

# Hepatitis C Genotype Determination by Melting Curve Analysis with a Single Set of Fluorescence Resonance Energy Transfer Probes

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**Background:** The genotype of hepatitis C virus (HCV) is a predictor of antiviral therapeutic response. We describe an approach for HCV genotype determination by real-time PCR and melting curve analysis.

**Methods:** After automated nucleic acid extraction, we used reverse transcription-PCR in a block cycler to amplify nucleotides 6–329 of the 5'-untranslated region of HCV. The product was further amplified by single-tube real-time seminested PCR in a LightCycler™ instrument (Roche). The final product was analyzed by melting curves with the use of fluorescence resonance energy transfer (FRET) probes. The FRET sensor probe was directed at nucleotides 151–170 of type 1 HCV and was designed to distinguish types 1a/b, 2a/c, 2b, 3a, and 4, with melting temperatures ( $T_m$ s) predicted to differ by 1 °C. Genotypes were compared in a blinded fashion with those of the INNO-LiPA™ test (Bayer Diagnostics) on 111 serum samples.

**Results:** In preliminary experiments, the  $Mg^{2+}$  concentration was found to be critical in allowing clear separation of melting points, with the best separation at a  $Mg^{2+}$  concentration of 2 mmol/L. The results for 111 samples clustered at expected  $T_m$ s for genotypes 1a/b ( $n = 78$ ), 2a/c ( $n = 2$ ), 2b ( $n = 11$ ), 3a ( $n = 14$ ), and 4 ( $n = 2$ ). Of the 111 samples, results for 110 were concordant with the comparison method at the level of type 1, 2, 3, or 4. Subtyping results were discordant for two samples, both of type 2. For 108 samples concordant with INNO-LiPA at the genotype and subtype levels, the mean  $T_m$ s were 64.1, 59.5, 54.2, 52.6, and 50.1 °C for types 1a/b, 2a/c, 4, 2b, and 3a, respectively, with SDs of 0.2, 0.3, 0.3, 0.2, and 0.3 °C. All 78 samples identified as type 1 were concordant with results of the comparison method.

**Conclusions:** Melting analysis with a single pair of FRET probes can rapidly provide information about HCV genotypes and identifies type 1 samples with high specificity.

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In 1997, an estimated 4 million people in the US and 150 million people worldwide were reported as chronically infected with hepatitis C virus (HCV),<sup>1</sup> and many were unaware of the infection. In the US, ~30 000 acute new infections occur each year and ~10 000 people die annually from sequelae of the infection (1, 2). HCV infection is the most common cause of chronic viral hepatitis, often leading to end-stage liver disease and/or hepatocellular carcinoma; thus, the effects of HCV infections have become the leading indication for liver transplantation in adults in the US (3, 4). Although the use of universal precautions and screening of the blood supply for HCV has decreased the incidence of newly diagnosed HCV infections in the US, the annual mortality attributable to the slowly progressive liver disease caused by HCV is expected to triple in the next 10–20 years (2).

Soon after isolation of the first cDNA clone of HCV (5), sequencing of isolates from several geographic locations (6–11) revealed extreme variability in sequence (12). The regions of the viral genome that encode the envelope proteins were the most variable (13), and the 5'-untranslated region (UTR) was the most conserved region among strains (14). Comparison of published sequences of HCV has led to the identification of at least 11 genotypes and >70 subtypes (12). Distinct genotypes differ from each other by as much as 33% over the entire viral genome, and within subtypes, the sequence identity is only 75–86% (4). In the US, genotypes 1a, 1b, 2a/c, 2b, and 3a are the most

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<sup>1</sup> Nonstandard abbreviations: HCV, hepatitis C virus; UTR, untranslated region; RT-PCR, reverse transcription-PCR; FRET, fluorescence resonance energy transfer; FITC, fluorescein isothiocyanate; and  $T_m$ , melting temperature.

common (4); the other genotypes are rarely found. Genotypes 4, 5, and 6 are most prevalent in North Africa and the Middle East, South Africa, and Hong Kong, respectively. Genotypes 7, 8, and 9 have been identified only in Vietnamese patients, and genotypes 10 and 11 have been identified in patients from Indonesia (4).

The determination of HCV genotype provides clinically important information that can be used to direct the duration and type of antiviral therapy and to predict the likelihood of sustained HCV clearance after therapy (1, 2, 15, 16). Because patients with HCV genotype 1 may benefit from a longer course of therapy and genotypes 2 and 3 are more likely to respond to combination interferon-ribavirin therapy, the common, clinically relevant distinction in the US population is between genotype 1 and types 2 and 3 (17–19). Insufficient data currently exist to determine the likelihood of therapeutic response for HCV genotypes 4, 5, 6, and 7.

In clinical laboratories, HCV genotypes are determined by sequencing or by the INNO-LiPA™ test (Bayer Diagnostics), often referred to as the line-probe assay. The latter test requires reverse transcription-PCR (RT-PCR) of the viral genome with biotinylated primers. Any resulting biotin-labeled amplicons are hybridized to an array of genotype/subtype-specific probes that have been immobilized to strips of nitrocellulose membrane. The immobilized amplicons are detected by use of an enzymatic colorimetric detection system. Although less technically demanding than sequencing, the procedure involves multiple steps and is time-consuming. High-throughput sequencing (DupliType™; Quest Diagnostics) has been developed for HCV genotyping, but the methodology and equipment are best suited to large laboratories with high test volumes.

We describe an HCV genotyping assay to distinguish genotypes 1, 2, 3, and 4 by use of melting curve analysis with a using a single set of fluorescent resonance energy transfer (FRET) probes.

## Materials and Methods

### PATIENT SAMPLES AND CONTROLS

Excess sera from samples submitted between January and May of 2001 to the University of Virginia Medical Laboratories for HCV genotyping were stored at  $-20^{\circ}\text{C}$  for 12–24 months after independent genotype determination by American Medical Laboratories (Chantilly, VA), using INNO-LiPA. Samples previously identified as HCV genotypes 1a, 1b, 2a/c, 2b, 3a, and 4 were used to develop the real-time PCR with melting curve genotyping assay on the Roche LightCycler™. At least one positive patient sample and a reagent control were processed in parallel with each batch of samples. Samples were processed in groups of 8, 16, or 32.

For validation of the developed genotyping assay, 124 samples were selected without conscious bias from the stored samples. These samples were genotyped with the

developed genotyping assay; melting curves were interpreted by three investigators blinded to the previously determined genotypes. Discrepant samples were sent to Quest Diagnostics, Nichols Institute (San Juan Capistrano, CA) for genotype verification by the DupliType sequencing assay. The DupliType assay sequences across the NS-5B and core regions of HCV (Quest Diagnostics).

### PRIMERS AND PROBES

RT-PCR was performed using two primers with complementary sequences located in the 5'-UTR that are conserved among known HCV genotypes (20). Nucleotide designation is according to Choo et al. (5). Primers for RT-PCR consisted of the forward primer NAF1 (nucleotides 6–26; 5'-GGCGACTCCACCATAGATC-3'), and the reverse primer NAR1 (nucleotides 329–309; 5'-GGTGCACGGTCTACGAGACCT-3'). Primers and probes for seminested PCR and HCV genotype determination in the LightCycler consisted of the forward primer NAF1, the reverse primer NAR3 (nucleotides 289–269; 5'-CCCTATCAGGCAGTACCACAA-3'), the FRET anchor probe HCVG-fluorescein isothiocyanate (FITC; nucleotides 125–148; 5'-GCCATAGTGGTCTGCGGAACCGGT-FITC-3'), and the FRET sensor probe RED-HCVG (nucleotides 151–170; 5'-LCRed640-GTACACCGGAATTGCCAGGA-phosphate-3').

PCR primers and FRET probes were purchased from Idaho BioChem. Solutions of each PCR primer and probe were prepared in nuclease-free Tris-EDTA buffer (10 mmol/L Tris, pH 8.3, containing 0.1 mmol/L EDTA) provided with the primers. Working dilutions (1:10; 20  $\mu\text{L}$  of stock plus 180  $\mu\text{L}$  of buffer) were prepared in the same buffer. FRET probes were protected from light and high centrifugal forces to prevent degradation or precipitation of the fluorophores.

### NUCLEIC ACID ISOLATION AND RT-PCR

Total nucleic acid was extracted from 200  $\mu\text{L}$  of serum by use of the Roche MagNA Pure LC Instrument and the MagNA Pure LC Total Nucleic Acid Isolation Kit, version 1 (cat. no. 3038505; Roche Molecular Biochemicals) and eluted with 100  $\mu\text{L}$  of elution buffer according to the manufacturer's instructions. We combined 10  $\mu\text{L}$  of extracted total nucleic acid with 40  $\mu\text{L}$  of RT-PCR master mixture. RT-PCR was performed in the Applied Biosystems GeneAmp 9600 PCR instrument. Each 50- $\mu\text{L}$  RT-PCR reaction contained 1 $\times$  AmpliTaq PCR buffer and 2 U of AmpliTaq polymerase (cat. no. N808-0166; Applied Biosystems); 40  $\mu\text{M}$  each of dATP, dCTP, dGTP, and TTP (cat. no. N808-0007; Applied Biosystems); 0.2  $\mu\text{M}$  forward primer NAF1; 0.2  $\mu\text{M}$  reverse primer NAR1; 10 units of RNAsin (cat. no. N2111; Promega); and 1.5 U of AMV reverse transcriptase (cat. no. M5101; Promega). Reverse transcription was performed at  $42^{\circ}\text{C}$  for 30 min, followed by denaturation at  $95^{\circ}\text{C}$  for 5 min. Subsequent PCR amplification consisted of 25 cycles of denaturation at

95 °C for 30 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 7 min.

#### REAL-TIME PCR WITH MELTING CURVE ASSAY

Seminested, "hot start" PCR reactions were performed in a final volume of 10  $\mu$ L, using the LightCycler-FastStart DNA Master Hybridization Probes Reaction Kit (cat. no. 3003248; Roche). We combined 2.5  $\mu$ L of RT-PCR reaction product with 7.5  $\mu$ L of master mixture in glass capillaries, and seminested PCR was performed in the Roche LightCycler. Each 10- $\mu$ L seminested PCR reaction contained 1 mM MgCl<sub>2</sub>, 0.25  $\mu$ M forward primer NAF1, 0.25  $\mu$ M reverse primer NAR3, 0.2  $\mu$ M HCVG-FITC probe, 0.2  $\mu$ M RED-HCVG probe, and 1 $\times$  LightCycler FastStart DNA Master Hybridization Probes Mix (contributing an additional 1 mM MgCl<sub>2</sub> so that the final MgCl<sub>2</sub> concentration was 2 mM per reaction). After a preincubation step at 95 °C for 10 min to activate the FastStart polymerase, PCR amplification was 50 cycles of denaturation at 95 °C for 3 s and a temperature transition rate of 20 °C/s, annealing at 56 °C for 10 s with a temperature transition rate of 20 °C/s, a single fluorescence measurement taken at the end of the annealing step, and extension at 72 °C for 12 s with a temperature transition rate of 0.5 °C/s. After amplification, melting curve analysis was performed by heating to 95 °C for 5 s with a temperature transition rate of 20 °C/s, cooling to 40 °C with a temperature transition rate of 20 °C/s, holding at 40 °C for 30 s, and then heating the sample at 0.1 °C/s to 80 °C. In two experiments, we detected no improvement in the sharpness of peaks or in melting temperature ( $T_m$ ) differences between genotypes (1 vs 2b or 3a) when slower (0.05 °C/s) or faster (0.2 and 0.5 °C/s) transition rates were used (not shown). Fluorescence data were collected continuously during this heating to monitor the dissociation of the RED-HCVG sensor probe. The derivative melting curves were obtained with the LightCycler data analysis software, Ver. 3.5 (Roche).

Mg<sup>2+</sup> titration experiments were done with patient samples representing genotypes 1b, 2a/c, 2b, and 3a as determined by the line-probe assay. The RT-PCR, seminested PCR, and melting peak analysis were performed as described above except that the MgCl<sub>2</sub> concentration used during the seminested PCR and melting curve analysis was varied from 1 to 8 mM (eight concentrations total).

#### LIMITS OF DETECTION

Viral titers, where known, were determined by the Roche Monitor<sup>TM</sup> assay. For determination of the lower limits of amplification, a sample with a viral titer of 125 987 IU/mL was diluted with uninfected human serum. The diluted samples (1:10, 1:100, 1:1000, and 1:10 000, each at 50  $\mu$ L plus 450  $\mu$ L of uninfected serum) were extracted on the MagNA Pure and subjected to RT-PCR followed by seminested real-time PCR and melting curve analysis.

## Results

#### PRIMER AND PROBE DESIGN

For optimal discrimination, the FITC-labeled anchor probe was designed to anneal to a nonvariable region of the 5'-UTR. The FRET sensor probe was designed to allow discrimination of HCV genotypes 1a/b, 2a/c, 2b, 3a, and 4 during melting curve analysis because of hybridization with different affinities to a region of the 5'-UTR that varies among the different HCV genotypes (Table 1). The FRET sensor-probe sequence was identical to the sequences of HCV subtypes 1a and 1b. The last 3' nucleotide shown in Table 1 is not contained in the FRET sensor probe, but sequence variation at this position will influence FRET sensor probe hybridization (21, 22). The lowercase letters in Table 1 represent genotype-specific nucleotide mismatches that reduce the affinity of the FRET sensor probe for the target sequences. Table 1 also shows the expected genotype-specific  $T_m$  ranges determined in preliminary experiments using serum samples infected with HCV of known genotype.

#### CATION TITRATION

Preliminary seminested real-time PCR with melting curve genotyping experiments suggested that hybridization of the FRET probes required optimization for genotype discrimination during this portion of the procedure. Mg<sup>2+</sup> titration experiments were done to determine the optimal cation concentration for detection and segregation of HCV genotype-specific melting peaks (Fig. 1). The reactions with 2 mM MgCl<sub>2</sub> provided the greatest discrimination among the melting peaks for the genotypes tested. Fig. 1A shows the data for a type 3a sample at the eight MgCl<sub>2</sub> concentrations tested (for the other HCV types, see Data Supplement available with the online version of this article at <http://www.clinchem.org/content/vol48/issue12/>). Fig. 1B shows the segregation of melting point peaks with samples for genotypes 1b, 2a/c, 2b, and 3a at 2 mM MgCl<sub>2</sub>.

#### COMPARATIVE TESTING

To compare this developed method with the INNO-LiPA, we blindly tested 124 patient samples that had been genotyped previously by this method. Genotype assignments based on all 124 first-derivative melting curves made independently by three investigators were in full agreement. Fig. 2 shows a representative experiment. The

**Table 1. HCV genotype-specific 5'-UTR sequence variation in the FRET sensor probe target region.**

Genotype	Sensor probe target	$T_m$ , <sup>a</sup> °C
1a/b	GTACACCGGAATTGCCAGGAC	63–64
2a/c	-----g---a	59–60
4	-----c---g---t	54–56
2b	-----a--g-a-a	52–53
3a	-----c--tg--gt	48–49

<sup>a</sup>  $T_m$ s were determined in preliminary experiments.

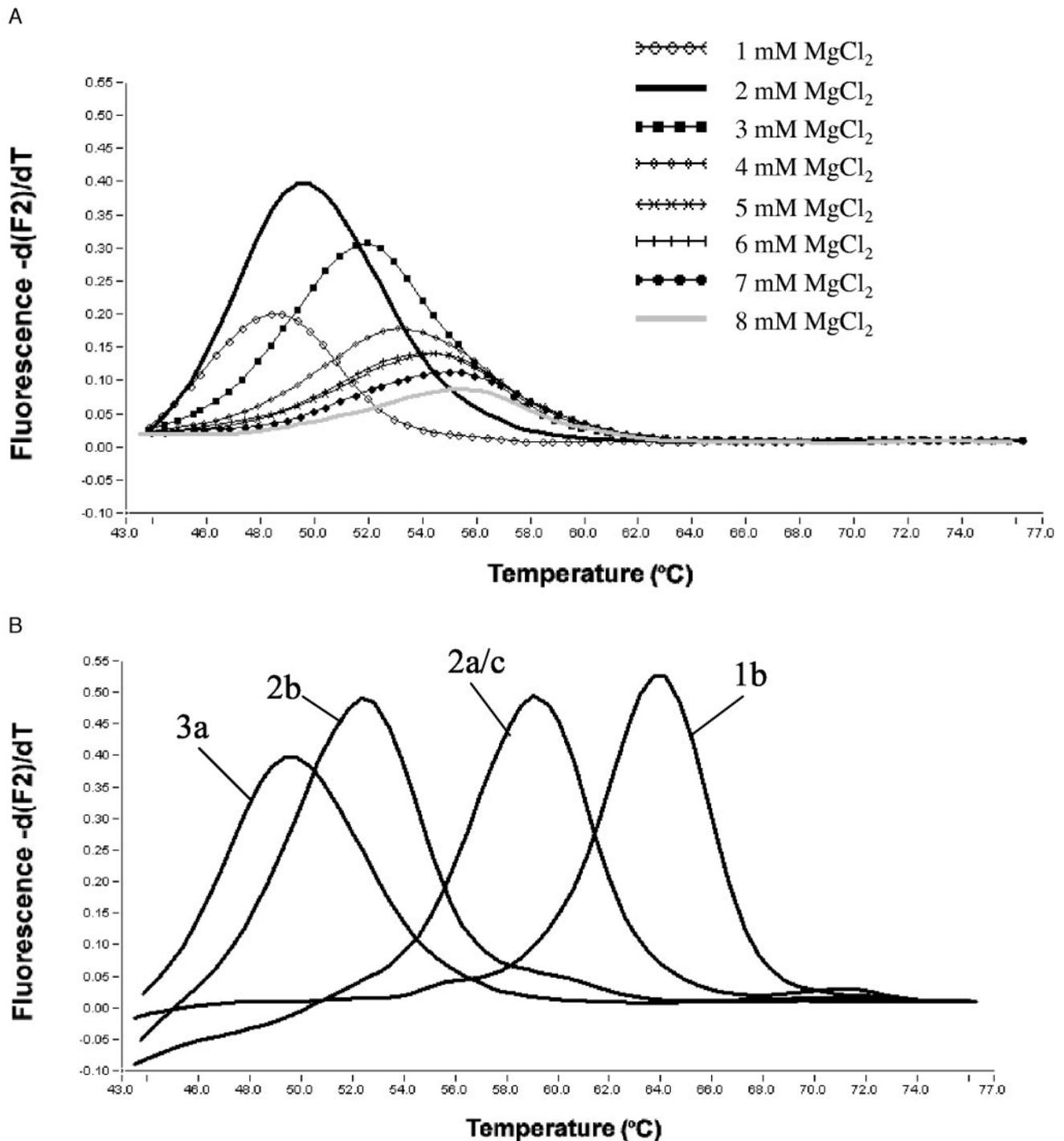


Fig. 1. Mg<sup>2+</sup> titration experiment using samples from patients infected with HCV genotypes 1b, 2a/c, 2b, and 3a.

(A), derivative melting curve plots of LCRed640 fluorescence [ $-d(F2)/dT$ ] vs temperature were obtained with serum from a patient with type 3a HCV in 1, 2, 3, 4, 5, 6, 7, or 8 mM MgCl<sub>2</sub> (MgCl<sub>2</sub> concentration is indicated in the *inset*). (B), comparison of genotype-specific melting transitions for four samples in 2 mM MgCl<sub>2</sub>. Data were obtained by monitoring the fluorescence of the LCRed640-labeled FRET sensor probe during heating from 40 to 80  $^{\circ}\text{C}$  at a temperature transition rate of 0.1  $^{\circ}\text{C}/\text{s}$ .

sample from patient 1 did not produce a detectable signal in this experiment, but it did on repeat analysis and was typed as 1a/b (data not shown). The samples from patients 1, 2, 3, 4, 7, and 8 were typed in the real-time PCR with melting curve assay as 1a/b, 1a/b, 3a, 2b, 1a/b, and 1a/b, respectively, and were concordant with the INNO-

LiPA. Patient 5 was typed as 2a/c three times by the new method, but as type 2b by INNO-LiPA. The 1a/b genotype from patient 6 was confirmed by sequencing because the sample was not typable by INNO-LiPA.

Among 124 samples tested, 13 were nontypable by either method (9 by line-probe, 7 by real-time PCR, and 2

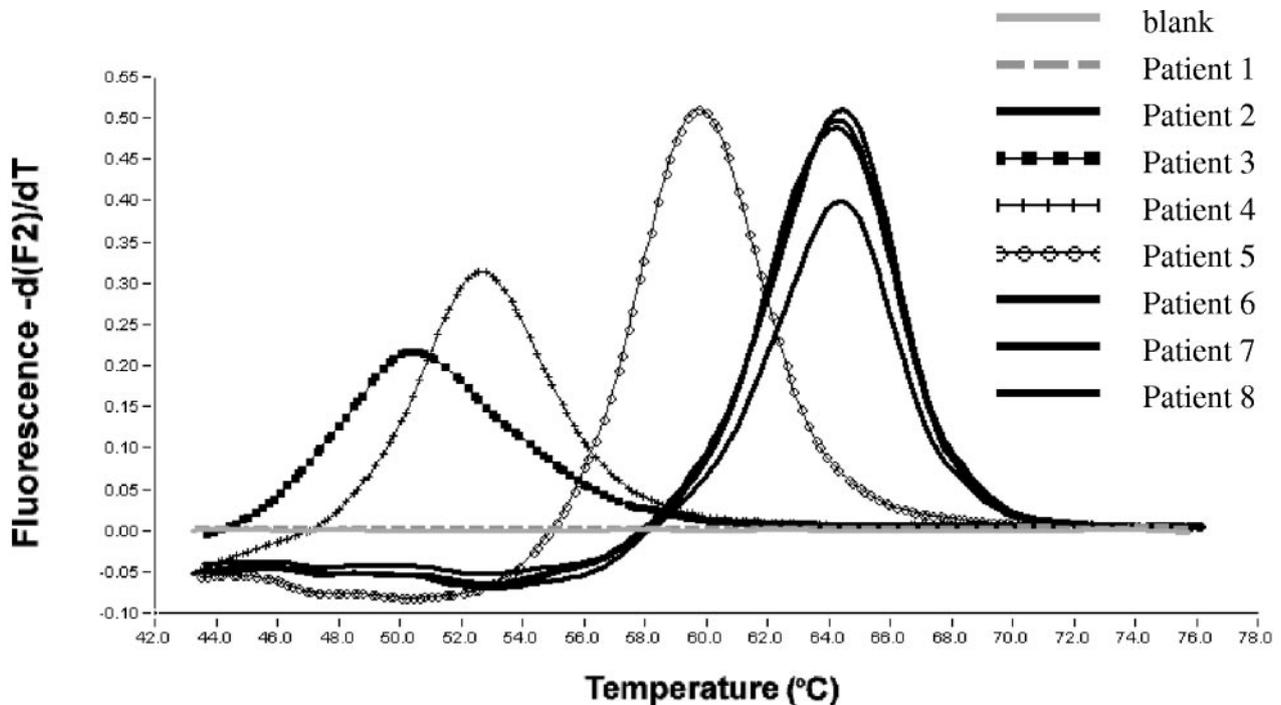


Fig. 2. Representative genotyping experiment using real-time PCR and melting curve analysis. Eight patient samples were genotyped with 2 mM MgCl<sub>2</sub>. Derivative melting curve plots are shown. A water blank was included to control for reagent contamination.

by both methods). Discrepancies between the real-time PCR with melting curve analysis and the line-probe method occurred in 3 of the remaining 111 samples (Table 2), and all 3 were type 2a/c by the new method. Two of these samples, including the sample from patient 5 in Fig. 2, were type 2b by INNO-LiPA; the latter was confirmed by sequencing. The other discrepant sample was type 1 by INNO-LiPA. In addition, one sample typed as 2a/c by the new method was typed only as type 2 by INNO-LiPA. The patient sample that was classified as type 1 by INNO-LiPA (confirmed by sequencing) and type 2a/c by real-time PCR could not be confirmed by repeat testing because of limited sample volume. Therefore, the possibility of clerical or sampling error could not be ruled out.

VARIABILITY OF  $T_{m,s}$  FOR GENOTYPES 1a/b, 2a/c, 2b, 3a, AND 4

The observed range of  $T_{m,s}$  found for each HCV genotype tested (120 determinations on 108 samples) is indicated in

Table 2. Concordance of the genotypes determined by melting curve analysis and INNO-LiPA.

This study	n	INNO-LiPA							
		1	1a	1b	2	2a/c	2b	3a	4
1a/b	78	10	41	27					
2a/c	6		1		1	2	2		
2b	11						11		
3a	14							14	
4	2								2
Total	111	10	42	27	1	2	13	14	2

Table 3. The maximum within-run variation was determined from runs with more than one sample of a specific genotype; this analysis included all samples with concordant results by the two assays. Within-run variation of  $T_{m,s}$  for genotype 4 was not determined because the number of samples was limited. The greatest total (all-run) range of  $T_{m,s}$  was 1.1 °C for genotype 1a/b samples.

The distribution of  $T_{m,s}$  and the  $T_m$  ranges for each genotype are shown in Fig. 3 and demonstrates the ability of the FRET sensor probe to discriminate genotypes of HCV found in patient samples.

LIMITS OF DETECTION FOR GENOTYPING

Two approaches were used to estimate the minimal viral load required for the described genotyping analysis: serial dilution of a patient sample with a high viral titer in virus-free serum and review of viral titers of the stored specimens used during the validation of the new method. In the dilution study, the assay was able to identify the genotype of the samples that contained ~126 000 and ~12 600 IU/mL, but was unable to genotype samples that contained concentrations of type 1a HCV ≤1260 IU/mL. Review of viral titers for the samples tested in the method-comparison study showed that the lowest viral titers that were genotyped by the new method were 10 400, 11 700, and 9700 IU/mL, although two samples with similar concentrations (10 900 and 14 700 IU/mL) failed to amplify. The specimens with titers of 11 700 and 14 700 IU/mL were both nontypable by INNO-LiPA. All samples with HCV concentrations >20 000 IU/mL amplified. At the other extreme, six of the samples that were

**Table 3. Within- and between-run variation of  $T_m$ s for samples of indicated genotypes by both INNO-LiPA and melting curve analysis.**

HCV genotype	Variation of $T_m$ , °C				
	Between runs			Within run <sup>a</sup>	
	Interval	Range	Mean (SD)	Interval	Range
1a/b	63.5–64.6	1.1	64.1 (0.2)	63.9–64.5	0.6
2a/c	59.2–60.1	0.9	59.5 (0.3)	59.3–59.6	0.3
4	54.0–54.6	0.6	54.2 (0.3)	ND <sup>b</sup>	ND
2b	52.0–52.9	0.9	52.6 (0.2)	52.0–52.8	0.8
3a	49.7–50.6	0.9	50.1 (0.3)	49.7–50.3	0.6

<sup>a</sup> Data only from runs with more than one sample of specific genotype.  
<sup>b</sup> ND, not determined.

successfully analyzed had viral loads of  $>8.5 \times 10^6$  IU/mL, and in at least one the HCV titer was  $>8.5 \times 10^7$  IU/mL.

### Discussion

The present study demonstrates that melting curve analysis can be used to identify type 1 HCV with high specificity and can classify other HCV types/subtypes with considerable confidence. The primary goal in the design of the assay was to identify type 1 HCV. Type 1 is treated differently from other common types of HCV and has a poorer prognosis. Reliable identification of type 1 samples by melting curve analysis has the potential to rapidly complete the testing of more than one-half of the genotyping samples in a typical US laboratory. To meet this goal, the sensor probe was synthesized with a sequence identical to the type 1 sequence so that type 1 virus was associated with a high  $T_m$ . We took advantage of the ability of melting analysis to detect sequence

variation(s) (from the probe sequence) signaled by a lower  $T_m$ . A lower  $T_m$  may represent a different genotype or perhaps a minor sequence variation in type 1, but finding the high  $T_m$  associated with the type 1 sequence indicates identity of the target sequence with the probe sequence and the presence of type 1 HCV. We do not envision other mechanisms (short of technical error) that might produce the high  $T_m$  in the narrow interval (63.5–64.6 °C) associated with the type 1 sequence. The experimental data confirmed that this strategy was successful in achieving complete specificity for type 1 HCV among the samples tested.

Although the approach used for this study was designed to discriminate type 1 HCV from other genotypes of HCV commonly seen in the US, it was important to consider less common genotypes as well. Sequences of types 5–11 were compared for sequence homology in the region of the sensor probe (see Table 1 in the Data Supplement) and showed that only three of the subtypes (6b, 7b, and 11a) were identical in sequence to type 1 in this region. However, when the primers used for amplification were compared with the sequences of these subtypes (see Table 2 in the Data Supplement), there was sufficient lack of identity with the forward primer (38% identity for type 6b and 74% identity for types 7b and 11a, with the five 3' nucleotides being different from type 1) that amplification is unlikely. This would prevent any false type 1 results and supports our conclusion that the procedure described allows for the identification of type 1 HCV with high specificity.

In examining reported sequences of common HCV genotypes and subtypes, it became apparent that a single probe could be designed to produce a different predicted  $T_m$  for each major type/subtype of HCV (Table 1). This strategy identified most samples correctly at the genotype level, although the correct subtypes of two were in question. At the genotype level (type 1, 2, 3, or 4), there was 99% concordance with the line-probe method; the single discordant sample could not be reanalyzed by our method because of insufficient remaining sample. When subtype was considered, the concordance dropped to 97% because of two additional discordant specimens that were

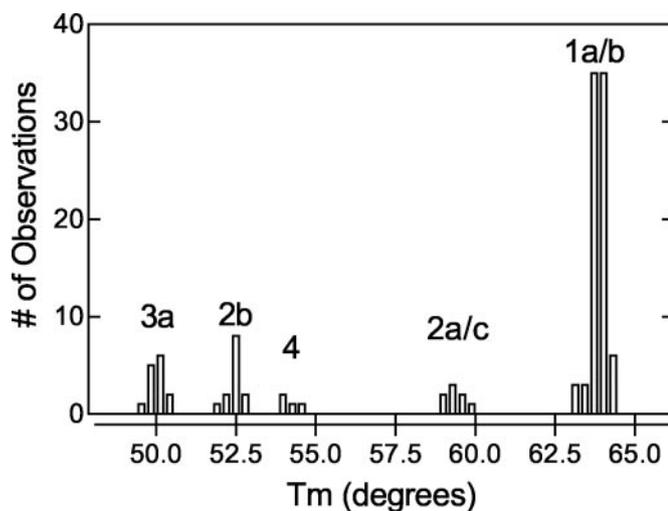


Fig. 3. Histogram illustrating the spread (interval) and range of each genotype-specific  $T_m$  for 120 observations on 108 samples with genotypes confirmed by INNO-LiPA.

The  $T_m$  ranges for genotypes 1a/b, 2a/c, 2b, 3a, and 4 were determined from results of 82, 8, 13, 14, and 4 determinations, respectively. Each bin is 0.3 °C wide; 0.3 °C was the minimum within-run variability of any genotype (Table 3).

typed as 2a/c by the new method and as 2b by the line-probe assay. This discrepancy is of little clinical importance.

The reference standard for genotyping uses the sequence of the NS-5B region of the HCV genome. The NS-5B region is too variable to be suitable for PCR, and most clinical assays use PCR amplification of the 5'-UTR for genotyping. It has been suggested that inconsistencies between 5'-UTR and NS-5B genotyping results may represent new subtypes (23). The discrepancies noted above between type 2a/c and 2b determinations may reflect atypical variation(s) in the 5'-UTR sequences: i.e., the single 5'-UTR region amplified by our assay may be identical to the sequence of a typical type 2a/c virus, whereas the other regions of the 5'-UTR tested with INNO-LiPA have a type 2b sequence. This is clearly possible in a virus with a high mutation rate, as has been documented for HCV (12).

The range of input viral particles that were successfully genotyped by this procedure ( $10^4$  to  $>8.5 \times 10^7$  IU/mL) appears to be comparable to the line-probe assay because there was approximately the same amplification failure rate in both assays (<8%). Some of the failures with the real-time PCR assay may be attributable to the prolonged storage of the samples used in the comparison study, whereas preanalytical variable(s) associated with transportation to a referral laboratory may have contributed to INNO-LiPA failures. In further work using freshly collected samples, we have found a 2% failure rate. In addition to the lack of prolonged storage before analysis, genotyping is now being performed earlier in the treatment course when HCV titers are high (as recommended by NIH guidelines), which may be contributing to the higher than predicted success rate.

To increase the amplification at the lower range of the assay, we performed several experiments using a two-stage, full-nested block-cycler PCR (20) and subsequent melting curve analysis with the FRET probes described. In the dilution experiment, this increased the ability to detect the HCV genotype down to a titer of 130 IU/mL. For the samples that were nontypable by the seminested method described in the present study, however, the yield was only 20% additional typings with the full-nested PCR, suggesting no significant improvement to the utility of the analysis.

The procedure described in this study requires ~1 h of hands-on time: 15–20 min to set up the extraction on the MagNA Pure, 15–20 min to set up the block cycler, 10–15 min to set up the LightCycler, and 5 min for data analysis, with an overall time from initiation of the procedure to results of 5.5 h. This compares with ~4 h of hands-on time for the line-probe assay, with a slightly longer time to results of 8 h. Additionally, we estimate that reagent and consumable supplies costs for the described procedure are approximately one-quarter to one-third those of the line-probe assay.

While this report was in preparation, Schroter et al.

(24) described another protocol for HCV genotyping by real-time PCR and melting curve analysis. Their method allows for the discrimination between types 1, 2, 3, and 4 by use of a single real-time RT-PCR amplification and three sets of FRET probes. As with our method, results for up to 32 patient samples and controls are available within minutes of completion of PCR, whereas a similar workload requires several hours after PCR when performed by sequencing or line-probe assays.

In conclusion, the present report and that of Schroter et al. (24) suggest that HCV genotypes can be determined rapidly with FRET probes. The present study further demonstrates that high-specificity identification of the common type 1 virus can be accomplished with a single sensor probe.

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