Ribonuclease-resistant RNA Controls (Armored RNA) for Reverse Transcription-PCR, Branched DNA, and Genotyping Assays for Hepatitis C Virus

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Background: Comparison and evaluation of molecular diagnostic assays for the detection and quantification of hepatitis C virus (HCV) RNA have been limited by the lack of RNA controls and calibrators. Armored RNA® technology is a means for producing RNA that is completely protected from plasma ribonucleases. This method produces recombinant pseudoviral particles that are noninfectious and contain predefined RNA sequences.

Methods: A consensus 412-base sequence from the 5’NCR/Core region of HCV subtype 2b was derived from 34 individually sequenced HCV genotype 2b variants. A DNA fragment encoding the consensus HCV-2b sequence was synthesized de novo, cloned, and expressed as an Armored RNA control. The resulting HCV-2b Armored RNA (AR-HCV-2b) contained the complete HCV-2b consensus RNA sequence encapsidated within a protective protein coat.

Results: AR-HCV-2b was fully recoverable from human plasma incubated at 4 °C for >300 days. The particles were tested in three clinical assay formats: Amplicor™ HCV Monitor 1.0, Quantiplex™ HCV RNA 2.0, and INNO-LiPA™ HCV II. When added into seronegative, nonviremic plasma, AR-HCV-2b showed reproducible signals and linear dilutions in both the Amplicor and Quantiplex assays. AR-HCV-2b was correctly identified as subtype 2b in the INNO-LiPA line probe assay.

Conclusion: The HCV-2b Armored RNA control is a versatile, durable, ribonuclease-resistant viral RNA control that is compatible in three different clinical assay formats.

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Hepatitis C virus (HCV) 3 is a global health issue, infecting millions of people around the world (1, 2). HCV is a genetically heterogeneous RNA virus, with a genome consisting of a positive polarity, single-strand 9.4-kb RNA. Variation within the genome sequence has led to a classification scheme of 6 major genotypes with more than 30 minor subtypes (3). The rate of progression to chronic hepatitis as well as the effectiveness of HCV antiviral drugs is influenced by HCV genotype. For example, interferon-α alone or in combination with ribavirin has had limited success in treating HCV genotype 1b (4, 5).

Two competing technologies for quantitatively detecting HCV RNA are currently used in clinical laboratories [reviewed in (6)]. The Amplicor™ HCV Monitor (Roche Diagnostic Systems) uses reverse transcription-PCR (RT-PCR) to coamplify HCV RNA and an internal RNA quantification calibrator. Viral quantification is achieved by comparison to the internal RNA quantification calibrator. The Quantiplex™ HCV RNA (Chiron Diagnostics) uses external DNA calibrators and direct hybridization of DNA probes to HCV RNA followed by chemiluminescent signal amplification. Quantification is achieved by generating an external calibration curve with the DNA calibrators. Direct comparison of viral quantification results from these two technologies is difficult and can be sensitive to HCV genotype (7, 8).

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3 Nonstandard abbreviations: HCV, hepatitis C virus; RT-PCR, reverse transcription-PCR; and bDNA, branched DNA.
No uniform ribonuclease-resistant clinical calibrators are available for research and clinical laboratories to assess the sensitivity, specificity, accuracy, and precision of these assays other than HCV derived from a single donor (9). Plasma-derived HCV is genetically diverse and prone to lot-to-lot variability. Furthermore, the wild-type virus is potentially infectious. The ideal control or calibrator for clinical assays for HCV would be stable in plasma, homogeneous, contain an RNA target, carry no risk of infection to laboratory workers, and be useful across several assay platforms for assay assessment and comparison.

Armored RNA® technology was developed to meet these criteria. Previously, an HIV Armored RNA control (AR-HIV-B) was developed and clinically tested (10). The encapsidated HIV RNA control consisted of a consensus 172-base portion of the gag sequence for subtype B viruses encompassing the target amplification region for the Ampli- copor HIV-1 Monitor assay. The AR-HIV-B control was stable in plasma and worked well in the RT-PCR assay. However, the AR-HIV-B control was not compatible with the Quantiplex HIV assay because the length of inserted HIV sequence was insufficient to act as a positive control in the branched DNA (bDNA) assay. In developing the present Armored RNA HCV control, we had three objectives: (a) to increase the amount of packaged recombinant RNA; (b) to develop a control that could be used in both the RT-PCR and bDNA HCV assays; and (c) to validate our approach to producing HCV genotype-specific Armored RNA controls.

In this report, we present the development of an HCV Armored RNA control for genotype 2b (AR-HCV-2b). The AR-HCV-2b control was constructed around the 5’NCR/Core region because the region is highly conserved among HCV genomes (2). Additionally, two commercially available clinical assays for HCV viral load quantification have either PCR product capture probes (Amplicor HCV Monitor) or hybridization capture probes (Quantiplex HCV RNA) targeted to the 5’NCR/Core region. Therefore, it was possible to make a single RNA control for use in both assay formats and to provide a method to directly compare the two assays. Lastly, whereas the 5’NCR is the most conserved genomic region, there are genotype-specific insertions, deletions, and single nucleotide polymorphisms available to differentiate the various HCV genotypes. Therefore, it was possible to produce genotype-specific Armored RNA controls for use in non-sequencing-based methods to determine HCV subtype, such as the INNO-LiPA assay.

**Materials and Methods**

**PREPARATION OF ARMORED RNA HCV GENOTYPE 2b (AR-HCV-2b) CONTROL**

Construction of HCV genotype 2b consensus sequence. A consensus 412-bp DNA sequence containing the HCV subtype 2b 5’NCR and a portion of the Core gene (nucleotide nos. −321 to +91; for a reference sequence see GenBank accession no. M62321) (11) was designed from the analysis of 34 individual HCV sequences of the 5’NCR/Core gene regions. Individual HCV viral sequences were obtained from the Mayo Clinic and had been independently verified for genotype by sequencing both the 5’NCR and portions of the NS5 gene. The HCV-2b consensus sequence included the 244-bp target for the Amplicor HCV Monitor (12). Additionally, the consensus sequence contained the capture probe regions for the Quantiplex HCV RNA 2.0 (13, 14) and INNO-LiPA HCV II assays (15). De novo construction of the HCV-2b consensus 5’NCR/Core fragment was performed using a modified ligase chain reaction developed for synthetic gene construction (16). Specifically, polyacrylamide gel electrophoresis-purified sense and antisense oligodeoxynucleotides 70–120 bases in length were designed with 20–30 bases of overhanging nucleotides. Ligation of the properly annealed double-stranded oligonucleotides was performed by a thermostable ligase (Ampligase™; Epicenter) by controlled incubation from 70 to 40 °C, decreasing by 2 °C every 5 min. After construction and cloning into the Armored RNA packaging vector, the consensus sequence was verified by dye-terminator chemistry (ABI 377). Fig. 1 shows the genomic organization of HCV, the HCV-2b consensus region as well as the relative positions of the primer and probe binding sites for the three clinical assays used in this work. The HCV-2b consensus sequence is shown in Fig. 2.

**Production of AR-HCV-2b particles.** The HCV-2b Armored RNA (AR-HCV-2b) control was expressed and purified as described previously (10). A diagram of the production of Armored RNA particles is shown in Fig. 3.

**FUNCTIONAL EVALUATION OF AR-HCV-2b CONTROL**

**HCV viral load and genotyping assays.** To determine viral copy number of AR-HCV-2b-supplemented plasma, the Amplicor HCV Monitor version 1.0 (RT-PCR; Roche Diagnostic Systems) and Quantiplex HCV RNA 2.0 (bDNA; Chiron Diagnostics) assays were performed according to the manufacturers’ instructions. To evaluate control specificity, AR-HCV-2b and AR-HIV-B (100,000 RNA equivalents, respectively) were individually added into plasma and evaluated in both the Amplicor HCV Monitor and Amplicor HIV Monitor (Roche Diagnostic Systems) (17) assays. AR-HCV-2b genotype was assessed by the INNO-LiPA HCV II assay (Innogenetics) using the kit instructions and Amplicor HCV Monitor denatured amplicon.

**Plasma stability of AR-HCV-2b.** AR-HCV-2b was examined for stability in human EDTA-preserved plasma. Initially, the purified AR-HCV-2b preparation was quantified, in duplicate, with the Amplicor HCV Monitor assay. EDTA plasma from a single donor (TriMar Hollywood, Inc.) was clarified by centrifugation at 5000 rpm for 30 min, and 1 g/L NaN₃ was added as an microcide. Naked HCV-2b RNA was generated by incubating AR-HCV-2b particles at 70 °C for 10 min. This procedure denatures the coat
protein, releasing the encapsidated RNA. The heat disruption of Armored RNA particles was monitored by ethidium bromide staining of intact and denatured preparations fractionated within an agarose gel. The dissociated or naked RNA displays a higher mobility rate than intact Armored RNA. For each stability study, a single batch of plasma was made with added AR-HCV-2b or naked HCV-2b RNA and aliquoted into single time-point samples of 100 μL, which is the volume used in the Amplicor HCV Monitor assay. The samples were then incubated at 4 °C for the experimental time period and then placed at −80 °C until the completion of the experiment. All samples were quantified, in duplicate, using the Amplicor HCV Monitor assay.

AR-HCV-2b as a positive control in clinical assays. For long-term examination of AR-HCV-2b as a clinical assay positive control, a single large pool of AR-HCV-2b-supplemented normal human plasma was aliquoted into several hundred 100-μL samples and stored at 4 °C. The samples were assayed along side patient samples and the Amplicor HCV Monitor kit positive control in regular clinical runs for HCV viral load determination. Both the AR-HCV-2b and the Amplicor HCV Monitor positive controls were assayed 2–4 times a week over the course of >300 days.

Results

AR-HCV-2b plasma stability
The stabilities of AR-HCV-2b and naked HCV-2b RNA were examined in human plasma. Ten thousand RNA equivalents of either AR-HCV-2b or naked HCV-2b were each incubated in EDTA-preserved human plasma at 4 °C for 30 days. The AR-HCV-2b samples were resistant to the nucleases found in human plasma, whereas the naked HCV-2b samples were degraded instantaneously in the ribonuclease-rich plasma (Fig. 4). AR-HCV-2b incubated at 4 °C in human plasma was stable over a 4-week challenge. The CV between duplicate samples was 13%. Additionally, AR-HCV-2b particles incubated in a mixture of RNAse A and T1 showed no degradation of the protected RNA control (data not shown). AR-HCV-2b-supplemented plasma was frozen at −20 °C and thawed to room temperature five times and evaluated. No loss of signal was observed.

To test for control specificity, plasma supplemented with AR-HCV-2b or AR-HIV-B was assessed in the Amplicor HIV Monitor and Amplicor HCV Monitor assays.

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Fig. 2. The HCV genotype 2b consensus sequence in AR-HCV-2b.

The subtype 2b consensus sequence packaged into Armored RNA was generated by the alignment of 34 individual HCV variants that were independently classified as subtype 2b (see Materials and Methods). The consensus sequence encompasses the 5' NCR and 91 bases of the Core protein region of the HCV genome. KY80 (nucleotides 46–69) and KY78 (nucleotides 261–289) primers are underlined (12).
AR-HIV-B produced no detectable signal in the HCV Monitor assay but was correctly quantified in the HIV Monitor assay. The AR-HCV-2b samples produced no signal in the HIV Monitor assay.

**Linear dilution of AR-HCV-2b in the bDNA assay**

The Quantiplex HCV RNA 2.0 (bDNA) assay for HCV viral quantification is a direct viral RNA hybridization technique. This assay is a sandwich nucleic acid hybridization assay in which DNA probes bind to the HCV RNA target, followed by chemiluminescent signal amplification of the probes (18). A twofold dilution series of AR-HCV-2b particles was individually added into human plasma and quantified using the bDNA assay. Triplicates samples and negative controls (normal human plasma) were evaluated for each dilution, and the quantification values were averaged. Twofold dilutions of AR-HCV-2b produced linear results, \( r^2 = 0.99 \) (Fig. 5). The negative controls were undetectable, and the mean CV for the AR-HCV-2b dilutions was 3.9% (range 1.8%-5.2%). These results show that the AR-HCV-2b control is capable of functioning in the bDNA assay as well as the RT-PCR assay for the clinical quantification of HCV viral load.

**AR-HCV-2b as a genotyping control**

Other than direct sequence analysis of individual isolates, the INNO-LiPA HCV II assay (Innogenetics) is the most widely used clinical assay for genotyping HCV in clinical plasma specimens. The assay uses the reverse hybridization technique and is based on sequence variation found within the 5'NCR of the various HCV genotypes (15). The line probe assay involves immobilized genotype-specific capture probes that differentially capture a 244-bp PCR product generated from the KY78/80 primer set (see Fig. 1) (12). The AR-HCV-2b control consensus sequence includes the 244-bp amplified region on which genotype discrimination is based. Samples were amplified and denatured with the Amplicor HCV Monitor kit components and then evaluated using the INNO-LiPA HCV II assay (Fig. 6). AR-HCV-2b was correctly genotyped as 2b by the INNO-LiPA HCV II assay, thus validating AR-HCV-2b as a genotype control. The negative control was not detected, and the HCV-positive patient sample genotyped as 2b.
AR-HCV-2b used in a clinical laboratory setting
To assess the long-term stability of the AR-HCV-2b in EDTA-preserved human plasma and its potential as a positive control in clinical viral load determinations, AR-HCV-2b was added into plasma and stored at 4 °C. Approximately 40,000 copies/mL of AR-HCV-2b was added to a single pool of negative human plasma and aliquoted into single-use samples (100 μL); the samples were stored at 4 °C and quantified by the Amplicor HCV Monitor assay in regular clinical runs (2–3 times per week), over the course of 300 days. There was no diminution of the copy number for the AR-HCV-2b positive controls (Fig. 7).

Positive controls from the Amplicor HCV Monitor kit were assayed in alternate clinical runs for comparison with the AR-HCV-2b control. The Amplicor HCV Monitor positive control consists of an in vitro-transcribed RNA encoding an HCV sequence. Because the HCV Monitor positive control is a naked RNA, it is degraded immediately if added directly to plasma. Therefore, the HCV Monitor positive control is added first to lysis buffer (per the manufacturer’s instructions) and then added to plasma for evaluation. The CVs for the two sets of positive controls were comparable: 48% for the AR-HCV-2b, and 46% for the Amplicor HCV Monitor positive control.

Discussion
Accurate and reproducible HCV RNA viral load measurements are essential for monitoring antiviral therapy. The two most widely used commercial assays for determining HCV RNA concentration from clinical specimens are the Amplicor HCV Monitor and the Quantiplex HCV assays, based on the RT-PCR and bDNA technologies, respectively. The RT-PCR assay uses an internal naked RNA calibrator, whereas the bDNA assay uses a series of external DNA calibrators for calculating HCV RNA concentrations. Both technologies contain nonideal components. Naked RNA can easily be degraded by ubiquitous RNases, and external DNA calibrators do not accurately reflect events that occur during RNA isolation. Armored RNA technology was developed as a direct response to the inherent weaknesses of the current controls and calibrators used in RNA-based diagnostics. The technology is a plasmid-driven packaging system that produces noninfectious recombinant MS2 bacteriophage containing the desired RNA sequence (19). The packaged RNA is able to withstand adverse clinical conditions under which unprotected RNA would usually degrade.

In this study, the technology was applied to quantitative HCV RNA determinations in human plasma. We constructed an HCV Armored RNA to act as a positive control and demonstrated its utility in both the RT-PCR and bDNA assay formats. AR-HCV-2b was stable when incubated directly with purified RNases as well as when
incubated in human plasma at 4 °C over the course of >300 days. The control endured five freeze-thaw cycles in plasma. AR-HCV-2b was reproducible, with CVs equivalent to the positive control for the Amplicor HCV Monitor assay. The present evaluation of AR-HCV-2b was performed in EDTA-anticoagulated human plasma as the biological matrix. Limited testing of serum supplemented with AR-HCV-2b showed equivalent results; however, the long-term stability of the control in serum or other bodily fluids may not be directly analogous to data obtained in plasma.

The specificity of the AR-HCV-2b control was demonstrated in that it was detected only in HCV quantification assays. For the present work, Armored RNA was used as an external positive control; however, the technology has the flexibility to produce calibrators for RNA quantification. For example, internal quantification Armored RNA calibrators may be added directly to clinical specimens, thereby eliminating the need to have external DNA calibrators in signal amplification-based formats.

The Armored RNA HCV control contained an RNA consensus sequence for HCV genotype 2b. Along with serum alanine aminotransferase concentrations, HCV genotype has been shown to be one of the factors predictive of a beneficial response to antiviral therapy (20). Accurate evaluation of the HCV genotype with defined RNA controls in a reverse hybridization-based assay eliminates laborious, direct sequence-based genotyping, which is currently considered the gold standard. The AR-HCV genotype 2b control was successfully validated in the most widely used clinical HCV genotyping assay, INNO-LiPA HCV II.

At present, the international HCV standard consists of plasma-derived HCV of a single genotype (type 1) from a single donor (9). Because amplification efficiencies vary widely according to the HCV genotype present (21), neither the AR-HCV-2b control nor the HCV international standard may be appropriate quantification calibrators for non-type 1/non-type 2 specimens. However, because of the flexibility of Armored RNA technology, other AR-HCV genotype-specific controls can be constructed rapidly. Panels of Armored RNA HCV genotype-specific controls may be used as a tool to uncover potential genotype bias in future diagnostic assays formats. Moreover, as new HCV genotypes are uncovered, genotype-specific Armored RNA controls could be quickly designed and added to assay development schemes.

HCV continues to present a threat to the safety of blood-based therapeutics, despite advances in sensitive antibody screening, because of inclusion of “window period” donations (nonseroconverted, recently infected individuals) and donations from sustained RNA-positive, seronegative individuals. The use of automated, sensitive nucleic acid tests for screening blood products promises to reduce this incidence rate (22). Because the vast majority of donors will be nonviremic and generate no positive signal, the performance of any diagnostic system will be difficult to assess, even with a large number of external controls. The use of internal controls in every specimen allows the sample-to-sample variability of the assay system to be tightly monitored and greatly reduces the chances of a false-negative result. Because Armored RNA is stable and can be added directly to each specimen before RNA isolation, it is amenable to high-throughput assay formats.

In summary, we have demonstrated that the Armored RNA technology can be used to produce ribonuclease-resistant RNA controls and calibrators. The technology allows the user to precisely define the control’s RNA sequence. We produced a HCV genotype 2b Armored RNA that includes the Amplicor HCV Monitor target region as well as hybridization sequences for both the Quantiplex HCV and INNO-LiPA capture probes. This single HCV control is the first engineered ribonuclease-protected RNA control capable of acting as a positive control for each of these assays. Armored RNA offers a method for comparing and contrasting not only current assays for precision, accuracy, specificity, and sensitivity, but it is easily reformatted to allow evaluation of newly emerging assay formats. In addition, because of their durability, Armored RNA controls and calibrators are ideal “on-board” reagents in automated assay platforms, which represent the future of molecular diagnostics.

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References


