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Robotic automation performs a nested RT-PCR analysis for HCV without introducing sample contamination

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Abstract

The Polymerase Chain Reaction (PCR) is a popular method to amplify and detect specific RNA and DNA sequences. To obtain maximum performance of PCR, it is best performed by highly skilled technologists because of the complexity of the assay and the potential for laboratory contamination from the amplification products produced. We chose to automate this nested RT-PCR for hepatitis C assay to significantly reduce the need for manual pipetting while preserving the excellent non-contamination performance of the corresponding manual test. A three axis cartesian robotic pipetting station was equipped to perform RT-PCR using an on-board automated thermal cycling device. 104 sera were analyzed using this modified pipetting station and we found a very close agreement (100% sensitivity and 98% specificity) with results previously obtained by corresponding manual RT-PCR analysis. This study demonstrated a user-programmed robotic pipetting system could successfully automate a complex PCR assay without contamination. Our results suggest that use of robotic pipetting station can provide cost efficient alternative to performance of molecular diagnostic assays while demonstrating minimal inter sample contamination. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Automation; Robot; RT-PCR; Hepatitis C; HCV; Molecular diagnostics; Contamination

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1. Introduction

The Polymerase Chain Reaction (PCR) (Hoffmann LaRoche, Inc., Nutley, NJ) has become a routine assay employed in many diagnostic and research laboratories [1]. It can amplify target DNA sequences a billion-fold and can be used either as a qualitative or quantitative technique to identify targets present in extremely small numbers. A number of diagnostic disciplines now routinely use PCR, including clinical microbiology and virology, genetics, oncology and forensics [2,3].

While PCR has become widely implemented, several alternative amplification techniques are beginning to challenge its dominant position as the in vitro amplification method of choice. These include Strand Displacement Amplification (SDA) [4] and Nucleic Acid Sequence Based Amplification (NASBA) [5], which require simpler instruments and fewer programming steps to perform compared to PCR. Because of the numerous steps involved in the PCR, there exists the potential for pipetting errors which can lead to cross contamination of samples [6]. Furthermore, it is difficult to determine with certainty that a negative control is not a false negative [7].

Few approaches to automate in vitro amplification procedures, including PCR, have been reported [8,9]. While Cartesian robots equipped with pipetting capabilities have been adopted for a wide variety of clinical laboratory techniques [10–12], use of a robot to automate PCR presents some unique challenges including on-board thermal cycling, reduction of amplicon contamination and coping with liquid volumes ranging from microliters to milliliters. Despite the promise of dedicated PCR instrumentation [13] and microfabricated chip-based PCR [14,15], flexible and affordable automation designed for the performance and flexible manipulation of the PCR remains elusive for clinical and research laboratories.

We further explored the automation of the PCR for two reasons. Firstly, since RT-PCR reactions contain more steps, they pose an increased potential for occurrence of contamination and hence are suitable for use as a mechanism to study contamination. In particular, the actual pipetting steps in each stage are likely to be the most prone to introduction of contamination. Secondly, the execution of RT-PCR is more complicated for a robotic pipetting station to perform and adds a level of complexity to the assay previously unexamined. The most critical stage for introduction of contamination is the initial sample processing, followed by the pre-amplification and amplification stages, then detection. For these reasons, we therefore chose to automate a nested reverse transcriptase (RT)-PCR assay that detects the hepatitis C (HCV) viral RNA to study the potential for corresponding contamination.

2. Materials and methods

2.1. RT-PCR hepatitis C virus assay

RT-PCR was performed according to a modified method used in the Clinical Molecular Diagnostic Laboratory at the University of Virginia Health Science Center [16]. Briefly, sera from peripheral blood samples were used for the detection of HCV after RNA isolation, and were selected on a random basis from those samples about to be discarded. HCV viral RNA was extracted from all sera in the Molecular Diagnostic Laboratory which is not connected to the robotics laboratory. For both the manual and automated PCR assays, HCV virions were manually extracted from 0.2 ml serum samples using a commercial column device (QIAamp Viral RNA kit, QIAGEN, Valencia, CA). Purified viral RNA extracts in the column eluates were stored at −80°C until used as the primary patient sample. For both versions of the nested RT-PCR HCV assay, paired duplicate aliquots of each serum sample were assayed. Following amplification, each pair of amplified aliquots was analyzed using agarose gel electrophoresis. A total of seven runs were performed: two per week for three weeks and one run in the fourth week.

2.2. Pipetting robot

The RT-PCR assay was automated on a Cartesian pipetting robot (MultiPROBE 104-DT, Packard Instruments, Meriden, CT) with three degrees of freedom. The MultiPROBE was equipped with a robot-accessible deck area of 1800 cm^2, four pipetting mandrels with liquid-level sensing capability, waste/wash station, moveable pipette tip racks, tip disposal system and a controlling computer (Fig. 1). Each pipetting mandrel was designed to accommodate either 200 μl or 20 μl tips (VersaTip). During the course of these studies, the MultiPROBE was programmed to pipet from 10 μl to 50 μl. The deck of the MultiPROBE was organized to minimize the possibility of amplicon contamination of reaction vessels (Fig. 2).

2.3. Automated thermal cycler

The MultiPROBE was outfitted with an automated thermal cycler (‘Progene SP7315’, Techne, Princeton, NJ) that was positioned at the left margin of the pipetting bed (Fig. 1). The thermal cycler holds 96 capless 0.2 ml thin-wall reaction tubes (0.2 ml PCR tubes, #1402-0200, USA Plastics, Ocala, FL) in an

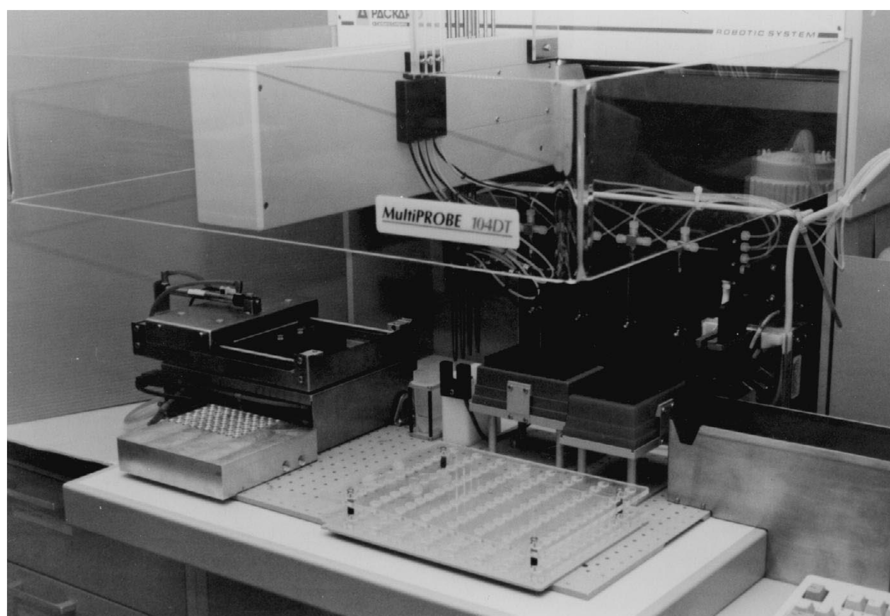

Fig. 1. Robotic pipetting station used for automating RT-PCR. The robot was equipped with a work surface of 1800 cm^2, four syringe pumps, four individually controllable mandrels, racks for disposable tips, automated thermal cycler (Techne Progene), automated tip disposal area and a tip wash station.

8×12 matrix. Cyclic heating and cooling were accomplished via peltier-based electronics and governed from a separate controller unit.

2.4. Assay design and performance

We automated the dispensing of both master mixes, RT-PCR products and mineral oil addition to the assembled 0.2 ml reaction vessels located in the thermal cycler (Fig. 2). Mineral oil added to each PCR tube acted as a liquid cap that permitted the MultiPROBE to gain access to the underlying contents without human intervention. The system also transferred first-stage reaction products directly to the second-stage PCR vessels.

Two hundred microliter small conductive filter tips (Packard #6000613) and 20 μl micro conductive filter tips (Packard #6000615) were used for pipetting reagents and samples. These tips allowed liquid level sensing for all pipetting steps except those involving mineral oil which rendered the liquid level sense feature inoperable. To minimize the possibility of contamination, disposable tips were changed between any transfers that involved aspiration of or delivery into reaction tubes.

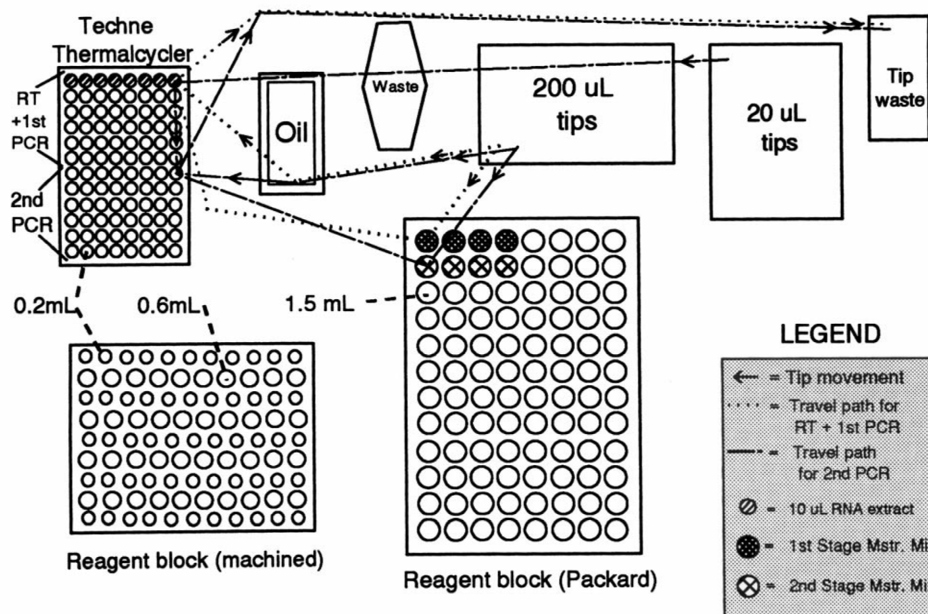

Fig. 2. Surface layout of pipetting station. Reagents were positioned to minimize small droplets of liquid that might adhere to pipet tip ends from falling into open reaction tubes. Sample movement was confined within the thermal cycler. The potential for contamination from tips has been minimized by dropping used tips on the (right) side of the robotic deck away from the location where samples are manipulated and amplified. Movements of reagents and tips are shown by arrows in the figure.

RT and First Stage Master Mix (without enzymes) were prepared manually in 400 μl aliquots using the following formulations (Note: Final concentrations listed in parenthesis): 10 X GeneAmp Buffer (1X), 10 mmol/l dNTPs (40 μmol/l), 50 μmol/l NAF1 and NAR1 amplimers (0.2 μmol/l), 40 U/ul RNAsin (8U/assay), 10 U/ul Reverse transcriptase (0.8 U/assay), and 5 U/ul Taq polymerase (1.0 U/assay). Four hundred microliter aliquots of Second Stage PCR Master Mix were prepared using identical reagents with the following substitutions: NAF1 and NAR1 were replaced with NAF3 and NAR3 at the same concentrations, RNAsin and Reverse Transcriptase were replaced with sterile distilled water. The sequences of the four oligonucleotide amplimers NAF1, NAR1, NAF3 and NAR3 are listed by Shindo et al. [16]. (Note: NAF1/NAR1 are the 'outer' amplimer set and create the amplicon from the first PCR, while NAF3/NAR3 are the 'inner' amplimer set that creates the 257 bp 'nested' amplicon observed in gel electrophoresis of positive samples). Both master mixes without enzymes were aliquotted into 1.5 ml Eppendorf tubes and stored frozen at −20°C until needed. Appropriate enzymes were manually mixed with thawed tubes of both master mixes, then placed in the Packard's 1.5

ml tube holder positioned on the robot's deck. Amplifications conditions for RT/1st PCR were: 42°C/30 min (reverse transcription), 95°C/5 min (initial denaturation), then 25 cycles of 95°C/30 s denature, 55°C/60 s anneal, 72°C/60 s extend, followed by 72°C/7 min (final extend) and 4°C soak. Amplification conditions for 2nd PCR were the same as 1st PCR, except reverse transcriptase step was deleted and amplification occurred for 35 cycles.

2.5. Procedure summary

The MultiPROBE delivered 40 μl of first stage master mix to duplicate 10 μl RNA sample extracts in PCR reaction tubes, then added an overlay of 50 μl of mineral oil (#M3516, Sigma Chemical Co., St. Louis, MO). Following a 30 min isothermal reverse transcription reaction, the first PCR amplification automatically commenced as a result of the computer-directed programming of the Techne thermal cycler controller. A 10 μl aliquot of this first stage amplification reaction was then used as starting material in the second-stage PCR with nested primers. The robot next dispensed 40 μl of second-stage master mix into clean 200 μl reaction tubes prepositioned in the thermal cycler, aspirated 10 μl of first-stage amplification products from under the mineral oil in the first-stage reaction vessels and delivered it into the second stage PCR tubes. Fifty microliters of fresh mineral oil were then added to each tube followed immediately by the start of second PCR amplification.

2.6. Amplicon detection

To focus exclusively on the robot's preparation and performance of the RT and subsequent PCRs, we used the same detection system for the robotic assay as had been used with the manually performed version. Amplicons from the second PCR amplification stage were size fractionated in 2% agarose (FMC Bioproducts, Rockland, ME) gels using 1X TAE buffer, and detected after staining with 0.5 μg/ml ethidium bromide and subsequent exposure to UV light (Fig. 3). PCR Molecular Weight Marker (#G3161, Promega, Madison, WI) was included in each gel for size calibration. The assay result was considered positive when there was a single 257 base-pair band observed in both lanes for each sample. For sera extracts which demonstrated 'equivocal' results (e.g., one lane is positive and its companion lane is negative), a second pair of aliquots were reassayed by the automated RT-PCR assay for HCV. The lanes were then rescored and the results recorded. True positive and true negative sample results were taken to be the corresponding results from the manually-performed nested RT-PCR assay for HCV.

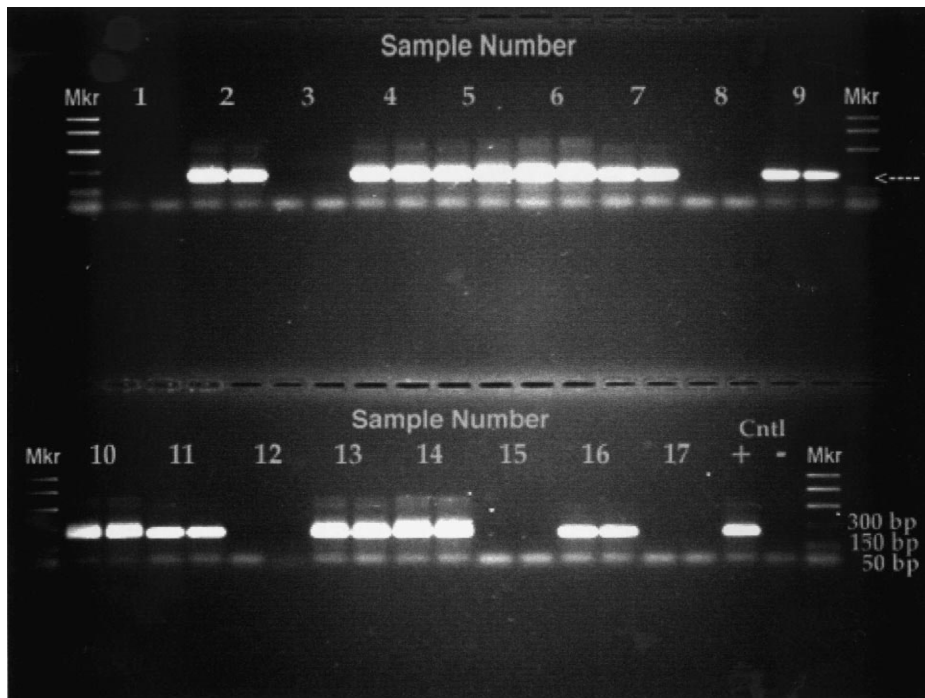

Fig. 3. Gel electrophoresis analysis of automated RT-PCR assay for HCV. A typical ethidium stained 2% agarose gel demonstrating the qualitative results from an automated RT-PCR analysis run. For a batch of 17 (#1–17) unknown samples, 25 μl of each amplified aliquot (2 aliquots/sample) were delivered into paired, adjacent lanes in the agarose gel along with a PCR molecular weight marker (Mkr) in the end lane. A control sample set (one known positive sample and one known negative sample), Cntl + and Cntl −, analyzed with that group of samples, was also included in that gel. A bright band with an estimated molecular size of 257 basepairs was demonstrated in all specimens determined to be positive by a similar PCR method run manually. In this gel, patient samples #2, 4–7, 9–11, 13, 14 and 16 were declared positive, while patient samples #1, 3, 8, 12, 15 and 17 were declared negative.

2.7. Assay controls

Each run included one positive and one negative control as well as both unknown positive and negative samples. The positive and negative controls consisted of extracted serum samples previously verified by manual PCR analyses.

2.8. Software development

The HCV pipetting programs were written in EasyPrep software (rev 1.916, Packard Instruments), which works in the GEM desktop operating system to

control MultiPROBE functions. As additional software from Packard was required to access the on-board thermal cycler, batch files were created to control the Techne Progene.

3. Results

3.1. RT-PCR results

Comparison of the results from analyzing 104 samples by the automated HCV assay and the manual version are shown in Table 1. These data indicate a 100% sensitivity and 98% specificity, which compares well with previous reports [9,17] of the performance of 'offline' thermocyclers coupled to pipetting stations. With duplicate assays, seventeen samples plus a single positive and single negative control (36 tubes total) were regularly assayed. Greater numbers of samples (up to 48 pairs of duplicates) could have been setup and amplified in the thermal cycler, however we were limited by the capacity of the gel electrophoresis apparatus.

The robot performed duplicate analyses from each serum extract to confirm the robustness of the automated assay. Of the 104 serum extracts analyzed, five samples gave equivocal results (one sample lane was positive while its companion was negative (results not shown)). For these five 'equivocal' samples, a second pair of aliquots from each sample were reassayed by the automated RT-PCR assay for HCV. Upon reassay, all five 'equivocal' samples were declared to be positive. This same type of result ambiguity was also observed for the manual assay (personal communication, Jim Bowden, University of Virginia). Fig. 3 also clearly illustrates the stark contrast between samples with positive results (samples #2, 4–7, 9–11, 13, 14 and 16) versus those with negative results (samples #1, 3, 8, 12, 15 and 17). Similar differences between positive and negative samples were observed in the gel photos from the manual

Table 1
Relationship between automated vs. manual performed nested RT-PCR results for HCV RNA[a]

Automated HCV results	Manual HCV results	
	Positive	Negative
Positive	59[b]	0
Negative	1	44

[a] Sensitivity = 100%; specificity = 98%; predictive value of a positive result = 100%; predictive value of a negative result = 98%.

[b] Note: Includes five samples that gave equivocal results on the first automated analysis. These samples on repeat automated analysis gave results that matched those from the manual assay.

HCV procedure. These results demonstrate the equivalence of the robotic and manual methods.

3.2. Control results

For the seven runs performed during 4 weeks, no bands were observed in the lanes from the negative controls. This signaled an absence of PCR contamination, eventhough the two PCR setups and subsequent gel electrophoresis of PCR amplicons occurred in the same room. Some of the robotic HCV runs were set up in the evening and were completed after employees departed. Gel electrophoresis analysis then occurred the following morning. Otherwise, analysis of samples set up for robotic HCV analysis in the morning could be completed within the same day after gel electrophoresis was finished in the afternoon.

4. Discussion

Contamination of PCR analyses by previously created PCR amplicons is considered a significant and continuous problem in molecular diagnostic laboratories. Historically, contamination in PCR reactions has resulted from carryover of PCR products (amplicons) into specimens, equipment or reagents with amplicons from previous reactions [18,19]. Kwok and Higuchi [20] recommended that the preparation of PCR analyses occur in a separate room from where the actual amplification and subsequent detection take place. Amplicon contamination of pipettors has been demonstrated to originate from amplicons that accumulate within pipettors and also within the air of molecular diagnostic laboratories [18]. It is likely that some PCR amplicon contamination results from human error in maintaining proper clean environment conditions. In order to curtail amplicon contamination associated with PCR-based amplifications, reactions based upon chemical means such as UV crosslinking with isopsoralen [21] and enzymatic reaction such as digestion with uracil-N-glycosylase [22] are commercially available for use by molecular diagnostic laboratories.

To further avoid false positives from the PCR, PCR tubes typically remain capped following amplification until they are transferred to another room for analysis. By contrast, we observed no contamination in the negative controls during our runs. This demonstrated that specimens could be assembled, amplified without caps (but with an oil overlay), and analyzed by gel electrophoresis in the same room. Although this analysis format differs from the current philosophy regarding control of amplicon contamination [20], another group who works with automated PCR has reported excellent results using this

'same room' philosophy [9,23]. We hypothesized that if we could integrate as many of the PCR assembly and amplification steps as possible using automation, we would reduce the likelihood of cross contamination.

Our method gave excellent correlation with results obtained using the manual method (i.e., 100% sensitivity and 98% specificity). For the only sample which gave discrepant results, (automated results positive and manual results negative), several explanations can be considered. Firstly, a sample mixup could have occurred. Since we observed identical results when this sample was reassayed by the automated method, we believe this is unlikely. A more plausible explanation is the automated method has a slightly lower limit of detection. This is reinforced when we examined the patient's prior manual results. We observed that from five serial samples from this patient, three manual results were positive while two were negative, suggesting that the patient's viral load was on the borderline of detection for the manual procedure. Thus the automated method gave results consistent with the manual procedure. One impact of the automated HCV analysis system on patient management could be lower costs due to a reduction in labor associated with performance of this procedure.

The absence of amplicon contamination in our study is probably the result of several robot-associated techniques. Firstly, the robot used an oil overlay on each reaction tube, preventing the escape of amplicons. Other automated PCR stations have also used oil [17,24] or liquid wax [23] as an overlay before PCR. With manual-based procedures, the addition of oil to each tube would be considered too labor intensive when compared to closing a plastic cap. However, the robot is ideal for performing tedious procedures that will improve assay performance. Secondly, we changed tips whenever there was a need to handle sample-associated liquids (e.g., RT and first stage PCR). We were able, however, to minimize tip usage when pipetting reagents and mineral oil as these were dispensed with the same tip before the samples were manipulated (e.g., second stage PCR). Finally, the MultiPROBE system's flexibility in the controlling delivery of fluids also contributed to the success of these experiments. The MultiProbe's liquid level sensing feature was used to pipet all aqueous liquids in these assays which minimized the contamination of the exterior surface of these pipette tips. Because the liquid level sensing system only needs a very small (e.g., 1–2 mm) penetration of a tip into the sample/reagent, the amount of residual material on the tip's exterior surface is minimized. These results suggest that as long as simple precautions are followed, the chance of amplicon contamination is relatively small in a laboratory performing automated PCR which is consistent with the earlier finding of $<0.1\%$ [9].

Other factors could also contribute to the successful automation of this molecular diagnostic assay. This includes the error rates which could be expected to be greater for the manually performed test due to the complexity of hand-assembling and pipetting of large number of components with volumes

ranging from a few microliters to 50 μl. Significant savings in laboratory overhead can result from being able to perform many of the steps involved in PCR in the same room. The flexibility of being able to handle small volume tips as well as large volume tips improves precision at the low volumes while delivering large volumes (e.g., 50–200 μl) in one pipetting step thus minimizing tip waste. The software control of sample aspiration and dispensing rate was important when manipulating mineral oil since its higher viscosity requires a much slower dispense speed compared with aqueous liquid.

Efforts to utilize pipetting robots to facilitate performance of molecular biology diagnostic assays have appeared recently [8,9,17,23–25]. In some cases, a thermal cycler was integrated within the pipetting robot to improve the efficiency of the sample throughput [9,23]. Other investigators placed the thermal cycler offline [17,25], believing this was less disruptive to the sample workflow. For our project, the need for an integrated thermal cycler was essential since different thermal reactions (e.g., RT vs. PCR) and multiple execution of the nested PCR were needed to successfully accomplish the RT-PCR analysis for HCV.

Based upon these previous accomplishments and our own results, there are additional enhancements that could be made to our automated system. For example, the robot could deliver the RNA extracts into the PCR tubes, transfer the nested HCV PCR amplicons into a microplate for further analysis [26], or a gel loading mechanism could be added to the robot's pipetting deck. This capability would be especially useful since quantification of viral load would be accomplished almost entirely automated. However, with the recent development of homogeneous detection methods such as TaqMan [27] and molecular beacons [28], there would be no need to perform gel analyses. By having a detection system such as TaqMan or molecular probes coupled to our automated method, there would be at least three advantages: 1) amplification tubes remain sealed and so minimize contamination potential; 2) HCV viral RNA could be quantified; and 3) elimination of a cumbersome detection system (i.e., gel electrophoresis).

5. Summary and conclusion

A Cartesian pipetting robotic system equipped with a robot-accessible thermal cycler successfully automated a tedious and technically challenging RT and nested PCR assay with 100% sensitivity and 98% specificity. Our results suggest that the robotic system provides laboratory performance equivalent to that in a manually-performed PCR assay. We believe that automation of PCR can result in significant cost savings in molecular diagnostic and research laboratories.

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