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Primer Sequence Modification Enhances Hepatitis C Virus Genotype Coverage

We applaud the efforts of Cook et al. to develop a real-time reverse transcription-PCR (RT-PCR) assay for ultrasensitive quantification of hepatitis C virus (HCV) RNA (4). The assay uses three forward primers, one reverse primer, and two TaqMan probes targeting the HCV highly conserved 5′ untranslated region (UTR) and has a linear dynamic range from 50 to 10^9 IU/ml plasma specimens, which is significantly superior to several currently available commercial kits (1). As the authors declared, the assay covers the main HCV genotypes encountered in the United States and detects genotypes 1a, 2a, and 3a with equal efficiency; however, the authors' experience in covering other genotypes was limited.

We have spent time adapting the assay and validating it initially on a panel of 12 plasma specimens (Table 1), including HCV genotypes/subtypes 1a, 1b, 2a, 2b, 3a, 4a, 6, and 6b and a negative control (genotypes/subtypes were determined by a sequencing-based TRUGENE HCV 5′NC genotyping kit [Bayer Diagnostics, Terrytown, NY] according to the manufacturer's instructions [5]). The 11 positive specimens had HCV viral loads from 58,300 to 4,870,000 IU/ml (viral loads were quantitated by a COBAS MONITOR HCV test [Roche Diagnostic Corporation, Indianapolis, IN] as described previously [5]). With the exception of one specimen (genotype 6b) that was collected from a patient whose HCV infection was contracted in South Korea, all 11 positive specimens were amplified correctly. An alignment of nucleotide sequences in the 5′ UTR of several HCV isolates circulating in Asia was performed (2, 3, 6–8). The results indicated that, in comparison to genotypes encountered in the United States, most of these Asian strains had a single-nucleotide truncation at the region where the forward primers were designed (4).

We then explored two solutions to extend the coverage of these Asian genotypes by modifying the forward primers used in the RT-PCR: (i) an additional forward primer (HCV-F4 [5′-GAC ACT CCR CCA TKA TCA CT-3′, where R is A or G and K is G or T]) was designed and added to cover the one nucleotide truncation, and (ii) the three original forward primers were replaced with one newly designed forward primer (KY80M [5′-AAG CGT CTA GCC ATG GCG T-3′]), which was modified from the original KY80 primer published previously (9).

Both implementations were able to cover the 6b genotype (specimen no. 3250) that was missed by the original primer/probe set (Table 1). Based on the values of the threshold cycle (C_T), i.e., the cycle number at which the amount of amplified gene of interest reached a fixed threshold, the analytical sensitivity was slightly decreased by the addition of the first new primer into the RT-PCR, but the difference between the C_T values was not statistically significant ($P > 0.05$). On the other hand, when the new single forward primer was implemented to replace the original three forward primers, there was a significant improvement in the test's analytical sensitivity, as indicated by decreased C_T values ($P = 0.016$) (Table 1).

Although the KY80M primer was based on a highly conserved region of the HCV genome, we hesitated to implement the primer in a real-time RT-PCR since the primer can form a four-pair nucleotide hairpin, which was probably the reason for exclusion by the Primer Express software (Applied Biosystems, Foster City, Calif.). However, when the annealing temperature reaches 60°C, the temperature used in the TaqMan real-time procedure, no hairpin formation is indicated (data not shown).

Based on the findings, we have replaced the original three forward primers in Cook's paper with the single KY80M forward primer for our routine HCV quantitation practice. Efforts are being spent to determine the analytical and clinical sensitivity of the TaqMan assay with the new forward primer for HCV quantitation on larger numbers of clinical specimens with a variety of genotypes.

TABLE 1. Threshold cycle values for HCV quantitation by TaqMan real-time RT-PCR

Specimen no.	Genotype/subtype	Viral load (IU/ml)	C_T value[a]		
			Use original forward primers	Add an additional primer	Implement a new forward primer
6872	1a	3,980,000	24.3	23.5	22.6
6804	1a	625,000	27.0	28.5	27.3
6855	1b	4,500,000	23.7	28.1	25.6
6761	1b	2,300,000	26.9	26.5	25.1
5959	2a	1,080,000	30.9	30.9	30.2
6408	2b	4,870,000	27.9	27.8	25.5
6608	3a	89,600	33.8	33.9	31.7
6097	3a	636,000	34.1	34.0	30.9
1802	4a	765,000	32.6	33.8	29.4
1687	6	2,130,000	28.0	26.2	23.3
3250	6b	58,300	45.0[b]	33.8	31.5
Mean ± SD[c]	NA[d]	NA	28.9 ± 3.7	29.3 ± 3.7	27.2 ± 3.2

[a] Each C_T value is an average of two values obtained when the experiment was performed in duplicate.
[b] The RT-PCR was run for 45 cycles; therefore, a C_T value of 45 indicates a negative finding.
[c] When means and standard deviations were calculated, data for specimen no. 3250 were omitted.
[d] NA, not applicable.

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Yi-Wei Tang*
Susan E. Sefers
Haijing Li
Departments of Medicine and Pathology
Vanderbilt University Medical Center
4605 TVC, 1161 21st Ave. S.
Nashville, TN 37232-5310

*Phone: (615) 322-2035
Fax: (615) 343-8420
E-mail: yiwei.tang@vanderbilt.edu