Allele-Specific PCR for Determination of IL28B Genotype

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The IL28B genotype is a critical determinant of interferon response in patients infected with hepatitis C virus genotype 1. We describe an allele-specific PCR assay for the IL28B genotype. The assay is simple and robust, uses commonly available real-time PCR instrumentation, and is well suited for clinical laboratories offering IL28B genotyping.

Interferon is used to treat hepatitis C virus (HCV) infections, but many patients do not respond. Several tightly linked single nucleotide polymorphisms (SNPs) in noncoding regions near the *IL28B* gene on chromosome 19 are strongly associated with response rates for HCV genotype 1 infections (5, 12, 15–17). The IL28B genotype is therefore useful in determining the appropriate therapy for these individuals (10). Here we describe a simple and robust allele-specific PCR assay for determination of the IL28B genotype.

The assay distinguished C versus T at rs12979860, which is associated with interferon response (5). The assay was performed in two wells, each containing the same 5' primer (CA AGCGGCGCTTATCGCATACGGCTA) and TaqMan probe (6-carboxyfluorescein [6-FAM]-CTGGCAGCGCACG-MGBNFQ). One well contained a C-specific 3' primer (GTGCAATTCAACC CTGGTACG), and the other contained a T-specific primer (GTG CAATTCAACCCTGGTACA). The 3' primers included a deliberate mismatch with the wild-type A at position –3 from the 3' end (Table 1) to further decrease the efficiency of the mismatched amplification reaction. Primers and probes for amplification of the internal control (EXOBS forward, 5'-AATTGGAAGTGGC GGAAGAA-3'; EXOBS reverse, 5'-GGAACCTAAGACAAGTGT GTTTATGG-3'; and EXOBS probe, 5'-VIC-AGCTATTGC AAACGCCATCGCACAA-6-carboxytetramethylrhodamine [TAMRA]) were included in the master mix (Life Technologies AmpliTaq DNA polymerase).

Two hundred microliters of whole blood was extracted using the Roche MagNA Pure LC (DNA isolation kit I) and eluted to a volume of 100 μ l. Extractions were done using lysis buffer spiked with internal control plasmid containing a 118-bp segment of jellyfish DNA at a concentration resulting in approximately 50 copies of plasmid in the final PCR. (For quality control [QC], the threshold cycle [C_T] for the internal control must equal 32 ± 1.5 .) Positive control plasmids contained a 261-bp amplicon with either the C or T allele at the rs12979860 SNP. (For QC, the C_T must equal 25 ± 1.5 .) PCR mixtures contained 10 μ l of DNA, 400 nM primers, 100 nM probe, and 40 μ l of master mix, using standard TaqMan conditions on an ABI 7500 real-time PCR instrument.

For genotype calls, raw PCR data were imported into an Excel macro that compared the SNP threshold cycle (C_T) for each allele-specific reaction. Samples with the C/C genotype were amplified faster with the C-specific primer as follows. If $C_T(C) - C_T(T)$ was ≤ -8 , the sample was considered C/C; if $C_T(C) - C_T(T)$ was ≥ 8 , the sample was considered T/T; finally, if $C_T(C) - C_T(T)$ was 2 to –2, the sample was considered C/T. All samples could have their genotypes definitely called using this algorithm.

We compared amplifications with 6 different 3'-allele-specific primers to determine those with maximum efficiency and minimum cross-reactivity for C and T genotypes. Primers containing an additional mismatched A at the 3' third to last base position in addition to the SNP-specific base provided the best specificity (Table 1; see Fig. S1 in the supplemental material). The –3 base mismatch did not substantially impact detection of the matched allele but reduced amplification of the mismatched allele by approximately 1,000-fold (9 cycles) (Table 1).

Assay performance was confirmed by testing DNA from 19 samples from a cohort previously described by one of us (12a). The new allele-specific assay gave identical results to those obtained at Duke University using the ABI TaqMan allelic discrimination kit (12a, 19) (6 C/C, 10 C/T, and 3 T/T).

We evaluated the impact of sample transportation and storage on assay performance, using EDTA-treated whole-blood samples transported to the laboratory at room temperature. The ability to clearly assign genotype was not impacted by additional storage of EDTA-treated whole blood at room temperature for 1 to 2 days, for up to 6 days at +4°C, or for a month or more at –20°C prior to testing (see Fig. S2 in the supplemental material). Validation on leftover EDTA-treated whole-blood samples submitted for routine hematological tests over a period of 1 month (transported at room temperature and stored up to 1 week at +4°C) gave unambiguous genotype results for all samples (Table 2). All positive, negative, and internal control reactions were within established quality control ranges.

Over the first 8 months of use, the assay was performed on 94 clinical samples in 35 once-weekly runs. Samples were transported at room temperature and stored for up to 1 week at 4°C prior to testing. All samples passed quality control criteria for the internal control and typing reactions. We observed a higher percentage of genotype T/T than was found in our general population (Table 2), probably because the test was more frequently ordered for patients who did not clear spontaneously or who had a poor treatment response. The mean C_T difference between the C and T reactions

Received 6 August 2012 Returned for modification 31 August 2012

Accepted 4 October 2012

Published ahead of print 10 October 2012

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Supplemental material for this article may be found at <http://jcm.asm.org/>.

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doi:10.1128/JCM.02084-12

TABLE 1 Candidate allele-specific primers and plasmids used in this study

Primer sequence ^a	<i>C_T</i> for plasmid	
	C genotype	T genotype
C allele primers		
GTGCAATTCAACCCTGGTTCG	27.6	31.4
GTGCAATTCAACCCTGGTTAG	35.4	>42
GTGCAATTCAACCCTGGTACG	28.7	>42
T allele primers		
GTGCAATTCAACCCTGGTTCA	34.2	30.0
GTGCAATTCAACCCTGGTTAA	>42	35.3
GTGCAATTCAACCCTGGTACA	>42	30.6

^a The underlined bases are those detecting the C and T alleles of the SNP. Boldface A bases are deliberately constructed mismatched bases.

for the C/C genotype was -9.4 (2 standard deviations [SDs]; range, -10.9 to -8.0). For the T/T genotype, the difference was 10.3 (2 SDs; range, 9.3 to 11.3), and for the C/T genotype, the difference was -0.1 (2 SDs; range, -1.3 to 1.2).

Quality control parameters indicated that the assay was stable over 8 months of clinical use; *C_T*s for the positive controls were very consistent, with excellent means and coefficients of variation (CV) (Table 3). The CV for patient samples was slightly higher, perhaps due to either differences in patient white blood cell counts or additional variation from the extraction step for the patient samples.

Given the utility of IL28B genotyping in choosing therapy for HCV-infected individuals, a simple, robust, and accurate assay is needed. Previously described SNP genotyping methods include restriction fragment length polymorphism (RFLP) (11), TaqMan allelic discrimination PCR (14, 18, 19), direct sequencing (7), the Invader assay (8), high-resolution melt (HRM) (9), tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) (4), pyrosequencing (2), the melt-mismatch amplification mutation assay (melt-MAMA) (3), and next generation sequencing (13). Ito et al. found good agreement between five methods (direct sequencing, Invader, TaqMan allele-discrimination, high-resolution melt, and hybridization probes) for the determination of the IL28B genotype (7). Cariani et al. compared sample types (swabs, blood, serum, and formalin-fixed paraffin-embedded [FFPE] liver biopsy specimens) for a probe-based allelic discrimination assay (1) and obtained identical results from all sources. These results suggest that the choice of method can be based largely on factors important in clinical laboratories, including simplicity and robustness.

We have designed an allele-specific PCR assay for the IL28B genotype and evaluated its performance over an extended period

TABLE 2 Genotype distribution in tested samples

Genotype	No. (%) of:	
	Uninfected clinical samples (<i>n</i> = 34)	Clinical tests of HCV-infected patients (<i>n</i> = 94)
C/C	15 (44)	29 (31)
C/T	17 (50)	45 (48)
T/T	2 (6)	20 (21)

TABLE 3 Assay stability in clinical use^a

Plasmid or genotype	No. of samples tested	Mean C _T (CV [%])	
		C allele mix	T allele mix
Plasmids			
C control	35	26.0 (2.2)	36.3 (2.3)
T control	35	36.7 (2.4)	26.6 (1.8)
Genotypes			
C/C patient	29	26.2 (3.2)	35.8 (2.2)
C/T patient	45	27.4 (3.3)	27.4 (3.3)
T/T patient	20	37.7 (8.8)	26.4 (3.3)

^a The assay was run once weekly over an 8-month period.

of clinical use. Our assay differs from the previously published allelic discrimination TaqMan assays because it evaluates success versus failure of the amplification reaction, rather than differential association of allele-specific probes. We believe that our allele-specific assay may offer advantages to clinical laboratories. Since mismatches between the probe and target, including previously unknown viral variants, can markedly affect *C_T* values for real-time PCR (6), allelic discrimination PCR may perform unpredictably if such viral variation exists anywhere within the probe region. Our assay should be robust for detection of rare viral variants, which would only affect amplification when present at the extreme 3' end of the allele-specific primers. In this case, the specific amplifications for both alleles would be affected and fall outside the control ranges. Such specimens could then be evaluated by sequencing. However, we did not observe this in the first 94 samples tested, so it is likely to be an uncommon occurrence. Finally, our allele-specific assay is simple to perform and utilizes only a simple Excel macro and standard real-time PCR software provided with most instruments. Thus, it could be readily incorporated into the work flow of any laboratory performing real-time PCR.

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