A rapid, duplex, high-resolution melting interleukin-28B gene (IL28B) genotyping assay, targeting both rs12979860 and rs8099917 polymorphisms, was developed and validated using 30 DNA samples from healthy volunteers. A linkage study on 300 healthy Singaporeans showed variable haplotypes. When the assay was applied to plasma DNA from 50 hepatitis C virus genotype-1 (HCV-1)-infected patients, five compound heterozygous types were detected.

The interleukin-28B gene (IL28B) polymorphism was recommended by the American Association for the Study of Liver Diseases (AASLD) as an important pretreatment predictor of sustained virological response (SVR) to pegylated interferon (PEG-IFN) and ribavirin (RBV), as well as to protease inhibitor triple therapy, in chronic hepatitis C virus genotype-1 (HCV-1) patients (5). Laboratory testing of IL28B polymorphism is currently more focused on rs12979860, which was the first single-nucleotide polymorphism (SNP) reported to be significantly associated with treatment-induced viral clearance (4), while rs8099917 genotyping is less available commercially (9). Both SNPs were reported to have high positive predictive values (PPV) for SVR in HCV-1 patients (6, 11). In our laboratory, a single-tube high-resolution melting (HRM) assay for simultaneous genotyping of rs12979860 and rs8099917 was developed. We genotyped the IL28B polymorphisms of 50 HCV-1-infected patients and analyzed the linkage disequilibrium (LD) of rs12979860 and rs8099917 in 300 healthy subjects from three major ethnic groups (Chinese, Malays, and Indians) residing in Singapore. Sample collection for research was approved by the National Healthcare Group Domain Specific Review Board in Singapore.

The HRM duplex assay was developed and evaluated using nucleic acid samples extracted from both plasma/serum (n = 50) and peripheral blood (PB) (n = 300). Real-time duplex PCR and HRM were carried out using a LightScanner 32-instrument (Idaho Technology, Inc., Salt Lake City, UT) on a 10-μl reaction mixture containing 1× LightCycler FastStart reaction mixture containing 1× LCGreen plus (Idaho Technology, Inc.), and 1 μl of the extracted nucleic acid template. The thermal cycling conditions include an initial hold at 95°C for 10 min, followed by 50 or 70 cycles (95°C for 10 s, 66°C for 15 s, and 72°C for 20 s) for DNA extracted from PB or plasma/serum, respectively. Subsequent HRM was performed with a continuous fluorescence acquisition mode from 70°C to 92°C at a ramp rate of 0.3°C/s. The normalized melting regions for rs8099917 and rs12979860 were 76°C to 82°C and 86°C to 92°C, respectively. The normalized melt peaks of the 3 variants were clearly discernible for both SNPs (Fig. 1).

Five of nine possible compound genotypes were observed in the 50 HCV-1-infected patients: (i) rs12979860 CC rs8099917 TT (n = 31; 62%), (ii) rs12979860 CT rs8099917 TG (n = 11; 22%), (iii) rs12979860 CT rs8099917 TT (n = 5; 10%), (iv) rs12979860 CT rs8099917 GG (n = 2; 4%), and (v) rs12979860 TT rs8099917 TT (n = 1; 2%). Both observed genotype and haplotype frequencies were determined in 100 Chinese, 100 Malay, and 100 Indian healthy subjects (Table 2) by direct genotype count and compared by χ² test with

### Table 1: Primer sequences for duplex HRM, presequencing PCR, and cycle sequencing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5′–3′)</th>
<th>Amplicon length (bp)</th>
<th>Corresponding human genomic region (NT_011109.16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs12979860N1F</td>
<td>CCTGGACGTGGATGGGTA</td>
<td>102</td>
<td>12006931–12006948</td>
</tr>
<tr>
<td>rs12979860N1R</td>
<td>GCGCGGAGTGCAATTCAA</td>
<td>120</td>
<td>12007015–12007032</td>
</tr>
<tr>
<td>rs8099917HRMF</td>
<td>CACGTGTCCTCCTTTGTTTCC</td>
<td>292</td>
<td>12006870–12006889</td>
</tr>
<tr>
<td>rs8099917HRMR</td>
<td>CATAAAAAGCAGCTACCAACTG</td>
<td>31</td>
<td>12007142–12007161</td>
</tr>
<tr>
<td>rs12979860SeqR</td>
<td>GCCATGTCAGGCTGCCTAGGG</td>
<td>89</td>
<td>12011436–12011368</td>
</tr>
<tr>
<td>rs8099917SeqR</td>
<td>TCCATACAAAAATCATACCAATGGGA</td>
<td>172</td>
<td>12011283–12011287</td>
</tr>
<tr>
<td>rs8099917SeqR</td>
<td>CATAAAAAGCAGCTACCAACTG</td>
<td>120</td>
<td>12011436–12011368</td>
</tr>
</tbody>
</table>

**Footnote:** Cycle sequencing primer.

The table provides primer sequences used for duplex HRM, presequencing PCR, and cycle sequencing, along with the amplicon lengths and corresponding human genomic regions.
the values predicted by the assumption of Hardy-Weinberg equilibrium. The coefficient of linkage equilibrium ($D^*$), $r^2$, and haplotype prevalence of the three major ethnic groups in the Singapore population were calculated using web-based software (http://www.oege.org/software) as described elsewhere (3, 10). Three of the 4 possible haplotypes with C-T (C allele for rs12979860 and T allele for rs8099917) were observed in the Singapore study cohort as the major haplotypes: two among the Chinese (C-T, 92%; T-G, 8%) and three among both the Malays (C-T, 89.5%; T-G, 6.5%; T-T, 4%) and the Indians (C-T, 77%; T-G, 10%; T-T, 13%). The C-G haplotype was not found among these three main ethnic groups in the Singapore population. In Singaporeans of Chinese descent, the two SNPs were observed to be in perfect LD ($D^* = 1.0$, $r^2 = 1.0$), with the rs12979860 C allele linked to rs8099917 T and the rs12979860 T allele linked to rs8099917 G. Three of 4 possible haplotypes (C-T, T-G, and T-T) were observed in the Malays ($D^* = 1.0$, $r^2 = 0.5926$) and Indians ($D^* = 1.0$, $r^2 = 0.3720$).

The accuracy of the HRM assay results was determined by Sanger sequencing (with two sets of sequencing primers; Table 1), using 30 randomly picked samples from the healthy cohort. All 30 genotypes obtained from the duplex HRM assay corresponded with their respective Sanger sequencing results.

HRM approaches have recently been adopted in IL28B genotyping (2, 7). Ito et al. evaluated monoplex HRM assays for the genotyping of four IL28B SNPs (7), while Fonseca-Coronado et al. used a melt-mismatch amplification mutation assay (melt-MAMA) which required multiple sets of primers with GC tails for genotyping the two IL28B SNPs (2). The use of a duplex HRM format in our assay requires only two sets of primers without any modifications for the simultaneous determination of rs12979860 and rs8099917 within a single tube. It is less labor intensive and more cost effective than Sanger sequencing and TaqMan genotyping assays, as expensive fluorescence-labeled probes are not involved and only a saturating dye is needed for detection. In addition, the single closed-tube reaction format minimizes the risk of carryover contaminations, and the high sensitivity attainable by this duplex HRM assay makes it a good option for routine diagnostic testing of the IL28B genotype.

As rs12979860 is located close to rs8099917 on chromosome 19 (only 4,378 bases apart), the two SNPs are in partial LD. High LD has been reported in Hispanics ($r^2 = 0.78$) and Caucasians ($r^2 = 0.52$), but very low linkage was found in African Americans ($r^2 = 0.07$) (4). In this study, using the duplex HRM assay, the two SNPs were simultaneously genotyped in 300 healthy individuals, a representative cross-section of the Singapore population. Both SNPs were observed to be in perfect LD ($r^2 = 1.0$) in the Chinese, and high linkage values were observed in the Malays and Indians. Interestingly, the T-T haplotype was observed, in addition to C-T and T-G, among Indians. This phenomenon is not predictable, by virtue of the rs12979860 unfavorable T allele being linked with the rs8099917 favorable T allele. When the rs12979860 and rs8099917 genotypes of an HCV-1 patient are not in agreement in predicting the treatment outcomes, other relevant clinical factors, such as baseline viral load, should be considered when making treatment decisions.

IL28B polymorphisms were also reported to be associated with high SVR in HCV-1-infected patients receiving treatments involving direct-acting antiviral agents (DAAs) (1). While overall SVR increases, patients are also subjected to adverse effects of the DAAs. Hence, HCV-1-infected patients should be stratified based on their IL28B polymorphisms into those with higher likelihood of SVR and those with non-SVR, to tailor treatment accordingly.
However, when factors concerning potential of medical adherence, cost, tolerability, and availability of alternate DAA medications are considered, the knowledge of *IL28B* polymorphisms will be of less relevance (8).

In summary, we developed a sensitive, cost-effective, and less-laborious duplex HRM assay to genotype two *IL28B* polymorphisms (rs12979860 and rs8099917) simultaneously within a single tube and showed that it is applicable to cell-free nucleic acids from plasma and sera. This assay can be readily adopted by any molecular diagnostic laboratory with HRM capability and will be clinically useful in predicting treatment response in HCV-1-infected patients.

**REFERENCES**