Long-Range (17.7 kb) Allele-Specific Polymerase Chain Reaction Method for Direct Haplotyping of R117H and IVS-8 Mutations of the Cystic Fibrosis Transmembrane Regulator Gene

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Genotyping of genetic polymorphisms is widely used in clinical molecular laboratories to confirm or predict diseases due to single locus mutations. In contrast, very few molecular methods determine the phase or haplotype of two or more mutations that are kilobases apart. In this report, we describe a new method for haplotyping based on long-range allele-specific PCR. Reaction conditions were established to circumvent the incompatibility of using allele-specific primers and a polymerase with proofreading activity. Haplotypes are determined by post-PCR analysis using different detection methods. The clinical application presented here directly determines the phase of two mutations separated by 17.7 kilobases in the cystic fibrosis transmembrane conductance regulator gene. Each mutation, the missense mutation R117H in exon 4 and the 5T polymorphism in intron 8 (IVS-8), have mild phenotypic effect unless they are present on the same chromosome (in *cis*). If an individual is heterozygous for both R117H and the IVS-8 5T variant, *cis/trans* testing is required to completely interpret results. The molecular method presented here bypasses the need to perform family studies to establish haplotypes. We propose use of this assay as a reflex clinical test for R117H- 5T-positive samples. (J Mol Diagn 2004, 6:264–270)

Detection of locus single mutations affecting gene function has been very successful in providing molecular diagnostics for human genetic diseases. In contrast, detection of two or more mutations on the same gene and determination of their phase (or haplotype) is more challenging. Advances in the field of human genome mapping,¹ the search for complex disease determinants,² pharmacogenomics,³ and accumulation of data from mu-

tation screening programs⁴ underline the need to develop additional molecular haplotyping methods. Current technologies require physical separation of chromosomes or cloning or are limited to analysis of single nucleotide polymorphism (SNPs) present in the range of 1 kilobase (kb).^{5–14} One recent exception is the development of a long-range molecular technique using a two-step PCR/ligation procedure.¹⁵ Here we describe an assay that uses long-range allele-specific amplification to haplotype two mutations separated by 17.7 kb in the cystic fibrosis transmembrane regulator (CFTR) gene.

The CFTR gene encodes a chloride channel and mutations in this gene are responsible for classic cystic fibrosis (CF) and atypical forms of the disease. Two of these mutations, R117H in exon 4 and the 5T polymorphism of the polythymidine tract in intron 8 (IVS-8 5T polymorphism) have a phenotypic synergic effect. The mutation R117H which accounts for approximately 0.8% of mutant alleles, changes an arginine to an histidine in a transmembrane domain of the protein, altering the conductance of the ion channel.¹⁶ The IVS-8 polymorphism affects the splicing efficiency of intron 8.^{17,18} A tract of 7T or 9T at the 3' end of intron 8 insures proper splicing of the intron while a 5T results in a majority of mRNA lacking exon 9.^{19–22} Each mutation is independently considered mild because in both cases, residual activity of the ion channel remains. Therefore, an individual carrying the R117H mutation and the IVS-8 5T polymorphism on two different chromosomes (in *trans*) is not affected by, nor considered a carrier of classic CF²³ although this individual might present with atypical CF. In contrast, a gene with both R117H and IVS-8 5T (in *cis*) is severely affected.²⁴ Both R117H and the IVS-8 5T variants have been found at higher frequencies in individuals with atypical CF than in the normal population.^{22,25–27}

If an individual is heterozygous for both R117H and the IVS-8 5T variant, it is necessary to establish if both mutations are in *cis* or in *trans* to correctly analyze the

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Table 1. Cycling Parameters

Steps	Hold		10 Cycles		25 Cycles		Hold	
	Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time
Denaturation	92°C	2 min	92°C	10 sec	92°C	15 sec		
Annealing			66.8°C	30 sec	66.8°C	30 sec		
Elongation			68°C	15 min	68°C	15 min*	68.0°C	7 min

*Each cycle increases by 20 seconds.

individual's CF status and provide adequate genetic counseling. Current technologies determine the status of both loci independently, and therefore do not provide information on the phase of both mutations. Currently, haplotyping of these two mutations is established by study of the parental lineage when available or inferred from genotypes in rare cases of homozygosity or known compound heterozygotes.²⁷ To analyze these cases directly, we have developed a molecular test that haplotypes the mutations, bypassing the need to obtain DNA from parents or relatives. Since the R117H and IVS-8 loci are separated by 17.7 kilobases (Cystic Fibrosis Mutation database at <http://genet.sickkids.on.ca/cftr/>), the assay described here is based on long-range allele-specific PCR. Allele-specific amplification has so far been restricted to amplification of fragments of DNA less than 2 kb and which do not require the use of an enzyme with proofreading activity. We show here that under optimal PCR conditions allele-specific PCR can be applied to long fragments.

Materials and Methods

Patient Samples

Genomic samples previously genotyped as R117H heterozygous were selected for the development of this assay and de-identified following Institutional Review Board protocol. DNA samples were extracted from whole blood on the MagNA Pure LC instrument (Roche Applied Science, Indianapolis, IN) with the MagNA Pure LC DNA Isolation Kit I (Roche Applied Science).

Long-Range Allele-Specific PCR

All reactions are performed in 25 μ l final volume using the LA PCR kit, version 2.1. (Takara Mirus Bio, Madison, WI). Reactions contain 1X LA PCR buffer II (with 2.5 mmol/L MgCl₂ final), 400 μ mol/L each dNTP ("dNTP mixture"), 200 nmol/L of each primer, 1.25 units of *TaKaRa* LA TaqTM, and 50 to 100 ng of genomic DNA. Desalted primers were synthesized by the DNA-peptide core facility at the University of Utah. Primer sequences and their predicted melting temperatures (T_{ms}) (determined at <http://biotoolsidtdna.com> using the default setting of 250 nmol/L primer concentration and 50 mmol/L monovalent salt) are presented in the Results section. The three forward primers were designed using the GenBank Sequence Number M55109 as a template (RH; 5' at nucleotide 248, H and R also at nucleotide 309) and the

reverse primer was designed using GenBank Sequence Number M55114 (nucleotide number 620).

Reactions were performed on a GeneAmp PCR System 9700 instrument (Applied Biosystems, Foster City, CA) using the 9600 ramp mode. The cycling parameters are listed in Table 1. Notice the increment of elongation time at each cycle.

Determination of R117H and IVS-8 Haplotype Using Oligonucleotide Ligation Assay

At the end of the PCR, products are analyzed using the Cystic Fibrosis Assay version 3.0 (Celera Diagnostics, Alameda, CA). This assay is an Oligo Ligation Assay (OLA).²⁸ In OLA, polymorphic sites are detected by using a set of three oligonucleotides. One is common and hybridizes with both alleles. The two others hybridize specifically to one allele or the other. These allele-specific oligonucleotides have 5' modifier tails of different lengths. After hybridization, the common oligonucleotide is ligated with the immediately adjacent specific oligonucleotide producing allele-specific ligation products. No ligation occurs if there is a mismatch at the 3' end of the allele-specific oligonucleotide. Ligation products are analyzed by size. The Cystic Fibrosis Assay version 3.0 from Celera provides all reagents and primers to amplify and analyze 32 CF mutations in addition to the IVS-8 polymorphism following American College of Medical Genetics recommendations.²⁹ We use the assay for detection only and not for amplification. The R117H and the IVS-8 polymorphism are analyzed concurrently using as template 1.5 μ l of the 17.7-kb PCR product previously diluted with 3.5 μ l of "diluent for purified DNA" buffer. Oligonucleotide ligation is performed by adding 5 μ l of "OLA mix" containing CF OLA reagent v3 ASR and rTth DNA ligase (P/N 4338109 and P/N 4338114, respectively, Celera Diagnostics) and 0.5 μ l of CF v3 5/7/9T OLA ASR (P/N4338111). Products of the ligation reactions are run on the ABI Prism 3100 DNA sequencer instrument (Applied Biosystems) and analyzed using CF Genotyper version 3 from ABI. The R117H and the IVS-8 loci are analyzed in the green channel. Other mutation sites can be examined in the yellow channel.

IVS-8 Analysis Using Hybridization Probes

As a confirmatory detection method, we developed fluorescent resonance energy transfer (FRET) hybridization probes to analyze the IVS-8 locus by derivative melting

Figure 1. Experimental design. The three PCRs are represented under the map of the relevant section of the *CFTR* gene. "RH" is the PCR that amplifies both the wild-type and the R117H mutant allele. Ralsp and Halsp amplify the wild-type allele (R) and the mutant allele (H), respectively. Names of the primers are located next to **thick arrows** indicating their directions.

curve analysis on the LightCycler instrument (Roche Applied Science). The IVS-8 polymorphism is detected in the F2 channel using probes designed from GenBank Sequence Number M55114; 5'-(507) CTATTGTTATTGTTT-TGTTTTGCTTCTCAAATAATCCCCAAA-LCRed640-(464)-3' and 5'-(462)-FAM-CCCTGTTAAAAACACACACACAC-phosphate-(438)-3'. The probe is an exact match with the 5T allele (underlined). FRET probes were synthesized at Idaho Technology (Salt Lake City, UT).

At the end of PCR, 8 μ l of allele-specific long-range products are mixed with 1 μ l of each FRET probe at 2.4 μ mol/L (final concentration of each probe is 0.2 μ mol/L in 12 μ l final reaction) in a capillary tube. The melting cycle profile is as follows: reaction mixtures are denatured 5 minutes at 95°C and cooled to 40°C for 2 minutes. Temperature is then increased to 70°C with a transition rate of 0.1°C/second. Fluorescence is monitored continuously during this melting phase. Derivative melting curves distinguish between the 5T, 7T, and 9T alleles based on T_m .

Results

Long-Range Allele-Specific PCR

Three independent long-range PCRs are performed per sample: one reaction amplifies both R117H alleles of the heterozygous R117H sample, while the other two are allele-specific (Figure 1). Primer sequences are shown in Table 2. The three PCRs use a common reverse primer (IVS8-R, in exon 9), but three different forward primers. The forward primer of the "RH" PCR (R117H-F) anneals in exon 4, 5' of the mutation site. The allele-specific forward primers (Ralsp-F and Halsp-F) differ at the 3' end by the mutation-specific nucleotide. This assay differs from allele-specific PCRs in which both alleles are amplified in a single reaction with differently labeled primers. Here, only one of the alleles is amplified per allele-specific reaction.

Figure 2. Effect of annealing temperature on specificity of long-range allele-specific PCR. Derivative melting curves of the IVS-8 locus obtained from the melting profile of allele-specific long-range PCR products with FRET probes. The curves centered on 54°C indicate a 7T allele. The curves centered on 61°C indicate a 5T allele. **Interrupted lines** represent the "Halsp" reactions and continuous lines are from the "Ralsp" reactions. **Plain lines** indicate reactions performed with an annealing temperature at 60.2°C, and **lines with added filled circles** at 66.8°C. The **thin black line** represent the "no template" control.

Long-range PCR requires a polymerase with high fidelity. This property is provided by the 3' to 5' exonuclease activity of the enzyme.³⁰ This activity is presumed incompatible with allele-specific amplification because the mismatched 3' nucleotide is removed by the enzyme's proofreading activity. Several different polymerases with proofreading activity and ability to amplify long fragments were tested. Although allele-specific amplification of the 17.7 kb was observed with several kits, the TaKaRa LA *Taq* kit showed the most reproducible results (see Material and Methods). As shown in Figure 2, we found that the reaction specificity for one allele is improved by higher temperature. The sample shown in Figure 2 is heterozygous for the R117H mutation and carries the 5T/7T IVS-8 polymorphisms. The FRET probe set that detects the IVS-8 locus dissociates from a 5T allele at 60°C, a 7T allele at 54°C, and a 9T allele at 49°C (data not shown). When primer annealing is performed at 60.2°C during the long-range PCR, two derivative melting curves are detected (plain and dotted lines) indicating amplification from both alleles. When the annealing temperature is increased to 66.8°C, (lines with filled circles), only the allele-specific to the primer is amplified. Table 2 details the predicted T_m s for each primer (see also Material and Methods). The optimal annealing temperature for allele-specific amplification is approximately 9°C higher than the predicted T_m s of the perfectly matched allele-spe-

Table 2. Primers Sequences

PCR	Primer	Sequence	Predicted T_m (°C)	
			R allele	H allele
"RH"	R117H-F	5'-TG TAGGAAGTCACCAAGCAGTAC	58.2	58.2
"Halsp"	Halsp-F	5'-CCCGGATAACAAGGAGGAACA	55.8 (1.9)	<u>57.7</u>
"Ralsp"	Ralsp-F	5'-CCCGGATAACAAGGAGGAACG	<u>58.1</u>	55.8 (2.3)
	IVS8-R	5'-CAACCGCCAACAACGTGCC	57.7	57.7

Bold characters indicate the allele-specific nucleotide.

Underlined numbers indicate the predicted T_m of the allele-specific primer with the perfectly matched allele.

Delta T_m s between perfectly matched and mismatched primer/allele pairs are indicated in parenthesis.

Table 3. Genotypes and Haplotypes of R117H Samples

Sample	Genotype	Haplotype		Interpretation
		R chromosome (wild type)	H chromosome (mutant)	
1	R117H-5T/7T	R-5T	H-7T	<i>Trans</i>
2	R117H-5T/7T	R-7T	H-5T	<i>Cis</i>
3	R117H-5T/9T	R-9T	H-5T	<i>Cis</i>
4	R117H-5T/7T	R-5T	H-7T	<i>Trans</i>
5	R117H/Del F508-7T/9T	R-9T	H-5T	<i>Cis</i>
6	R117H-7T/7T	failed	H-7T	na
7	R117H-7T/7T	R-7T	H-7T	na
8	R117H-7T/9T	R-9T	H-7T	na
9	R117H-7T/7T	R-7T	H-7T	na
10	R117H-7T/7T	failed	H-7T	na
11	R117H-7T/7T	R-7T	H-7T	na
12	R117H-7T/7T	R-7T	H-7T	na
13	R117H-7T/7T	R-7T	H-7T	na
14	R117H-7T/9T	R-9T	H-7T	na
15	R117H-7T/9T	R-9T	H-7T	na
16	R117H/DelF508-7T/9T	R-9T	H-7T	na
17	R117H/G542X-7T/9T	R-9T	H-7T	na

na, not applicable.

cific primers. At this high temperature amplification from the mismatched primer, presumably shortened by one nucleotide by the enzyme proofreading activity and less stable (delta Tms around 2°C, Table 2), is minimal.

Haplotyping of R117H Samples

Sixteen samples with a variety of IVS-8 genotypes were haplotyped (Table 3). Three samples were heterozygous 5T/7T, one was 5T/9T, five were 7T/9T, and seven were homozygous 7T. The four samples with a 5T allele were haplotyped with both detection systems (OLA and FRET probes). Figure 3 shows OLA (A and B) and FRET (C and D) analysis of selected samples. In addition to the R117H and IVS-8 loci, the OLA analysis reveals results for other CF mutation loci included in the CF version 3.0 kit from Celera. In the same channel as R117H and IVS-8, the two extra peaks correspond to the wild-type alleles of the 621 + 1G > T and 711 + 1G > T mutations. In the yellow channel, the following loci can be examined: R347, I148, R334, and 1078. This is expected, since all these sites are found in the 17.7-kb amplified fragment. In the sample shown in Figure 3A all these loci are wild type. Mutations at these positions (621 + 1G > T, 711 + 1G > T, R347P, I148T, R334W, and 1078delT) could be detected and associated with one of the haplotypes. In this analysis, the samples shown in A and C (samples 1 and 4) carries the 5T allele on the wild-type chromosome (R117) therefore R117H and 5T are in *trans*. In sample 2 (B) and sample 3 (D), both mutations are in *cis*. Therefore these two individuals are carriers of a severe mutation in the *CFTR* gene. Results from all samples are summarized in Table 3. Five of the six possible allelic combinations were found in these samples. In the six samples with a 9T allele, this polymorphism is on the wild-type chromosome. Genotypes of R117/R117H locus and the 5T/7T/9T locus were confirmed by sequencing the extremities of each PCR obtained from sample 1 and 2 (data not shown).

Accuracy and Reproducibility

Several methods are available for genotyping independently both R117H and IVS-8^{27,31} but determination of the phase relies on family studies or unequivocal results such as homozygosity of the IVS-8 genotype or compound heterozygous with delF508-9T.^{24,27} Although R117H and the 5T IVS-8 polymorphism are found at relatively high frequency in populations affected with CF or with atypical CF,^{22,25-27} they are relatively rare in the normal population and we found only five R117H/5T-positive samples in more than 10000 CF tests. In only one case, analysis of parental DNA was possible and confirmed the molecular haplotyping data. The molecular assay, analyzed by OLA determined the haplotype of one sample to be R-5T, H-7T. The parent carrying the R117H mutation is IVS-8-7T homozygous and the other parent has the 5T allele (data not shown). Thus, this linkage study confirms the molecular diagnosis that R117H and 5T are in *trans* in this sample. Additionally, sample 5 and 16 (Table 3) and the Coriell cell line control GM13591 (Bernacki SH, Beck JC, Muralidharan K, Schaefer F, Shrimpton AE, Pont-Kingdon G, Stenzel TT, submitted) are compound heterozygous R117H and DelF508. DelF508 has been shown to be in *cis* with a 9T-allele.²⁴ At the IVS-8 locus, sample 16 is 7T/9T and sample 5 and GM13591 are 5T/9T. Molecular haplotyping determined the haplotypes as R117H-7T/DelF508-9T (sample 16) and R117H-5T/DelF508-9T (sample 5 and GM13591). In all cases, the assay associated correctly DelF508 with the 9T variant.²⁴

Specificity of the reaction to one allele is controlled by the PCR conditions and we tested within- and between-run reproducibility of the PCR. Within-run comparison was estimated by setting a triplicate PCR of two samples with a 5T allele. Haplotypes, analyzed by OLA provided identical results (data not shown). Additionally, four to five independent PCRs were performed on three samples and gave identical data. This suggests that the results

Figure 3. Analysis of long-range allele-specific PCR by OLA and FRET. **A** and **B**: OLA analysis. The three PCRs from a sample are analyzed in the "green" channel and organized from top to bottom as "RH", then "Halsp", and then "Ralsp". The horizontal axis represents fragment size in bp. The vertical axis indicates fluorescence. The bottom graph in **A** shows signals detected in the "yellow" channel that correspond to other positions (see text) on the 17.7-kb PCR fragment. **C** and **D**: Melting curve analysis of the IVS-8 polymorphisms. Amplification from both alleles ("RH" reaction) is in **gray**, wild-type allele-specific in **black**, and mutant allele-specific in **interrupted lines**. "No template" controls (**thin lines**) for the three reactions are also shown.

(and thus the haplotypes) are not artifacts from the allele-specific amplification reaction.

Discussion

This test allows the direct haplotyping of the R117H mutation and the 5T polymorphism of intron 8 of the *CFTR* gene. Both the R117H mutation and the IVS-8 variant have been found at a higher frequency in individuals affected with classic or atypical cystic fibrosis. This test will aid in the assessment of individuals undergoing CF carrier screening, diagnostic confirmation of affected individuals, and molecular testing of patients with atypical CF. If an individual is found to have the R117H mutation and the IVS-8 5T polymorphism, the molecular test can establish the *cis/trans* status of both mutations without the need to obtain DNA from parents.

Haplotyping technologies have rarely been applied to clinical testing for several reasons: methods are labor intensive and highly sophisticated,^{12,32} rely on extreme dilution of DNA not practical in a clinical setting,^{5,11} are limited to short fragments of DNA,^{6,8-10,13} or are not accurate enough to determine haplotypes from specific individuals.³³ A recent, elegant molecular approach that

uses post-PCR ligation has been described and would also allow the amplification and haplotyping of the R117H mutation and the IVS-8 5T polymorphism.¹⁵ This approach has the disadvantage of requiring additional post-PCR steps before analysis. Allele-specific amplification by PCR is another powerful and relatively easy method, but has typically been limited to small DNA fragments.¹⁴ Allele-specific amplification is commonly used in genotyping assays where both alleles are amplified with differently labeled primers and analyzed. One report describes the use of allele-specific amplification to analyze a 10-kb DNA fragment.³⁴ In this case, both alleles differ by an Alu insertion, providing a long specific sequence for the allele-specific primer. To our knowledge, our assay is the first report of long-range allele-specific PCR using a polymerase with proofreading activity and a single nucleotide polymorphism as a site for the specific primer. To haplotype, amplification of the DNA from only one allele is required per reaction and the annealing temperature during PCR is crucial to achieve complete specificity of the reaction. It is possible that the proofreading activity of the polymerase shortens the mismatched primer by one nucleotide giving the longer matched primer enough thermodynamic advantage to

participate in the final product. Both detection methods (OLA and FRET probe) are accurate. However, the OLA method has the advantage of analyzing both loci simultaneously. The analysis of the R117H loci from each PCR provides an internal control. An additional probe set, distinguishing the R117H mutation from the wild type, could also be added to the FRET probe assay and multiplexed with the IVS-8 probe. Another advantage of the OLA is the ability to address other loci in the amplicon, therefore examining additional association of mutations to an haplotype. Of particular interest is the analysis of the (TG) tract found adjacent to the T- tract in IVS-8. Length polymorphism of the TG sequence is reported to influence splicing efficiency of intron 8 which might explain the partial penetrance of the 5T tract.^{18,21,22,35,36} Detection systems other than the two described in this publication can also be used to analyze the long-range PCR products.

The demonstration of the compatibility between long-range and allele-specific PCR provides an easy approach to molecular haplotyping of other target genes. Since long-range PCR can synthesize fragments up to 40 kb, multiple loci can be analyzed on these long fragments of DNA, identifying defined haplotypes. As the average size of haplotype blocks in the human genome is less than 20 kb,¹ long-range allele-specific PCR can be designed to study specific haplotype blocks.

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