neoplasia type 2 (MEN2) is a cancer syndrome with well-characterized causative mutations. Missense mutations in [~]15 codons of the RET gene have been linked to disease phenotypes in the vast majority of cases. These missense mutations range from very simple single nucleotide base changes to more numerous changes at a given codon; they therefore are often tested for by more than one DNA-based diagnostic method. We developed and evaluated a PyrosequencingTM technology-based approach for MEN2 mutation testing that allows both simple and complex mutations to be analyzed on one platform. Methods: Archived DNA from peripheral blood of patients referred to the Mayo Clinic Molecular Genetics laboratory for MEN2 testing was selected. One to all of codons 609, 611, 618, 620, 630, 634, 768, 804, and 918 were analyzed by Pyrosequencing technology to match the original analysis of each patient. Template PCRs were set up using an automated liquid handler; the subsequent post-PCR preparation step was performed manually, and the sequencing was performed by a PSQ 96 instrument. Samples were tested in batch sizes expected to occur routinely. Results: We analyzed samples from 217 patients who previously tested negative for MEN2 and 230 patients who previously tested positive, for a total of 1449 sequencing reactions. One discrepant result was found (100% concordant for negatives and 99.6% concordant for positives). A total of 37 unique mutations or alterations of unknown significance were analyzed. Conclusion: Pyrosequencing technology offers an accurate, nonisotopic, simple, and rapid method for the analysis of DNA from patients suspected of having MEN2.
Background: Recent studies have demonstrated the existence of circulating mitochondrial DNA in plasma and serum, but the concentrations and physical characteristics of circulating mitochondrial DNA are unknown. The aim of this study was to develop an assay to quantify mitochondrial DNA in the plasma of healthy individuals. Methods: We adopted a real-time quantitative PCR approach and evaluated the specificity of the assay for detecting mitochondrial DNA with a cell line (rho0) devoid of mitochondria. The concentrations and physical characteristics of circulating mitochondrial DNA were investigated by experiments conducted in three modules. In module 1, we evaluated the concentrations of mitochondrial DNA in plasma aliquots derived from four blood-processing protocols. In module 2, we investigated the existence of both particle-associated and free forms of mitochondrial DNA in plasma by subjecting plasma to filtration and ultracentrifugation. In module 3, we used filters with different pore sizes to investigate the size characteristics of the particle-associated fraction of circulating mitochondrial DNA. Results: The mitochondrial DNA-specific, real-time quantitative PCR had a dynamic range of five orders of magnitude and a sensitivity that enabled detection of one copy of mitochondrial DNA in plasma. In module 1, we found significant differences in the amounts of circulating mitochondrial DNA among plasma aliquots processed by different methods. Data from module 2 revealed that a significant fraction of mitochondrial DNA in plasma was filterable or pelletable by ultracentrifugation. Module 3 demonstrated that filters with different pore sizes removed mitochondrial DNA from plasma to different degrees. Conclusions: Both particle-associated and free mitochondrial DNA are present in plasma, and their respective concentrations are affected by the process used to harvest plasma from whole blood. These results may have implications in the design of future studies on circulating mitochondrial DNA measured in different disease conditions.


Background: Genetic risk factors associated with venous thrombosis include mutations in the factor V (Leiden), factor II (prothrombin), and methylenetetrahydrofolate reductase (MTHFR) genes. We evaluated a method using electronically addressable microarrays for the detection of mutations in these genes that have been associated with vascular disease. Methods: The NanoChip(R) Molecular Biology Workstation (Nanogen) uses electronic microarrays for mutation detection. Factor V, factor II, and MTHFR genotypes identified in the NanoChip system on 225 samples were compared with genotypes from LightCycler(R) assays (Roche). We determined within- and between-cartridge signal and ratio variation and analyzed the effect of additional mutations at or near the detection area used for the NanoChip assays. Results: Genotypes determined for all three mutations on the NanoChip platform were in complete concordance with LightCycler results. Within-cartridge signal variation as measured by the CV of fluorescence signals was <10% for each allele when present. The within-cartridge CV for heterozygous mutant/wild-type ratios was <8.5%, and between-cartridge CV was <18%. A dilution study showed that results could be obtained in this assay with 6 ng of nucleic acid per PCR, the lowest input tested. The presence of additional sequence variations near the expected mutations can produce equivocal or discrepant results. Conclusions: Mutation detection using the NanoChip Molecular Biology Workstation was accurate and reproducible for the three assays evaluated.

Background: Malignant hyperthermia (MH) is a fatal autosomal dominant pharmacogenetic disorder characterized by skeletal muscle hypertonicity that causes a sudden increase in body temperature after exposure to common anesthetic agents. The disease is genetically heterogeneous, with mutations in the gene encoding the skeletal muscle ryanodine receptor (RYR1) at 19q13.1 accounting for up to 80% of the cases. To date, at least 42 RYR1 mutations have been described that cause MH and/or central core disease. Because the RYR1 gene is huge, containing 106 exons, molecular tests have focused on the regions that are more frequently mutated. Thus the causative defect has been identified in only a fraction of families as linked to chromosome 19q, whereas in others it remains undetected. Methods: We used denaturing HPLC (DHPLC) to analyze the RYR1 gene. We set up conditions to scan the 27 exons to identify both known and unknown mutations in critical regions of the protein. For each exon, we analyzed members from 52 families with positive in vitro contracture test results, but without preliminary selection by linkage analysis. Results: We identified seven different mutations in 11 MH families. Among them, three were novel MH alleles: Arg44Cys, Arg533Cys, and Val2117Leu. Conclusion: Because of its sensitivity and speed, DHPLC could be the method of choice for the detection of unknown mutations in the RYR1 gene.


http://www.clinchem.org/cgi/content/abstract/48/11/1873

Background: Electronic microarrays comprise independent microelectrode test sites that can be electronically biased positive or negative, or left neutral, to move and concentrate charged molecules such as DNA and RNA to one or more test sites. We developed a protocol for multiplexed gene expression profiling of mRNA targets that uses electronic field-facilitated hybridization on electronic microarrays. Methods: A multiplexed, T7 RNA polymerase-mediated amplification method was used for expression profiling of target mRNAs from total cellular RNA; targets were detected by hybridization to sequence-specific capture oligonucleotides on electronic microarrays. Activation of individual test sites on the electronic microarray was used to target hybridization to designated subsets of sites and allow comparisons of target concentrations in different samples. We used multiplexed amplification and electronic field-facilitated hybridization to analyze expression of a model set of 10 target genes in the U937 cell line during lipopolysaccharide-mediated differentiation. Performance of multiple genetic analyses (single-nucleotide polymorphism detection, gene expression profiling, and splicing isoform detection) on a single electronic microarray was demonstrated using the ApoE and ApoER2 genes as a model system. Results: Targets were detected after a 2-min hybridization reaction. With noncomplementary capture probes, no signal was detectable. Twofold changes in target concentration were detectable throughout the ([~]64-fold) range of concentrations tested. Levels of 10 targets were analyzed side by side across seven time points. By confining electronic activation to subsets of test sites, polymorphism detection, expression profiling, and splicing isoform analysis were performed on a single electronic microarray. Conclusions: Microelectronic array technology provides specific target detection and quantification with advantages over currently available methodologies for targeted gene expression profiling and combinatorial genomics testing.


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http://www.clinchem.org/cgi/content/abstract/48/10/1668

Background: Enzymes of the cytochrome P450 3A (CYP3A) family are responsible for the metabolism of >50% of currently prescribed drugs. CYP3A5 is expressed in a limited number of individuals. The absence of CYP3A5 expression in [~]70% of Caucasians was recently correlated to a genetic polymorphism (CYP3A5*3). Because CYP3A5 may represent up to 50% of total CYP3A protein in individuals polymorphically expressing CYP3A5, it may have a major role in variation of CYP3A-mediated drug metabolism. Using sequencing, have been identified (Hustert et al. Pharmacogenetics 2001;11:773-9; Kuehl et al. Nat Genet 2001;27:383-91) variant alleles *2 through *7 for CYP3A5. Detection of CYP3A5 variant alleles, and knowledge about their allelic frequency in specific ethnic groups, is important to establish the clinical relevance of screening for these polymorphisms to optimize pharmacotherapy. Methods: In a group of 500 healthy Dutch Caucasian blood donors, we determined the allelic frequency of the CYP3A5*2, *3, *4, *5, *6, and *7 alleles by use of newly developed PCR-restriction fragment length polymorphism assays.
Results: The frequency of the defective CYP3A5*3 allele in the Dutch Caucasian population was 91%, followed by the CYP3A5*2 (1%) and CYP3A5*6 (0.1%) alleles. The CYP3A5*4, *5, and *7 alleles were not detected. Conclusions: On the basis of its allelic frequency, screening for the CYP3A5*3 allele in the Caucasian population is extremely relevant. In addition, screening for the CYP3A5*2 allele may be taken into consideration in individuals heterozygous for the CYP3A5*3 allele. The CYP3A5*4, *5, *6, and *7 alleles have low allelic frequencies that do not support initial screening.


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http://www.clinchem.org/cgi/content/abstract/49/10/1642

Background: fms-related tyrosine kinase 3 (Flt3) is the most commonly mutated gene in human acute myeloid leukemia (AML) and has been implicated in its pathogenesis. Because screening of Flt3 in AML patients by PCR followed by gel electrophoresis is time-consuming and fails to detect some very small internal tandem duplications (ITDs), we developed a method for screening of FLT3 receptor mutations with PCR plus denaturing HPLC (D-HPLC). Methods: Total mRNAs extracted from 34 AML patients were first analyzed for the presence of juxtamembrane length mutations and tyrosine kinase domain point mutations by a conventional method involving PCR amplification, restriction enzyme digestion, and agarose gel electrophoresis (PCR-RED-AGE). Subsequently, the same patient panel was analyzed by D-HPLC, using specifically designed primers and optimized running temperatures for the length and point mutation analysis. Results: Thirty-four patients were analyzed by PCR-RED-AGE; 9 were positive for known Flt3 mutations: 6 of 34 (18%) for ITDs in exon 14 and 3 of 34 (9%) for point mutations in exon 20. The same patient panel was analyzed by D-HPLC, and additional nucleotide changes were discovered; in total, 14 sequence variations were identified: 7 of 34 (21%) for ITDs in exon 14; 2 of 34 (6%) for point mutations in exon 20; 1 of 34 (3%) for a new point mutation in exon 16; and 4 of 34 (12%) for polymorphisms in exons 13 and 14. Direct sequencing analysis identified nucleotide alterations in each of the "D-HPLC positives" but in none of the "D-HPLC negatives", yielding a specificity and sensitivity of 100% for D-HPLC-based screening. Conclusions: This novel D-HPLC-based procedure, which is optimized for identification of new point mutations in the catalytic and regulatory domains of FLT3 receptor, could potentially be useful for studies involving precise genotype determination, which could be critical for selection of innovative AML therapies targeting the FLT3 protein.


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http://www.clinchem.org/cgi/content/abstract/48/9/1406

Background: Methods for analysis of the single-nucleotide polymorphism (SNP) known as factor V Leiden (FVL) are described. The technique provides rapid, highly accurate detection of the point mutation that encodes for replacement of arginine-506 with glutamine. After formal assay qualification, 758 clinical samples that had previously been analyzed by the InvaderTM Monoplex Assay were tested as research samples in a commercial clinical laboratory. Methods: Primers specific for factor V (FV) were prepared, and PCR was performed. Samples were analyzed using the NanoChip(R) Molecular Biology Workstation with fluorescently labeled reporters for wild-type and SNP sequences. Results: Of the 635 samples classified by the Third WaveTM assay as FV wild type, 10 were identified as heterozygous FVL by the NanoChip technique. Similarly, of the 114 putative heterozygous samples, 4 were wild type, and of the 9 reported homozygous samples, 6 were homozygous, 2 were heterozygous, and 1 was FV wild type by the NanoChip assay. All 17 results that were discordant with the Third Wave analysis were confirmed by DNA sequencing to be correctly classified by the NanoChip technology. The Nanochip system was 100% accurate in characterizing wild-type, heterozygous, and homozygous samples compared with accuracies of 99.2%, 90.2%, and 100% for the comparable Third Wave analysis.

Conclusions: The NanoChip microelectronic chip array technology is an accurate and convenient method for FVL screening of research samples in a clinical laboratory environment.


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http://www.clinchem.org/cgi/content/abstract/48/9/1551

Background: 2',5'-Oligoadenylate synthetases (2-5AS) are type I interferon (IFN)-induced proteins with antiviral capacity. Three major forms of 2-5AS with distinct enzymatic activities have been described in IFN-treated human cells. We measured distinct forms of 2-5AS mRNA to analyze the relationship with its enzymatic activity and response to IFN therapy in chronic hepatitis C.

Methods: We established a method to quantify p40/p46 and p69/p71 forms of 2-5AS mRNA by use of reverse transcription followed by competitive PCR. The 2-5AS mRNA concentrations were measured in peripheral blood mononuclear cells from 40 patients with chronic hepatitis C and 28 control individuals.

Results: Reconstitution experiments and comparison with Northern blot analyses revealed that our method accurately and linearly quantified 2-5AS mRNA. 2-5AS mRNA
concentrations and 2-5AS enzymatic activity were correlated (P <0.03). Our data demonstrated a correlation in 2-5AS mRNA between p40/p46 and p69/p71 (P <0.02), indicating a similar regulation of the expression of these genes. Our data also demonstrated that pretreatment concentrations of 2-5AS mRNA correlated with responses to IFN therapy in chronic hepatitis C. Conclusions: Our method for measuring 2-5AS mRNA concentrations could provide an important marker for selecting patients for IFN therapy and may be useful for the development of more effective therapeutic strategies for chronic hepatitis C.


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http://www.clinchem.org/cgi/content/abstract/49/9/1458

Background: Inappropriate quality management of reverse transcription-PCR (RT-PCR) assays for the detection of blood-borne prostate cancer (PCa) cells hampers clinical conclusions. Improvement of the RT-PCR methodology for prostate-specific antigen (PSA) mRNA should focus on an appropriate numeric definition of the performance of the assay and correction for PSA mRNA that is not associated with PCa cells. Methods and Results: Repeated (RT-)PCR tests for PSA mRNA in single blood specimens from PCa patients and PCa-free controls, performed by four international institutions, showed a large percentage (approx 50%) of divergent test results. The best estimates of the mean, \( \lambda \) (SD), of the expected Poisson frequency distributions of the number of positive tests among five replicate assays of samples from PCa-free individuals were 1.0 (0.2) for 2 x 35 PCR cycles and 0.2 (0.1) for 2 x 25 PCR cycles. Assessment of the numeric value of the mean can be considered as a new indicator of the performance of a RT-PCR assay for PSA mRNA under clinical conditions. Moreover, it determines the required number of positive test repetitions to differentiate between true and false positives for circulating prostate cells. At a predefined diagnostic specificity of \[ IMG ] = "BORDER="0">98%, repeated PCRs with \( \lambda \) of either 1.0 or 0.2 require, respectively, more than three or more than one positive tests to support the conclusion that PSA mRNA-containing cells are present. Conclusions: Repeated nested PCR tests for PSA and appropriate handling of the data allow numeric quantification of the performance of the assay and differentiation between analytical false and true positives at a predefined accuracy. This new approach may contribute to introduction of PSA RT-PCR assays in clinical practice.


http://www.clinchem.org/cgi/content/abstract/50/9/1544

Background: Transthyretin-associated hereditary amyloidosis (ATTR) is an inherited disease in which variants in the primary structure of transthyretin (TTR; prealbumin) lead to the extracellular
polymerization of insoluble protein fibrils, causing organ failure and ultimately death when major organs are involved. We have developed an integrated approach to molecular diagnosis with initial analysis of intact plasma TTR by electrospray ionization mass spectrometry (MS) and referral of positive samples for DNA sequence analysis and real-time PCR to confirm the common Gly6Ser polymorphism. Methods: Samples from 6 patients previously diagnosed with ATTR and from 25 controls with (n = 15) or without (n = 10) polyneuropathy were analyzed in a blinded fashion for the presence of variant TTR. TTR protein was extracted with an immunoaffinity resin from 20 \( \mu \text{L} \) of archived plasma samples. The purified TTR was reduced with tris(2-carboxyethyl)phosphine and analyzed by MS. The appearance of two peaks (or a single peak shifted in mass indicative of a homozygous variant), including the wild-type mass of 13 761 Da, was indicative of the presence of a variant, and the individual was referred for DNA sequence analysis. Results: MS analysis of intact reduced TTR correctly identified each of six samples known to contain variant TTR. These results were corroborated by subsequent DNA sequence analysis. Additionally, all Gly6Ser polymorphisms were correctly called based on the +30 mass shift and an equal relative abundance of the +30 polymorphism relative to wild-type TTR. No false-positive results were seen. Conclusions: This referral method eliminates the necessity of sequencing most samples and allows screening for the familial forms of amyloidosis in a broad patient population in a timely fashion. This method correctly identified all previously known variants and also identified a novel variant, Val94Ala.


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