Validation and Clinical Application of a Locus-Specific Polymerase Chain Reaction- and Minisequencing-Based Assay for Congenital Adrenal Hyperplasia (21-Hydroxylase Deficiency)

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Congenital adrenal hyperplasia is an autosomal recessive disorder caused by defective adrenal steroid biosynthesis, resulting in reduced glucocorticoid and increased androgen production. The majority of cases are due to inactivation of the 21-hydroxylase gene (*CYP21A2*), most commonly caused by genomic rearrangements with the adjacent, highly homologous pseudogene *CYP21A*. The most common deletions and gene conversion events have been defined and are typically detected by Southern hybridization detection of *CYP21* rearrangements and/or polymerase chain reaction (PCR). However, Southern hybridization is laborious, and allele-specific PCR results may be difficult to interpret. We have therefore developed a locus-specific, PCR-based, minisequencing method for detecting the 12 most common *CYP21A2* mutations. We validated the assay using a panel of 20 previously genotyped samples obtained from individuals who collectively have a broad spectrum of mutations causing 21-hydroxylase deficiency. We also used 19 control samples having no *CYP21* mutations. All validation samples were correctly typed, and we identified haplotypes that may be useful for clinical diagnosis. Results from 102 clinical samples demonstrate that this assay is a rapid, reliable, and robust method for locus-specific identification of mutations and is suitable for routine clinical use and prenatal diagnosis. (*J Mol Diagn* 2005, 7:236–246)

and nonclassic (NC) disease. Classic disease is more severe and includes salt-wasting and simple virilizing forms.¹ The salt-wasting form is associated with hyponatremia and hyperkalemia in both males and females with aldosterone deficiency. Both salt-wasting and simple virilizing forms include ambiguous genitalia in females. NC disease is less severe, with phenotypes including virilization in childhood, hirsutism and inconsistent menstruation in women, and infertility in both men and women.^{2–4} CAH varies in frequency in different populations, but classic CAH is estimated to affect ~1:13,000 births.^{5,6} The prevalence of the mild NC form is estimated at 0.1% of the general population,⁴ but has been reported to be as high as 1:100 in New York⁷ and 1:27 in the Ashkenazim.⁸ Prenatal diagnosis and treatment for fetuses at risk for classic CAH are feasible and effective at preventing virilization of the affected female,^{9–11} however the appropriateness, ethics, and outcomes remain controversial.^{12–14}

More than 90% of CAHs are caused by 21-hydroxylase deficiency, attributable to mutations in the gene coding for 21-hydroxylase (*CYP21A2*). The *CYP21A2* gene is located on chromosome 6p21.3, in a region containing genes coding for proteins that determine human leukocyte antigen types. The 21-hydroxylase gene locus also contains a pseudogene, *CYP21A*, which is located ~30 kb from *CYP21A2*, among the complement genes *C4A* and *C4B*. *CYP21A* is ~98% and 96% homologous to *CYP21A2* in its exons and introns, respectively; however, minor differences from the active *CYP21A2* gene render the *CYP21A* gene inactive. The proximity and homology of *CYP21A2* with *CYP21A* is thought to predispose the locus to meiotic crossovers between *CYP21A2* and *CYP21A*, which may result in duplication or deletion of entire segments of the gene region.^{2,15,16} The most common CAH-related mutations include: a 30-kb deletion or genomic rearrangement/conversion fusing *CYP21A2* with *CYP21A* (30%), IVS2-13 A/C→G (also called In2G, 28%), I172N (9%), V281L (9%), Q318X (4%), R356W (4%), exon 6 cluster mutation (I235N, V236E, M238K, 4%), G110Δ8nt (3%),

Congenital adrenal hyperplasia (CAH) is characterized by a defect in adrenal steroid biosynthesis, causing reduced glucocorticoid production and increased androgen production. CAH is manifested in a variety of phenotypic severities, which are broadly classified as classic

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Table 1. Assay Validation Panel

Sample	Expected genotype*	Observed genotype†	Phenotype
4110	In2G [‡] / F306 + 1 nt	In2G/ F306 + 1 nt	Unknown
5846	30-kb del/gene conversion	30-kb del/gene conversion	Salt wasting CAH
5686	Null/null	Null/null	Salt wasting CAH
5557	In2G/Q318X	In2G/Q318X	Severely virilized CAH
6025	In2G/R356W	In2G/R356W	Salt wasting CAH
7214	In2G/I172N	In2G/I172N	Severely virilized CAH
7215	In2G(A,C)/Q318X	In2G(A,C)/Q318X	Unaffected carrier
7227	In2G/null [§]	In2G/In2G [§]	Salt wasting CAH
7229	WT/In2G	WT/In2G	Unaffected carrier
7243	V281L/30-kb del	V281L/30-kb del	Nonclassic CAH
7257	V281L/V281L	V281L/V281L	Nonclassic CAH
3904	In2G/L433P	In2G/WT [¶]	Unknown
4451	In2G/P453S/R483 + C	In2G/P453S [¶]	Unknown
5467	G110Δ8nt /R408C	G110Δ8nt /WT [¶]	Unknown
6730	W407X/null	WT/WT [¶]	Unknown
RD03-1	V281L/V281L	V281L/V281L	Nonclassic CAH
RD03-3	V281L/V281L	V281L/V281L	Nonclassic CAH
RD03-5	WT/V281L	WT/V281L	Unaffected carrier
147	WT/V281L/gene conversion	WT/V281L/gene conversion	Unaffected carrier
46,500	WT/V281L/gene conversion	WT/V281L/gene conversion	Nonclassic CAH

The PCR/minisequencing assay correctly typed 19 samples as wild type (data not shown). Wild-type samples included one anonymized prenatal sample.

*Determined using Southern hybridization/allele-specific PCR, reflexed to a sequencing assay when indicated, or using a READIT assay (Promega Corp.).

†Determined by PCR/minisequencing assay.

‡ IVS2-13 A/C→G is designated In2G.

§Both the expected and observed genotypes were interpreted as being consistent with a CAH phenotype.

¶The expected genotype includes one or more mutations not detected in the PCR/minisequencing assay. These mutations were subsequently detected in an assay that sequences the entire *CYP21A2* gene.

P30L(2%), F306 + 1nt (1%), and P543S (1%).^{4,17,18} These events, with the exception of the P543S mutation, are derived from the *CYP21A* pseudogene, and together account for ~95% of 21-hydroxylase deficiency. The remaining ~5% of 21-hydroxylase deficiency is caused by rare sporadic mutations.^{4,16,19}

New diagnostic initiatives include heel-stick filter-paper screening of newborns,^{20,21} gas chromatography/mass spectrometry,²² and molecular techniques. Gene deletions and large conversions are typically detected by Southern hybridization¹⁶ or a recently described, semiquantitative polymerase chain reaction (PCR)/enzyme digestion-based method.²³ Techniques for molecular diagnosis of point mutations include locus-specific oligonucleotide hybridization,²⁴ amplification-created restriction sites,²⁵ single-stranded conformational polymorphisms,²⁶ locus-specific PCR,^{27–29} ligation detection reactions,³⁰ and multiplex minisequencing.³¹ These methods have been reviewed by White and Speiser.⁴ Allele-specific PCR results may be difficult to interpret, unless the mutations identified can be confidently assigned to the *CYP21A2* or pseudogene loci. The issue is complicated by the finding that back-conversion may occur, in which selected normal alleles and point mutations may cross back to the pseudogene. It is important, therefore, to establish initially whether gross recombination or conversion events have occurred, by examining the entire gene locus, before proceeding to mutation analysis. Locus-specific PCR, followed by a multiplex minisequencing step using 13 primers, has been shown to be a valid technique that can be used to detect the 10 most common point mutations.³¹ However, Southern blot analysis is still required for determination of genomic rearrangements for this method. Our approach is to perform locus-specific PCR using four primers external to the open reading frame to de-

termine whether or not genomic rearrangement or deletion has occurred, followed by minisequencing using internal primers to detect internal *CYP21A2* mutation.

Here, we describe a two-step molecular diagnostic approach, which is able to detect the most common gene conversions, point mutations, and deletions responsible for ~90% of 21-hydroxylase deficiency without need of Southern hybridization. We use four locus-specific, duplex PCR reactions to detect the presence or absence of *CYP21A2*, *CYP21A*, and two products of genomic rearrangement. After PCR, amplicons are further analyzed by single-base extension with fluorophore-labeled nucleotides and separation/sizing by capillary electrophoresis (CE), to detect 12 common point mutations and a small deletion. Assay design, validation, and clinical application are discussed.

Materials and Methods

Samples

Anonymized samples whose genotypes had previously been tested and obtained with appropriate informed consent, were used for validation (Table 1). These included samples from 15 patients with CAH (21-hydroxylase deficiency), 3 patients with NC CAH (21-hydroxylase deficiency), 2 patients carrying a *CYP21A2* mutation, and 1 wild-type individual (kindly provided by Veronica Mericq, M.D., Institute of Maternal and Child Research, University of Chile, Santiago, Chile). Samples from 19 patients were submitted to Quest Diagnostics for DNA testing to identify mutations associated with non-CAH disease. These indi-

Figure 1. Amplification scheme for *CYP21* genes and relative position of mutations within *CYP21*. **A:** Primers ME0008 and ME0066 amplify the functional *CYP21A2* gene (amplicon 1), whereas primers ME0059 and ME0067 amplify the *CYP21A* pseudogene (amplicon 2). Relative positions of the *EcoRI* sites used for demonstration of locus-specific amplification are designated by RI. **B:** Primers ME0059 and ME0066 amplify the *CYP21A/A2* fusion gene created when a 30-kb deletion occurs (amplicon 3). **C:** Primers ME0008 and ME0067 amplify the *CYP21A2/A* rearrangement product (amplicon 4). **D:** Relative positions of the exons, 10 common point mutations and a small deletion (G110Δ8nt) detected in the assay. IVS2-13 A/C→G is designated In2G.

viduals were presumed to be unaffected with CAH and were anonymized before inclusion in this study as negative controls. For clinical testing of 220 patient samples, DNA was extracted from peripheral blood samples or cultured amniocytes using the QIAamp DNA kit (Qiagen, Valencia, CA). Clinical indications for testing were obtained from the treating physician by a genetic counselor in our molecular genetics department.

PCR and Agarose Gel Electrophoresis

Four locus-specific oligonucleotides were designed that hybridize upstream and downstream of either *CYP21A* or *CYP21A2*. The four primers were combined into each of the four possible pair-wise reactions to obtain amplicons corresponding to: *CYP21A2* (ME0008 and ME0066), *CYP21A* (ME0059 and ME0067), the *CYP21A/A2* gene deletion/conversion product (ME0059 and ME0066), and the *CYP21A2/A* gene rearrangement product (ME0008 and ME0067) (Figure 1; A to C). Upstream primers hybridize 200 bp and 830 bp 5' of the initiation codons of *CYP21A2* and *CYP21A*, respectively, whereas downstream primers hybridize 440 bp 3' of the stop codon. Duplex PCR reactions contained 1× PCR buffer (Roche, Alameda, CA); 200 μmol/L each dNTP (Roche); 0.2 μmol/L each of the four primers (two *CYP21* primers and two internal control primers), according to the scheme below and as illustrated in Figure 1 (Qiagen, Palo Alto, CA); and 2.5 U Expand Long Template Enzyme (Roche). Primers for deletion analysis were: ME0008, 5'-GCTTCTTGATGGGTGATCAAT-3'; ME0059, 5'-TCCCAATCCTTACTTTTGTG-3'; ME0066, 5'-CCTCAATCCTCTGCAGCG-3'; and ME0067, 5'-CCTCAATCCTCTGCGGCA-3'. Internal control primers

amplify a fragment from within the unrelated *AVPR2* gene: ME0004, 5'-ACAGGCTCTGGCCAATTCTC-3'; and ME0027, 5'-ACCTGGCCGTGGCTCTGTTC-3'.

PCR master mixes contained the following primer combinations: amplicon 1 (*CYP21A2*), ME0008, ME0066, ME0004, and ME0027; amplicon 2 (*CYP21A*), ME0059, ME0067, ME0004, and ME0027; amplicon 3 (*CYP21A/A2* gene deletion product), ME0059, ME0066, ME0004, and ME0027; and amplicon 4 (*CYP21A2/A* gene rearrangement product), ME0008, ME0067, ME0004, and ME0027. Touchdown thermal-cycling conditions were: 95°C for 5 minutes followed by 10 cycles of 95°C for 30 seconds, 65°C for 30 seconds (temperature reduced by 1°C each cycle), 72°C for 2 minutes; and 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes, with a final extension at 72°C for 10 minutes. After PCR amplification, products were electrophoresed on a 1% agarose gel, for confirmation of PCR quality and discrimination of the *CYP21* and rearrangement fragments.

Restriction Analysis

To ensure locus-specific amplification of each of the *CYP21* genes, we performed restriction analysis of the four PCR amplicons. After PCR, each of the four reactions were digested with 10 U *EcoRI* (Amersham Pharmacia Biotech, Piscataway, NJ) in 1× Buffer H (Amersham) for 2 hours at 37°C. Digestion products were electrophoresed on a 2% agarose gel to determine the size of digestion products.

Single Base Extension and CE

After amplification and agarose gel discrimination, 2 μl of duplex PCR product was digested with 2 U of shrimp alkaline phosphatase (USB, Cleveland, OH) and 1 U of exonuclease I (USB) in 1× shrimp alkaline phosphatase buffer (USB) in a total volume of 6 μl, to remove PCR primers and unincorporated dNTPs. Each digestion reaction was incubated at 37°C for 2 hours followed by 15 minutes at 75°C. After incubation, 5 μl of SNaPshot ready reaction mix (Applied Biosystems, Foster City, CA) and 1 μl of primer extension (PE) primer mix (Table 2) were added to each digestion reaction; total reaction volume, 12 μl. The oligonucleotides designed for minisequencing are of increasing length and end one base before each mutation base change.

Thermal-cycling conditions were: 25 cycles of 95°C for 30 seconds, 50°C for 5 seconds, and 60°C for 30 seconds. Shrimp alkaline phosphatase (1 μl) was added to each extension reaction, and samples were incubated at 37°C for 1 hour followed by 15 minutes at 75°C, to digest unincorporated dNTPs. Two μl of the final digestion product was diluted with 36 μl of Hi-Di formamide (Applied Biosystems) and 2 μl of LIZ size standard (diluted 1:5 in formamide, Applied Biosystems), and samples were heated at 95°C for 5 minutes and cooled on ice for at least 5 minutes before electrokinetic injection into the ABI3100 CE instrument (Applied Biosystems).

Table 2. Primers for PE Mix

Primer name	Mutation locus	Sequence	Final concentration (μmol/L)	Peak size	Wild-type peak	Mutant peak
ME0243	P30L	5'-AGCCCCGGGGCAAGAGGC-3'	3	22 to 24	Blue	Green
ME0050	IVS2-13 A/C→G	5'-T ₃ CCAGCTTGCTGTCAGGAGGAG-3'	8	29 to 30	Red/blue	Black
ME0244	G110Δ8nt-F	5'-T ₁₄ ACCCGGACCTGTCSTTGG-3'	8	34 to 38	Blue	Red
ME0235	G110Δ8nt-R	5'-T ₁₇ GGGCTTTCCAGAGCAGRGA-3'	6	39 to 41	Blue	Black
ME0236	I172N	5'-T ₂₂ CTCCGAAGGTGAGGTAACAG-3'	8	45 to 46	Green	Red
ME0237	I235N	5'-T ₂₈ CCATAGAGAAGAGGGAYCAC-3'	11	50 to 52	Red	Green
ME0238	V236E	5'-T ₃₅ GCTGCCTCAGCTGCWTCTCC-3'	13	57 to 59	Green	Red
ME0239	M238K	5'-T ₄₀ CCTTGTGCTGCCTCAGCTGC-3'	12	61 to 63	Green	Red
ME0240	V281L	5'-T ₄₆ GGACAGCTCCTGGAAGGGCAC-3'	1	68 to 70	Blue	Red
ME0071	F306 + 1nt	5'-T ₄₇ CACCCTCTCCTGGGCCGTGGTTTTTTT-3'	0.5	76 to 78	Blue	Red
ME0241	Q318X	5'-T ₆₀ CCCCAGATTGAGCAGCGACTG-3'	8	80 to 84	Black	Red
ME0242	R356W	5'-T ₆₆ ATCGCCGAGGTGCTGCGCCTG-3'	10	88 to 90	Black	Red
ME0074	P453S	5'-T ₇₂ CTGCAGGCCTTCACGCTGCTG-3'	10	93 to 95	Black	Red

Sequencing

Five of the *CYP21A2* mutations provided for validation are not detected in the current assay, and were therefore cycle-sequenced in forward and reverse directions, to verify the results. PCR products were Exo/shrimp alkaline phosphatase-purified (as described above) and cycle-sequenced using Big Dye, Version 3.1 (Applied Biosystems), according to the manufacturer's protocol. For complete *CYP21A2* sequencing, the following primers were used: ME0096, 5'-CCAATGAGACTGGTGTCTATTC-3' (5' untranslated region, forward); ME0097, 5'-CAGCATA-GCAAGAACCCATC-3' (intron 2, reverse); ME0098, 5'-CCAAGAGGACCATTGAGGAAG-3' (exon 2, forward); ME0099, 5'-GCTGTGGAGAAACAGTGTGAG-3' (intron 3, reverse); ME0100, 5'-GGAAAGCCCAACAAGAAGCTC-3' (exon 3, forward); ME0101, 5'-AGCATGAGAATGCAGCTGTG-3' (intron 5, reverse); ME0103, 5'-GAAGGAGCCTTTTGCTTGTC-3' (intron 7, reverse); ME0104, 5'-CACTGAGACCACAGCAAACAC-3' (intron 7, forward); ME0105, 5'-CCTCCACCACATTTTCACG-3' (intron 9, reverse); ME0106, 5'-CACAGTCATCATCCGAACC-3' (exon 9, forward); ME0107, 5'-GACCAAGAACTTTTCGCTCC-3' (exon 10, reverse); ME0108 5'-TGTAACACAGTGCTGCGAG-3' (exon 10, forward); ME0117, 5'-CACCTCTGCAGGAGAGC-3' (intron 6, forward); ME0118, 5'-CTTGCTCAATGCCACCATC-3' (exon 8, forward); and ME0152, 5'-TAAAACTGGAGCCACTGG-3' (exon 5, forward). Sequences were aligned and examined using SeqScape software (Applied Biosystems).

Results and Conclusions

Assay Design

Four duplex PCR reactions produce 3.4-kb or 4.0-kb amplicons when analyzed by agarose gel electrophoresis (Figure 2). A 1.1-kb amplicon from within the functionally unrelated *AVPR2* gene allows internal control of PCR reaction conditions, thereby reducing the likelihood of false-negative results. Amplification primers ME0059 and ME0008 are locus-specific for the *CYP21A* and *CYP21A2* loci, respectively, and anneal 830 bp (ME0059) and 200

bp (ME0008) upstream of the start codon. Primer ME0008 overlaps a *TaqI* restriction fragment length polymorphism between *CYP21A2* and *CYP21A* that is typically used for *CYP21* deletion/rearrangement analysis by Southern hybridization.¹⁶ Primers ME0066 and ME0067 are locus-specific for the *CYP21A2* and *CYP21A* loci, respectively, and anneal within the 3'UTR, 440 bp downstream from the stop codon.

Our PCR method was examined for locus-specific amplification of the *CYP21A2* and *CYP21A* genes by *EcoRI* restriction mapping. Although the *CYP21A* locus contains two *EcoRI* sites within intron 2 and exon 4, *CYP21A2* has only the exon 4 site (Figure 1A). This *EcoRI* restriction fragment length polymorphism, like the *TaqI* site above, can be used for Southern analysis of *CYP21* for genomic deletions and rearrangement.³² As expected, *EcoRI* digestion of amplicon 1 (*CYP21A2*) produced 1198-bp and 2194-bp fragments, whereas digestion of amplicon 2 (*CYP21A*) produced 1315-bp, 505-bp, and 2194-bp fragments (data not shown). The *EcoRI* digestion pattern of

Figure 2. Examples of PCR data. **A:** Example of the PCR amplicons produced in a typical wild-type sample. M, molecular size standard (bp). **Lane 1:** amplicon 1, the *CYP21A2* amplicon runs at 3.4 kb, while the internal standard runs at ~1 kb; **lane 2:** amplicon 2, the *CYP21A* amplicon runs at 4.0 kb, while the internal standard runs at ~1 kb; **lanes 3 and 4:** amplicons 3 and 4, no rearrangement amplicons are present, but the internal standard is present at ~1 kb. **B:** Example of the PCR amplicons produced by a sample containing a pseudogene, 30-kb deletion and gene conversion rearrangement loci, but no *CYP21A2* fragment. M, molecular size standard (bp). **Lane 1:** amplicon 1, no *CYP21A2* amplicon present, but the internal standard is present at ~1 kb; **lane 2:** amplicon 2, the *CYP21A* amplicon migrates at 4.0 kb, while the internal standard migrates at ~1 kb; **lane 3:** the *CYP21A/A2* deletion amplicon migrates at 4.0 kb, and the internal standard migrates at ~1 kb; **lane 4:** the *CYP21A2/A* rearrangement amplicon migrates at 3.4 kb, and the internal standard migrates at ~1 kb.

[illegible] [illegible]

amplicons 3 (*CYP21A/A2*) and 4 (*CYP21A2/A*) depended on the location of the breakpoint of deletion or rearrangement relative to the polymorphic *EcoRI* site within intron 3.

After PCR, amplicons were analyzed using a SNaPshot multiplex minisequencing kit for 11 common point mutations and a small deletion that alter protein function. Typical results from minisequencing are shown in Figure 3, A and C. Because the SNaPshot ready reaction mix contains all four nucleotides, each labeled with a different color fluorophore, resulting single-base extension oligonucleotides are colored specifically according to the nucleotide that was added during the reaction. After CE and analysis with GeneScan and GenoTyper software (Applied Biosystems), nonoverlapping peaks with similar heights are produced that provide genotype information. Poly-T chains at the 5' end of most oligonucleotides (Table 2) prevent the resulting peaks from overlapping, and help reduce secondary structure formation due to the addition of other sequences. The small 8-bp deletion commonly found in exon 3 was analyzed by two single-base extension reactions that add bases in the forward and reverse directions (G110Δ8nt-F and G110Δ8nt-R), based on the method of Krone and colleagues.³¹ To facilitate rapid scoring, the genotyper software was programmed to label peaks representing wild-type alleles as black, and peaks representing mutant alleles as red. Our minisequencing method and reporting chart also provides easy-to-read break-point information for gene deletions and large conversion events when a series of black labels change to a series of red labels, or vice versa (Figure 3, B and D).

Typical samples from normal individuals produce amplicons 1 (*CYP21A2*) and 2 (*CYP21A*), but may also produce amplicon 4 (*CYP21A2/A*). Minisequencing results for wild-type amplicon 1 are nonoverlapping peaks of similar sizes with all-black labels. Minisequencing results for the pseudogene in amplicon 2 may be nonoverlapping mutant peaks, with all-red labels. However, we have observed much heterozygosity within the pseudogene amplicon, so the peaks and labels may reflect a mixture of wild type and mutant. Minisequencing results for wild-type amplicon 4, if present, produce an identical pattern to that of amplicon 1. Although the presence of amplicon 4 may be considered an unexpected result, it has been observed on multiple occasions and likely corresponds to *cis*-gene duplications detected by other groups,^{33,34} gene conversion at the 3' end of the gene, or polymorphism under the primer binding site, none of which cause coding sequence changes. Regardless of the mechanism that creates these fragments, downstream minisequencing (or complete sequencing) will inform the user whether or not these amplicons 4 contain any gene-deactivating mutation.

Typical samples from individuals affected by CAH or carriers may produce any combination of the four PCR

amplicons. Minisequencing results for amplicon 1 or amplicon 4 from mutant chromosomes exhibit nonoverlapping peaks, with one or more mutant red-labeled peaks. As in samples from normal individuals, amplicon 2 SNaPshot results indicate much heterogeneity. The presence of amplicon 3 indicates a 30-kb deletion allele or a large gene-conversion allele with the 5' end of *CYP21A* and the 3' end of *CYP21A2*. The presence of amplicon 4 in an affected individual may indicate an allele with a point mutation, a small deletion, or a gene-conversion event. These species, as well as the wild-type amplicon 4 detected in unaffected individuals and carriers, can be distinguished by the presence or absence of mutant alleles within the minisequencing results. Gene conversion or large deletion events and their breakpoint locations within amplicons 3 or 4 are indicated by a trend of red-labeled peaks followed by a trend of black-labeled peaks, or vice versa. Care should be taken when considering the peak for P453S, because this mutation is not transferred from the pseudogene and may be mistakenly identified as the tail end of a gene-conversion event.

Assay Validation

Twenty samples from CAH patients or carriers and 19 samples from healthy donors previously characterized for *CYP21* mutations were analyzed during assay validation (Table 1). Clinical subtypes of the patients (if available) were determined based on clinical manifestations and the levels of relevant steroid metabolites and electrolytes in plasma and urine.³⁵ Mutations from 15 of the CAH patient or carrier samples were previously determined using Southern hybridization for deletion analysis,¹⁹ allele-specific PCR for detection of the eight most common point mutations,³⁵ and sequencing for the detection of remaining mutations. Three samples with a V281L mutation were collected at Quest Diagnostics for validation of the CAH Ashkenazi Jewish allele assay, and were previously shown to carry V281L mutations using a READIT assay (Promega Corp., Madison, WI). Mutations from the two remaining samples from CAH patients were previously determined by allele-specific PCR only.³⁵ The validation panel was assessed for the presence of 13 mutations (the 30-kb deletion, 11 point mutations, and one small deletion), and had 11 different mutant *CYP21A2* genes. Each of the 13 mutations examined in the assay was detected at least once.

All validation samples were correctly typed, according to the expected genotypes. Results from seven samples warrant further discussion. Amplicon 1 of sample 7215 had A, C, and G alleles at the IVS2-13 locus, revealing the presence of at least three copies of the *CYP21A2* gene (Figure 4A). Although the individual carries both IVS2-13 A/C→G and Q318X mutations, they were suspected of

Figure 3. Examples of minisequencing data and reporting scheme. **A:** Minisequencing results for the amplicons obtained in Figure 2A. Peaks obtained for wild-type alleles in the *CYP21A2* amplicon are labeled black and mutant alleles are labeled red. Minisequencing results are not shown for amplicons 3 and 4, which were absent in the PCR. **B:** Reporting scheme for minisequencing data in **A**. Rows represent the four possible amplicons from the four PCR reactions. Columns report the presence or absence of each PCR amplicon and the genotype of each locus. Squares are colored black for wild-type alleles and red for mutant alleles. IVS2-13 A/C→G is designated In2G. **C:** Minisequencing results for the amplicons obtained in Figure 2B. **D:** Reporting scheme for minisequencing results in **C**.

A.

	PCR amp	P30L	In2G	G110 Δ8nt	I172N	I235N	V236E	M238K	V281L	F306+1nt	Q318X	R356W	P453S
1	+		A/C/G										
2	+												
3	-												
4	-												

B.

	PCR amp	P30L	In2G	G110 Δ8nt	I172N	I235N	V236E	M238K	V281L	F306+1nt	Q318X	R356W	P453S
1	-												
2	+												
3	+												
4	+												

C.

	PCR amp	P30L	In2G	G110 Δ8nt	I172N	I235N	V236E	M238K	V281L	F306+1nt	Q318X	R356W	P453S
1	-												
2	+												
3	+												
4	+												

Figure 4. Sample reporting scheme for clinical samples. **A:** Reporting for sample 7215, a WT/Q318X/IVS2-13 A/C→G (designated In2G) normal carrier. Wild-type alleles are illustrated as **black squares**, mutant alleles as **red squares**, and heterozygotes as **red cross-hatched squares**. This patient is known to be normal from biochemical data, as well as the presence of three *CYP21A2* alleles, demonstrated by multiple heterozygosity at the IVS2-13 locus. **B:** Reporting for sample 7257, a V281L/V281L-affected sample. Although amplicon 3 appears to be a 30-kb deletion fragment with an intron 7 breakpoint, this rearrangement fragment was shown to be a duplicated gene fragment (see text). **C:** Reporting for sample 7243, a V281L/30-kb del-affected patient. Heterozygosity in amplicon 3 reveals the presence of two rearrangement fragments, one with an intron 3 breakpoint and a second with an intron 7 breakpoint.

being unaffected because of the third copy of *CYP21A2*. The laboratory that contributed the sample confirmed the presence of the third normal allele. Although our assay easily detected the third copy of *CYP21A2*, it does not determine the copy number of such mutations, nor the phase of compound heterozygotes. Therefore, similar samples sent to our laboratory for molecular analysis are given preliminary results only, until family studies are completed.

Because the current assay does not allow determination of copy number, we originally reported sample 7227 as IVS2-13 A/C→G/IVS2-13 A/C→G (data not shown). However, the laboratory that contributed the sample pre-

viously determined it to be IVS2-13 A/C→G/null. Lack of polymorphism by complete gene sequencing and family studies further suggest that the sample has an IVS2-13 A/C→G/null genotype. It is of note that neither IVS2-13 A/C→G/IVS2-13 A/C→G nor IVS2-13 A/C→G/null contain functional copies of *CYP21A2* and are therefore consistent with a CAH phenotype.

Samples 3904, 4451, 5467, and 6730 all contain mutant alleles that are not detected in the currently reported assay but were subsequently detected by complete gene sequencing (data not shown). Similar samples from patients who present all of the classic biochemical and

Table 3. Summary of Clinical Experience Testing for *CYP21A2* Mutations

Clinical indication for testing	Number of patients	Negative*		Carrier [†]	No normal <i>CYP21A2</i>
		Neg2	Neg1		
Confirmed CAH [‡]	33	0	0	0	33
Prenatal [§]	12	1	2	5	4
Obligate carrier	26	1	0	23	2
Population screening [¶]	31	17	11	3	0
Totals	102	19	13	31	39

*Because the current assay does not always provide copy number information, patients negative for mutation were divided into Neg2 (individuals with two normal alleles, detected either by heterozygosity at the IVS2-13 A/C>G locus, or analyzed as amplicons 1 and 4) and Neg1 (individuals with only one normal allele detected, typically due to homozygosity at the IVS2-13 A/C>G locus).

[†]At least one normal allele and at least one mutant allele detected.

[‡]Clinically diagnosed children and adults, currently being treated for CAH.

[§]1:4 risk of classic CAH.

[¶]Partners of affected individuals and obligate carriers, no known family history of CAH.

clinical symptoms of 21-hydroxylase deficiency could be reflexed to analysis by complete gene sequencing. Samples 147 and 46500 are from an asymptomatic mother and her son with NC disease characterized by precocious pubarche and advanced bone age. Prior testing included all 12 point mutations examined in the current assay, but did not include deletion analysis. Both prior testing and the current assay detected the presence of a normal *CYP21A2* gene (data not shown), and were therefore unsuccessful at identifying the genetic cause of the boy's disease. Follow-up with complete gene sequencing also failed to find a *CYP21A2* mutation.

Summary of Clinical Experience

At the time of submission of this article, 220 clinical samples had been analyzed for *CYP21A2* mutations using the approach reported here. The majority of samples ($n = 208$, 95%) were from peripheral blood and 12 (5%) were prenatal samples (cultured amniocytes). Prenatal samples were tested only after confirmation of the indication for testing by a genetic counselor, and were tested in duplicate to confirm the analytical results.

Table 3 summarizes the results of 102 samples: 33 from individuals with confirmed CAH, 12 submitted for prenatal screening (one in four risk for classic disease), 26 from obligate carriers, and 31 submitted for general population screening (from partners of known affecteds and from obligate carriers with no known family history). The remaining 118 samples tested include samples from individuals for whom we were not able to obtain sufficient information (eg, baseline and stimulated 17-hydroxyprogesterone levels) to be confident of a 21-hydroxylase deficiency diagnosis, individuals with family history of CAH (not obligate), individuals submitted for simultaneous biochemical and molecular testing (from individuals who were later determined not to have 21-hydroxylase deficiency), and parents of suspected CAH patients. Simultaneous biochemical and molecular testing is a common occurrence in the commercial laboratory in contrast to the academic setting in which samples are submitted for molecular testing only after biochemical results confirm the clinical diagnosis.

The results shown in Table 3 were divided into three classes: no mutations detected, one mutation detected

(carriers), and no normal *CYP21A2* locus detected. Because the current assay provides evidence of two *CYP21A2* genes only when there is heterozygosity or when they are amplified separately (ie, amplicons 1 and 4), normal individuals were further classified as negative for mutation with one normal allele (Neg1) or negative for mutation with two normal alleles (Neg2).

All of the samples submitted for molecular testing from patients with clinically diagnosed and treated disease ($n = 33$) carried no normal copies of *CYP21A2*. There are likely samples from other clinically diagnosed and treated patients in our test population for which we were not able to confirm diagnosis with the treating physician. Confirmed cases include 21 simple virilizing or salt wasting patients and 16 patients with nonclassic disease. Disease chromosomes detected include 17 30-kb deletion or gene conversion, 14 IVS2-13 A/C→G, 11 I172N, 18 V281L, 4 G110Δ8nt, 1 Q318X, and 1 R356W mutations.

Twelve samples were from prenatal patients with a one in four risk of classic CAH. Parental samples are always genotyped before receipt of prenatal samples intended for mutation confirmation and transmission determination. Three of the prenatal cases had no mutations, five were carriers, and four had no normal copies of *CYP21A2*. Disease chromosomes detected include five 30-kb deletion or gene conversion, six IVS2-13 A/C→G, and two I172N mutations.

Twenty-six obligate carriers were tested using the current assay. Two of these had two *CYP21A2* mutations (IVS2-13 A/C→G/I172N and V281L/IVS2-13 A/C→G). Both individuals were pregnant. One is a previously known affected individual who is currently being treated with dexamethasone, and the other is asymptomatic and had no problems becoming pregnant. In a third obligate carrier, we found no *CYP21A2* mutant alleles. This individual is the mother of a boy with salt-wasting CAH and likely carries a rare mutation not detected by the current assay. This sample will be sequenced when our complete sequencing assay is ready for service. The remaining 23 patient samples had a single *CYP21A2* mutation. These mutations included nine 30-kb deletion or gene conversion, nine IVS2-13 A/C→G, four I172N, one V281L, two G110Δ8nt, and two Q318X mutations.

Thirty-one samples from individuals having no previous family history of CAH were submitted for mutation

screening (Table 3). Thus, the population screening category in Table 3 includes data only from partners of individuals with clinically diagnosed and treated disease and new partners of obligate carriers. Twenty-eight samples had no *CYP21A2* mutations and three had one mutation. Mutations detected in this population include two 30-kb deletion or gene conversion mutant alleles and one V281L mutant allele.

Within our test population of 220 patient samples (440 chromosomes), we detected 137 mutant chromosomes. These mutant chromosomes can be further divided into 42 30-kb deletion or gene conversion chromosomes (30.6%), 31 IVS2-13 A/C→G (22.6%), 19 I172N (13.9%), 29 V281L (21.2%), 6 Q318X (4.4%), 6 G110Δ8nt (4.4%), 2 P453S (1.5%), 1 F306 + 1nt (0.7%), and 1 R356W (0.7%). We detected no chromosomes with P30L or exon 6 cluster mutations alone, although we routinely detect these mutations within 30-kb deletion and gene conversion mutant chromosomes.

Haplotypes Observed and Their Significance for Clinical Diagnosis

The inclusion of 11 point mutations and one small deletion in our assay, as well as the testing of several families and triads for prenatal diagnostics, allowed us to observe several interesting haplotypes that maybe useful for clinical diagnosis. During validation, we tested seven individuals with V281L mutation (Table 1). Two of these, 7257 and 7243, were samples from Ashkenazi NC CAH patients originally diagnosed by Southern blot analysis and locus-specific PCR. Southern blot results indicate the presence of *CYP21A2*, *CYP21A*, and a duplicated gene fragment in both samples. In our assay, both samples produced amplicons 2, 3, and 4, with identical minisequencing results for amplicons 2 and 4, but different results for amplicon 3 (Figure 4, B and C). Amplicon 3 of sample 7257 is mutant for P30L, IVS2-13 A/C→G, G110Δ8nt, I172N, I235N, V236E, M238K, V281L, and F306 + 1nt, and wild-type for Q318 and R356, indicating a deletion/conversion fragment with an intron 7 breakpoint. In contrast, minisequencing results from the product of PCR reaction 3 for sample 7243 revealed heterozygosity and, therefore, the presence of two or more amplicon 3 fragments. These amplicons were homozygous mutant for P30L, IVS2-13 A/C→G, and G110Δ8nt; heterozygous for I172N, I235N, V236E, M238K, V281L, and F306 + 1nt; and homozygous wild-type for Q318 and R356, indicating that one has an intron 3 breakpoint and the second has an intron 7 breakpoint. Our amplicon 3 fragment with the intron 7 breakpoint likely corresponds to a gene duplication with a large conversion event. Whether the product of deletion or conversion, the presence of multiple mutant alleles within this fragment indicates that it will not produce functional protein.

Another haplotype that was observed involves a single chromosome with two copies of *CYP21A2*: one wild-type copy and a second carrying the Q318X mutation. This chromosome was first observed in sample 7215 during validation (discussed above), and was observed in a

further four individuals from two families. Although our assay facilitates observation of the second copy of *CYP21A2* through A/C heterozygosity at the IVS2-13 locus, the Q318X mutation could be misinterpreted in an assay that does not identify two separate chromosomes. This haplotype likely corresponds to a rare duplicated 21-hydroxylase haplotype recently described by Baumgartner-Parzer and colleagues.³⁶

Discussion

The 21-hydroxylase deficiency molecular diagnostic assay reported here detects common mutations within *CYP21A2*, including large genomic rearrangements, point mutations, and a small 8-bp deletion. This method allows rapid and accurate locus-specific identification of deletions and conversions. The assay functions reliably in routine clinical testing and is sufficiently efficient to allow prenatal testing. The assay allowed a rapid turnaround from receipt of the clinical blood sample or cultured prenatal sample to written report of the diagnostic result.

The structure of the *CYP21* locus has complicated mutation testing since the gene was first identified and associated with 21-hydroxylase deficiency. The high level of identity between *CYP21A2* and *CYP21A* makes PCR primer selection difficult because it is necessary to ensure that they correctly amplify the expected gene product. Therefore, most diagnostic groups currently test for mutations by Southern blot analysis and a separate PCR reaction for each point mutation or small deletion. However, the relatively compact size of the two genes (3.1 kb) allowed us to amplify each gene accurately by taking advantage of the limited heterogeneity outside of the coding region. The *cis*-arrangement of *CYP21A2* and *CYP21A* on a single chromosome allowed detection of large deletions or conversions without a Southern blot by combining the primers into all four possible pair-wise combinations. Furthermore, inclusion of an internal control amplicon decreases the likelihood of false-positives by controlling for PCR reaction conditions. Although four PCR reactions per patient sample can be cumbersome in the clinical laboratory, we find this method preferable to Southern blot hybridization analysis.

When designing this assay, we expected that amplicons 4 would consist only of genomic rearrangement fragments with 5' ends corresponding to the *CYP21A* pseudogene and 3' ends corresponding to the *CYP21A2* functional gene product, as illustrated in Figures 1C, 3C, and 3D. However, we have seen amplicons 4 with completely normal coding sequences when using minisequencing and sequencing. Other workers in the area have noted the polymorphic nature of the *CYP21* genomic locus,^{16,19} and it is possible that the source of these wild-type amplicons 4 is gene conversion or polymorphism at the primer binding site. However, minisequencing or sequencing of each locus-specific PCR product safeguards the user from incorrectly assigning functionality to an allele in question. We think this method is at least as informative as Southern hybridization (if not more so) in terms of identifying functional gene

products, because it directly tests for mutations within each product. When used in combination with family studies to determine phase of mutations, this assay provides informative haplotype data as is advocated by Koppens and colleagues.³³

The minisequencing portion of this assay is modified from the principles described by Krone and colleagues.³¹ These reactions are multiplexed, semiautomated, and efficient, providing simultaneous detection of 11 point mutations and a small 8-bp deletion within a single tube. CE detection of labeled oligonucleotides allows accurate sizing/discrimination, and color-coded labels aid mutation and breakpoint reporting. This approach is clinically appealing because it reduces the total number of PCR reactions, compared with other methods, and uses fluorescent labeling of oligonucleotides and CE instead of radioactive labeling, as is optimally required in Southern blots. We have improved on the method of Krone and colleagues³¹ by optimizing primer design, reordering the minisequencing peaks in order, relative to the exons, and color-coding the peak labels according to whether they are wild-type (black) or mutant (red). Additionally, we have validated this technique in the clinical laboratory, the results of which are presented in this report.

Our clinical experience is limited to date, but results are beginning to indicate the sensitivity and specificity of the assay. We have detected no normal copies of *CYP21A2* in 33 of the 33 known affected individuals tested to date. Results from our limited number ($n = 12$) of prenatal samples are close to the expected pattern of 1:2:1 for affected, carrier, and unaffected, noncarrier—we found 4 of 12 (33%) affected, 5 of 12 (42%) carriers, and 3 of 12 (25%) unaffected, noncarriers. Twenty-five of twenty-six (96%) obligate carriers had at least one mutant *CYP21A2* allele; this is slightly different from the 100% expected carrier rate. The one obligate carrier in whom we found no mutation is the mother of a boy affected with salt-wasting CAH, so paternity is not likely to be the cause of this outlier. A rare or *de novo* mutation may be causing CAH in this child; however, we were not able to test this theory. In individuals without a family history of CAH, we found 3 of 31 carriers (~10%), a slightly higher rate than the expected 5% carrier rate,⁴ but this is not out of range for such a small sample population.

Although our test population contains several individuals from families, and therefore does not represent a population ascertained without bias, our observed frequencies of the various mutant chromosomes is similar to that seen by other groups within the US population.^{4,17,18} We observed more V281L mutations than expected (21% versus 9%), but this may reflect a higher proportion of individuals from at-risk populations (eg, Ashkenazi) than was seen in other studies.

Examination of Ashkenazi V281L mutant chromosomes reveals the possibility that amplicon 3 may be interpreted as a 30-kb deletion when it is actually a gene duplication with a large conversion event. Therefore, it may be difficult, in practice, to identify deletions without analysis of an entire family. Southern blots using both the *CYP21* and

C4 probes may provide more information about the presence or absence of a deletion. Regardless of whether the rearrangement event is the product of conversion or deletion, the minisequencing component of our assay allowed us to determine that the amplicon 3 associated with the V281L allele is nonfunctional, by examining the pattern of mutations. Ultimately, the ease, speed, and accuracy of our new PCR method, compared with the Southern method, outweigh the potential lack of discrimination between a deletion or conversion event.

The assay we describe has been optimized for use as a qualitative diagnostic tool, and is not optimal for quantitative assessment of copy number or determination of the phase in which mutations lie. In instances in which the IVS2-13 A/C>G locus is not multiply heterozygous, or two *CYP21A2* genes do not amplify independently into amplification reactions 1 and 4, the presence of a functional gene may be difficult to detect. Therefore, difficult cases involving compound heterozygosity may require additional family studies. Prenatal diagnosis may also warrant further family studies, to establish the phase of multiple mutations.

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