

Molecular Beacons as Diagnostic Tools: Technology and Applications

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Molecular beacons are single-stranded, fluorophore-labeled nucleic acid probes that are capable of generating a fluorescent signal in the presence of target, but are dark in the absence of target. Molecular beacons allow multiplex detection of PCR products in real time in a homogeneous assay format. Real time detection is inherently quantitative and affords a greater dynamic range than end-point detection methods. Reactions in a homogeneous assay format are sealed before amplification takes place, providing improved contamination control. A single cyclor/reader instrument, coupled with automated sample preparation, results in higher throughput and greater ease of use. A multiplex qualitative assay that detects *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, along with an internal control, has been developed. High specificity is achieved through careful selection of primers, probes and assay conditions. Quantitative HIV, HCV, and HBV viral load assays, with sensitivities of 50 copies/ml, 20 IU/ml, and 50 copies/ml, respectively, are achievable. The viral load assays are designed to quantitate all subtype and genotype specimens equivalently. A molecular beacon assay has been designed to detect a single nucleotide polymorphism in the β_2 adrenergic receptor gene. Clin Chem Lab Med 2003; 41(4):468–474

Key words: β_2 adrenergic receptors; *Chlamydia trachomatis*; *Neisseria gonorrhoeae*; Polymerase chain reaction; Single nucleotide polymorphism; Viral load.

Abbreviations: BSA, bovine serum albumin; CT, *Chlamydia trachomatis*; EDTA, ethylenediaminetetraacetic acid; FAM, carboxyfluorescein; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; NG, *Neisseria gonorrhoeae*; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.

Introduction

Molecular beacons were developed to allow the detection of specific nucleic acid sequences in homogeneous assays and in living cells without the requirement to separate unhybridized probe molecules from probe:target complexes. A molecular beacon is a single-stranded oligonucleotide capable of forming a

stem-loop structure. The loop sequence is complementary to a target sequence and is flanked by short complementary arms that form a stem. The oligonucleotide is labeled at one end with a fluorophore and at the other end with a quencher molecule. In the stem-loop conformation, energy from the excited fluorophore is transferred to the quencher and released as heat instead of light. When the loop sequence is hybridized to a specific target sequence, the two ends of the molecule are separated and the energy from the excited fluorophore is emitted as light, generating a detectable signal (1).

Detection methods in amplified nucleic acid diagnostics have typically involved post-amplification processing of reaction products (2–5). Molecular beacons allow a homogeneous amplification and detection format, *i.e.*, one where detection reagents are added to the reaction before the start of amplification, along with the other reaction components. By removing the need for post-amplification manipulation of products, the contamination risk for subsequent reactions is greatly reduced. A homogeneous assay format is also a prerequisite for real time detection. Real time detection methods are inherently quantitative. In contrast to end-point quantitation, data is available over the entire course of the reaction, so that quantitation can take place at the optimal time point for each reaction, even though that time point may be different for each reaction in the run. The result is that the dynamic range of an assay is greatly increased.

Molecular beacons can be designed to either discriminate or tolerate mismatches between the loop and target sequences by modulating the relative strengths of the loop-target hybridization and stem formation. In certain applications, linear probes may be as effective as molecular beacons if random coiling of unhybridized linear probe brings the fluorophore in close proximity to the quencher.

Diagnostic applications of molecular beacon technology include the detection of pathogens (6–9) and the quantitation of viral loads (10–13). Molecular beacons have also been used to detect viral replication (14), the Y chromosome (15), and single nucleotide polymorphisms (SNPs) (16, 17). *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) are among the most common etiological agents of urogenital infections in men and women (18). Nucleic acid amplification methods make possible the direct detection of CT and/or NG DNA in specimens. Human immunodeficiency virus (HIV) is the etiological agent of Acquired Immunodeficiency Syndrome (AIDS) (19, 20). Hepatitis B virus (HBV) and hepatitis C virus (HCV) are major causes of chronic hepatitis (21). Quantitation of the viral load in peripheral blood is an indicator of disease progression and is an aid in the management of patients receiving antiviral therapy (22–24). Recently, a SNP in codon 16

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of the β_2 adrenergic receptor gene has been reported to be associated with variable response to β -agonist therapy. β -agonists such as albuterol bind to β_2 adrenergic receptor sites and are widely used to treat asthma. However, individuals homozygous for a SNP within codon 16 of the β_2 adrenergic receptor gene encoding arginine in place of the normal glycine residue may respond differently to such therapies (25–28).

Here we describe real time, homogeneous assays for CT and NG, quantitative viral load assays for HIV, HCV, and HBV, and an assay for the codon 16 SNP in the β_2 adrenergic receptor gene.

Materials and Methods

CT/NG assay

PCR reactions were carried out in a total volume of 50 μ l containing (final concentration) 1 \times PCR Gold buffer, 3 units of TaqGold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 14 mM $MgCl_2$ and 0.65 mM of each of the four deoxynucleotide triphosphates. The CT forward and reverse primers were present at 0.6 μ M each, the NG forward primer at 0.5 μ M, and the NG reverse primer at 0.4 μ M. The PCR reaction products were detected homogeneously with linear DNA probes using 0.2 μ M FAM-labeled CT probe, 0.3 μ M VIC-labeled NG probe and 0.4 μ M NED-labeled internal control probe (VIC and NED phosphoramidites were obtained from Applied Biosystems, Foster City, CA, USA). The CT primers amplify a 102 bp region of the CT cryptic plasmid while the NG primers amplify a 122 bp sequence located in the NG *Opa* gene. The internal control target DNA was present at 80 molecules per reaction in every reaction. The CT forward and the NG reverse primers were used to non-competitively amplify a 129 bp sequence in an unrelated internal control target DNA. Cycling was initiated with 9 min and 30 s at 95 $^{\circ}C$, to activate the TaqGold, followed by 92 $^{\circ}C$ for 30 s, 58 $^{\circ}C$ for 30 s, and 65 $^{\circ}C$ for 1 min for up to 50 cycles. The ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was programmed to read reactions at every cycle during the 65 $^{\circ}C$ phase.

The analytical sensitivity of the assay was determined by diluting CT and NG DNA (Advanced Biotechnologies, Inc. Columbia, MD, USA) from 10^8 molecules per reaction to 20 molecules per reaction. High concentrations of DNA were quantitated by OD260, and low concentrations of DNA (below 1000 molecules) were diluted from purchased DNA quantitated to be 1×10^4 per μ l (Advanced Biotechnologies, Inc. Columbia, MD, USA). The auxotypes/serovars sensitivity of the assay was determined by testing with 15 serovars of CT and 57 auxotypes/serovars of NG. CT serovars and six of the NG auxotypes/serovars were obtained from ATCC as noted in Table 1. One NG auxotype/serovar was obtained from Dr. David Farrell, formerly of Queensland Health Pathology Service Toowoomba Laboratory, Queensland, Australia, and the remainder was obtained from Dr. W. L. H. Whittington, Center for AIDS and STD and *Neisseria* Reference Laboratory, University of Washington, USA.

The specificity of the assay was tested by challenging the assay with 1×10^7 copies of potentially cross-reactive *Neisseria* spp and *Chlamydia* spp DNA listed in Table 2. Samples were obtained from ATCC unless otherwise noted. Several samples known to cross-react with commercially available NG tests were obtained from Dr. David Farrell (29). These potentially cross-reactive samples are indicated by footnotes in Table 2. NG samples obtained from within Abbott Laboratories (Abbott Park, IL, USA) are also noted.

HIV quantitative assay

PCR reactions were carried out in a total volume of 100 μ l containing (final concentration) 10% glycerol, 62.5 mM bicine, 143.7 mM potassium, 0.125 mM EDTA, 0.0125mg/ml bovine serum albumin (BSA), 0.078% Tween 20, 13 units rTth enzyme, 2.5 mM $MnCl_2$, 0.375 mM of the four deoxynucleotide triphosphates, 0.6 μ M forward primer, 1.6 μ M reverse primer, 0.2 μ M FAM-labeled HIV beacon probe, and 0.1 μ M VIC-labeled internal control beacon probe. The HIV primers and probe were selected from the highly conserved integrase region of the polymerase gene. PCR conditions were as follows: 1 cycle at 59 $^{\circ}C$ for 30 min, followed by 4 cycles at 95 $^{\circ}C$ for 30 s, at 54 $^{\circ}C$ for 30 s, and at 72 $^{\circ}C$ for 30 s, followed by 5 cycles at 90 $^{\circ}C$ for 30 s, at 59 $^{\circ}C$ for 30 s, and at 72 $^{\circ}C$ for 30 s, followed by 43 cycles at 90 $^{\circ}C$ 30 s, at 59 $^{\circ}C$ for 30 s and at 72 $^{\circ}C$ for 30 s, and at 35 $^{\circ}C$ for 30 s where fluorescence readings were taken.

A panel of HIV RNA was generated and quantified as described in Abravaya *et al.* (30). The panel corresponded to samples containing 10, 100, 1,000, 10,000, 100,000, and 1,000,000 copies of HIV RNA copies per ml, equivalent to 4, 40, 400, 4,000, 40,000, or 400,000 HIV RNA copies per reaction, respectively. To determine the analytical sensitivity of the assay, panels containing 4, 20, and 40 copies of HIV RNA per reaction were tested.

HCV quantitative assay

PCR reactions were carried out in a total volume of 100 μ l containing (final concentration) 8% glycerol, 50 mM bicine, 115 mM potassium, 0.1 mM EDTA, 0.01mg/ml BSA, 0.063% Tween 20, 10.5 units rTth enzyme, 2 mM $MnCl_2$, 0.3 mM of the four deoxynucleotide triphosphates, 0.1 μ M forward primer, 1.6 μ M reverse primer, and 0.25 μ M FAM-labeled HCV beacon probe. The HCV primers and probe were selected from the highly conserved 5' untranslated region of the HCV genome. PCR conditions were as follows: 1 cycle at 62 $^{\circ}C$ for 30 min, followed by 5 cycles at 95 $^{\circ}C$ for 30 s and at 58 $^{\circ}C$ for 30 s, followed by 40 cycles at 95 $^{\circ}C$ for 30 s, at 62 $^{\circ}C$ for 30 s, and at 55 $^{\circ}C$ for 30 s where fluorescence readings were taken.

A panel corresponding to samples containing 6, 12.5, 50, 500, 5,000, 5×10^4 , and 5×10^6 IU HCV RNA per ml, equivalent to 2.5, 5, 20, 200, 2,000, 2×10^4 , and 2×10^6 IU HCV RNA per reaction, respectively, was generated and tested. The HCV RNA target was supplied as a dilution of an Armored RNA construct prepared by Ambion (Austin, TX, USA). To determine the analytical sensitivity of the assay, panels containing 2.5, 5, and 20 IU per reaction were tested.

HBV quantitative assay

PCR reactions were carried out in a total volume of 100 μ l containing (final concentration): 1 \times PCR Gold buffer, 10 units of TaqGold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 0.4 mM of each of the four deoxynucleotide triphosphates, 3.5 mM $MgCl_2$, 1.6 μ M of an HBV forward primer, 1.6 μ M of an HBV reverse primer, 0.2 μ M FAM-labeled HBV beacon probe, 0.2 μ M VIC-labeled internal control beacon probe, and 1,000 copies of internal control target. The HBV primers and probe were selected from a highly conserved region of the HBV surface antigen gene. The HBV primers co-amplify an unrelated internal control target DNA sequence. PCR conditions were as follows: TaqGold activation at 94 $^{\circ}C$ for 10 min, followed by 45 cycles at 92 $^{\circ}C$ for 30 s and 58 $^{\circ}C$ for 1 min where fluorescent readings were taken. The "9600 emulsion" option on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was selected.

A panel consisting of 10, 100, 1,000, 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 , and 1×10^9 copies of HBV DNA per reaction was

prepared from a stock of linearized plasmid DNA containing the entire HBV genome. To determine the analytical sensitivity of the assay, dilutions of HBV DNA consisting of 2.5, 5, and 10 copies per reaction were prepared and tested.

β_2 adrenergic receptor gene polymorphism assay

PCR reactions were carried out in a total volume of 50 μ l containing (final concentration): $1 \times$ PCR Gold buffer, 1.25 units of TaqGold enzyme (Applied Biosystems, Foster City CA, USA), 0.2 mM of each of the four deoxynucleotide triphosphates, 3 mM $MgCl_2$, 0.3 μ M each for the forward and reverse primers (31), and 0.1 μ M or 0.2 μ M of the appropriate molecular beacon probe designed to be complementary to either variant (Arg) or wild-type (Gly), respectively. Primers were designed to allow amplification of the target sequence containing both wild-type and variant alleles of the human β_2 adrenergic receptor gene by oligonucleotide hybridization PCR. PCR conditions were as follows: TaqGold activation at 95 °C for 10 min, followed by 45 cycles at 94 °C for 30 s and 55 °C for 50 s, where fluorescent readings were taken, followed by 72 °C for 30 s.

Standard and unknown samples consisted of 125 ng DNA isolated from whole blood. DNA was extracted from whole blood using either the QIAamp® Blood Mini Kit (for samples less than or equal to 200 μ l) or the QIAamp® Blood Maxi Kit (for sample volumes from 200 μ l to 10 ml) (Qiagen, Valencia, CA, USA). The identity of standards and unknown samples were confirmed by the Sanger dideoxynucleotide sequencing method using an ABI PRISM 377 DNA Sequencer with BigDye™ terminator chemistry and AmpliTaqFS (all from Applied Biosystems, Forster City, CA, USA).

The ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used for both thermal cycling and homogeneous detection of the infectious disease assays, except the β_2 adrenergic receptor assay was performed on the ABI PRISM 7700 Sequence Detection System

(Applied Biosystems, Foster City, CA, USA). A reference dye was included in the reactions in order to normalize the fluorescence signals across the wells of the PCR tray.

Results and Discussion

CT/NG assay

Figure 1 illustrates the real time amplification curves generated with high and low amounts of CT DNA. dRn, the baseline-subtracted FAM signal divided by the reference dye signal, is plotted for each cycle. Similar results were obtained with NG PCR (data not shown). Eighty, forty, and twenty molecules of CT and NG DNA per reaction were detected 100% of the time (Table 3). Addition-

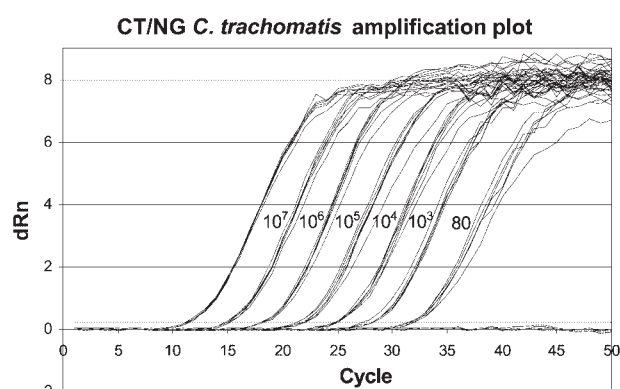


Figure 1 Real time amplification plot of reactions containing from 80 to 10^8 copies of *C. trachomatis* DNA.

Table 1 CT/NG assay serotype/auxotype sensitivity.

<i>C. trachomatis</i> serovars	<i>N. gonorrhoeae</i> auxotypes		<i>N. gonorrhoeae</i> serovars			
	Auxotype	Strain	Serovar	Strain	Serovar	Strain
LGV II	PAU	SK-95-197	IA-1,2	SK-96-503	IB-4/IB-15	SK-96-269
E	PAU	SK-95-279	IA-1,2	SK-96-543	IB-4/IB-15	SK-96-276
LGV I	PAU	SK-95-544	IA-1,2	SK-96-548	IB-4/IB-15	SK-96-293
B	PAU	SK-95-773	IA-1,2	SK-96-694	IB-4/IB-15	SK-96-372
F	PAU	SK-95-796	IA-1,2	SK-96-755	IB-4/IB-15	SK-96-485
LGV III	AHU	SK-96-011	IA-5	SK-94-139	PROTO	SK-99-004
I	AHU	SK-96-536	IA-5	SK-94-178	PROTO	SK-99-019
J	AHU	SK-96-575	IA-5	PIT 8396	PROTO	SK-99-039
K	AHU	SK-96-597	IA-5	NRL 32781	PRO	SK-99-031
Ba	AHU	SK-96-302	IB-1	SK-97-002	PRO	SK-99-051
D	AU	SK-95-046	IB-1	SK-97-009	AUO	SK-99-073
A	AU	SK-95-148	IB-1	SK-97-011	AUO	SK-99-095
H	AU	SK-95-239	IB-1	SK-97-017	IB-3	SK-99-104
G	AU	SK-95-246	IB-1	SK-98-361	IB-3	SK-99-124
C	AU	SK-95-257	IB-5	SK-96-441	IB-3	SK-99-125
	1	ATCC 27628	IB-5	SK-96-508	IB-2	SK-99-126
	5	ATCC 27629	IB-5	SK-96-526		
	9	ATCC 27630	IB-5	SK-96-539		
	22	ATCC 27631	IB-5	SK-96-545		
	12	ATCC 27632				
	16	ATCC 27633				
	DPNG	D. Farrell				

Table 2 CT/NG specificity panel.

1	<i>N. animalis</i>	23	<i>N. weaveri</i>	42	<i>N. subflava</i> (David Farrell)
2	<i>N. canis</i>	24	<i>C. psittaci</i> ^a	43	<i>N. subflava</i> ^b (David Farrell)
4	<i>N. caviae</i>	25	<i>C. pneumonia</i> ^a	44	<i>N. subflava</i> ^b (David Farrell)
5	<i>N. cinerea</i>	26	<i>C. pneumoniae</i> ^a	45	<i>N. subflava</i> (David Farrell)
6	<i>N. cuniculi</i>	27	<i>N. subflava</i>	46	<i>N. subflava</i> ^b (David Farrell)
7	<i>N. denitrificans</i>	28	<i>N. subflava</i>	47	<i>N. subflava</i> (David Farrell)
8	<i>N. elongata elongata</i>	29	<i>N. subflava</i>	48	<i>N. cinerea</i> (David Farrell)
9	<i>N. elongata glycolytica</i>	30	<i>N. subflava</i>	49	<i>N. sicca</i> (David Farrell)
10	<i>N. flavescens</i>	31	<i>N. subflava</i>	50	<i>N. sicca</i> ^b (David Farrell)
11	<i>N. iguanae</i>	32	<i>N. subflava</i> (Abbott)	51	<i>N. mucosa</i> (David Farrell)
12	<i>N. lactamica</i>	33	<i>N. subflava</i> (Abbott)	52	<i>N. lactamica</i> (David Farrell)
13	<i>N. macacae</i>	34	<i>N. mucosa</i> (Abbott)	53	<i>N. lactamica</i> (David Farrell)
14	<i>N. meningitidis-B</i>	35	<i>N. subflava</i> (David Farrell)	54	<i>N. meningitidis</i> (David Farrell)
16	<i>N. meningitidis-C</i>	36	<i>N. subflava</i> (David Farrell)	55	<i>N. lactamica</i>
18	<i>N. meningitidis-D</i>	37	<i>N. subflava</i> (David Farrell)	56	<i>N. lactamica</i>
19	<i>N. mucosa</i>	38	<i>N. subflava</i> ^b (David Farrell)	57	<i>N. lactamica</i>
20	<i>N. polysaccharea</i>	39	<i>N. subflava</i> (David Farrell)	58	<i>N. meningitidis-A</i>
21	<i>N. sicca</i>	40	<i>N. subflava</i> ^b (David Farrell)		
22	<i>N. subflava</i>	41	<i>N. subflava</i> (David Farrell)		

^apotential *C. trachomatis* cross reactors, ^bknown cross reactors with alternative PCR assays.

Table 3 CT/NG assay sensitivity.

<i>C. trachomatis</i> DNA copies/rxn	Number detected	Percent detected
80	6/6	100%
40	6/6	100%
20	6/6	100%
<i>N. gonorrhoeae</i> DNA copies/rxn	Number detected	Percent detected
80	6/6	100%
40	6/6	100%
20	6/6	100%

ally, all of the 15 serovars of CT and all of the 57 auxotrophs/serovars of NG listed in Table 1 could be detected at the 20-molecule level. The analytical specificity of the CT/NG assay was demonstrated by challenging the assay with potentially cross-reactive *Neisseria* spp and *Chlamydia* spp (Table 2). The PCR assay did not detect any of the potentially cross-reactive organisms at 10⁷ molecules per reaction, even those known to cross-react with another commercial assay (28).

Real time PCR technology provides a rapid, sensitive, and specific methodology for detection of CT and NG. PCR reactions can typically be completed in less than 2 hours. The demonstrated analytical sensitivity and specificity of real time PCR, coupled with automated sample preparation and a control for inhibition, ensures the reliable diagnosis of these important sexually transmitted diseases.

Viral load assays

In Figure 2A, real time amplification plots are shown across the dynamic range of HIV RNA levels from 10

copies/ml to 1,000,000 copies/ml. Four replicates were tested at each level including negatives, and the mean values are plotted. In Figure 3A, all four replicates are shown on a standard curve with a slope of -2.9969 , and a correlation of 0.9976. To further evaluate the sensitivity of the real time HIV quantitative assay, a larger number of replicates were run at the low HIV RNA levels. Reactions containing 40 copies, 20 copies, and 4 copies were run in 32 replicates. The real time HIV quantitative assay has high sensitivity; 40 and 20 copies per reaction were detected 100% of the time and 4 copies per reaction were detected 81% of the time (Table 4).

The amplification plots of six replicates of HCV RNA calibrators at concentrations throughout the dynamic range of the assay from 2.5 to 2×10^6 IU/reaction including negatives are shown in Figure 2B. The standard curve has a slope of -3.3317 and a correlation of 0.9993 (Figure 3B). Six out of six replicates of HCV RNA at 20, 5, and 2.5 IU per reaction were detected (Table 4), demonstrating the sensitivity of the assay.

HBV DNA calibrators ranging in concentration from 10 to 10⁹ copies per reaction and negatives were run in replicates of six. The amplification plots for the HBV DNA calibrators are shown in Figure 2C. All six replicates at the 10-copy per reaction level were detected. Figure 3C shows the standard curve generated from the HBV DNA calibrator data shown in Figure 2C. The standard curve demonstrates good PCR amplification efficiency with a slope of -3.1675 and a correlation of 0.9969, in the dynamic range from 10 to 10⁹ copies per reaction. A sensitivity study evaluated 20 replicates of 10, 5, and 2.5 copies per reaction of HBV DNA. Results in Table 4 show that HBV DNA is detected in 95% of the 10 copies, 100% of the 5 copies, and 75% of the 2.5 copies per reaction replicates.

Using molecular beacon technology in a real time PCR assay format for the detection and quantitation of viral nucleic acids results in assays that are sensitive,

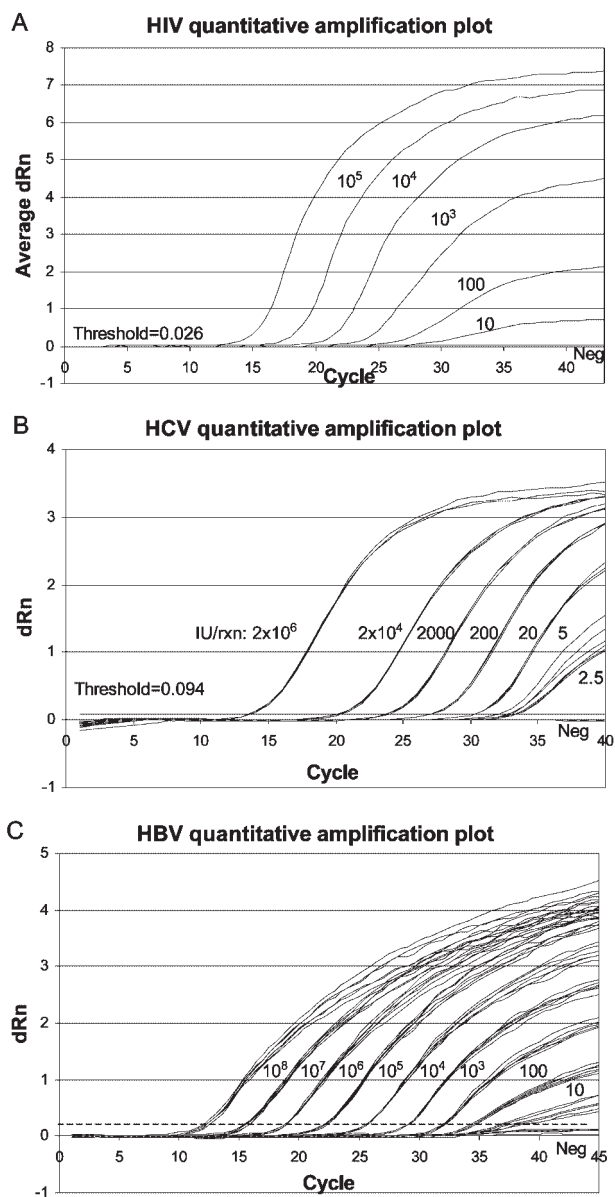


Figure 2 Real time amplification plots for the quantitative viral load assays.

Table 4 Sensitivity of viral load assays.

HIV RNA copies/rxn	Number detected	Percent detected
40	32/32	100%
20	32/32	100%
4	26/32	81%
HCV RNA IU/rxn	Number detected	Percent detected
20	6/6	100%
5	6/6	100%
2.5	6/6	100%
HBV DNA copies/rxn	Number detected	Percent detected
10	19/20	95%
5	20/20	100%
2.5	15/20	75%

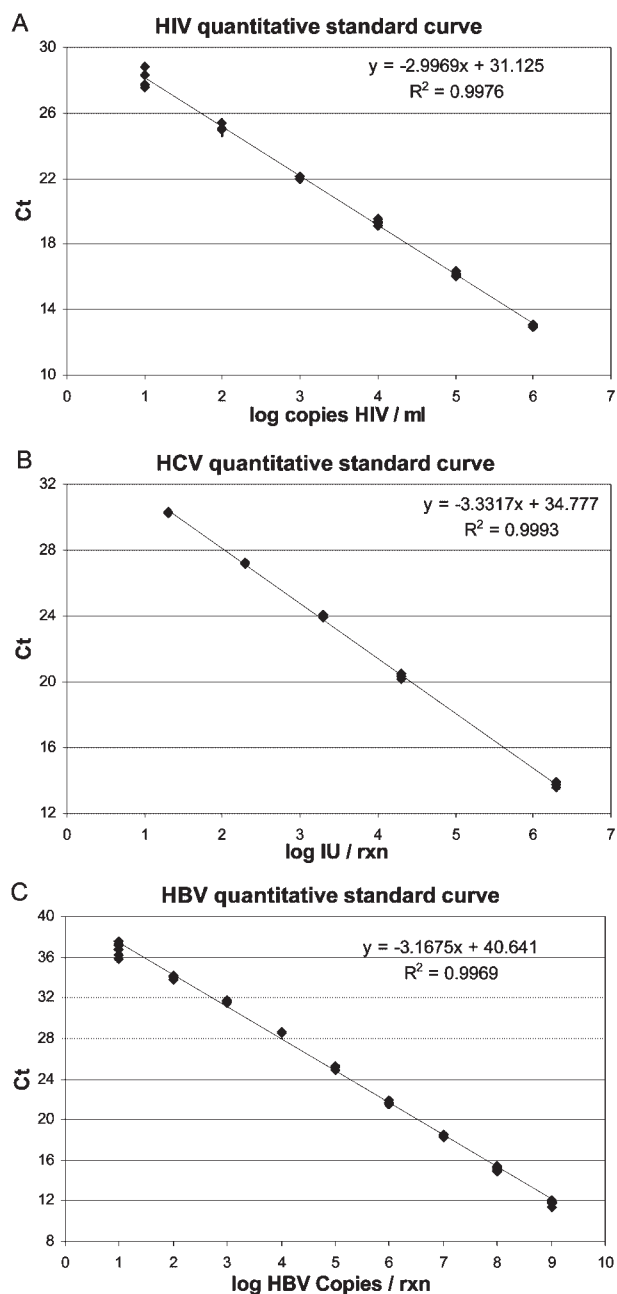


Figure 3 Standard curves for the quantitative viral load assays.

have wide dynamic range, and can be run rapidly. The HIV, HCV, and HBV assays will enable the effective monitoring of viral load in patients undergoing anti-viral therapy across a wide range of virus concentration in patient samples without need for dilution, and can achieve detection down to a very low limit. Also, due to the selection of primers and probes from conserved regions of the genomes, these viral assays have excellent subtype and genotype detection capabilities.

β₂ adrenergic receptor gene polymorphism assay

The read temperature for real time PCR analysis was selected as 55 °C based on the desire for suitable signal to noise, or discrimination ratio, for both the wild-type

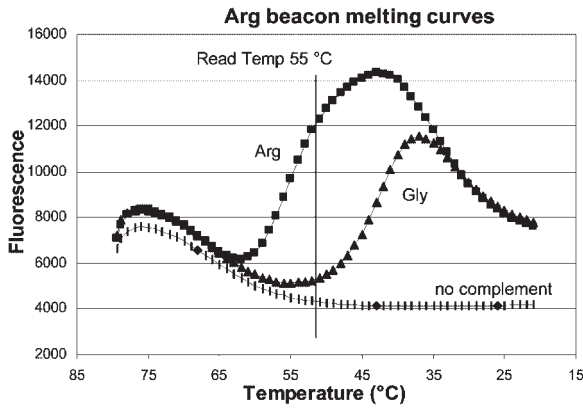


Figure 4 Melting curves for the Arg-specific beacon in the presence of Arg and Gly complements, compared to the beacon melting curve without complement present.

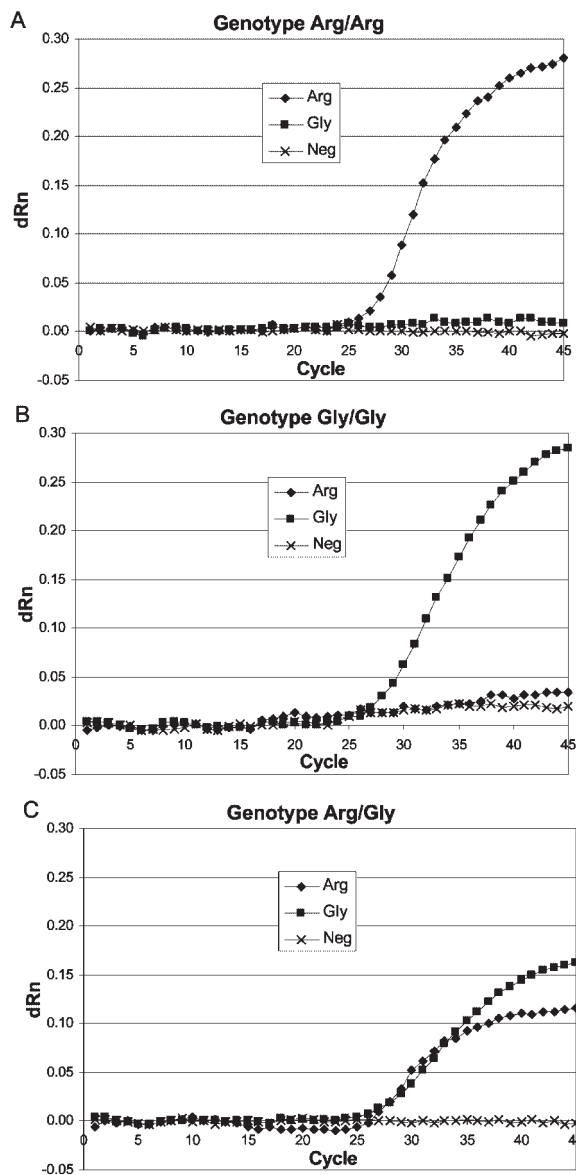


Figure 5 Real time amplification plots for (A) homozygous Arg/Arg genotype, (B) homozygous Gly/Gly genotype, and (C) heterozygous Arg/Gly genotype samples. For each sample, the results of individual reactions containing only the Arg-specific probe or only the Gly-specific probe were plotted, along with a no-beacon control reaction.

(Gly) and variant (Arg) beacons. The discrimination ratio is the ratio of the signal obtained using a probe for the allele targeted for detection in the presence of its complementary allele relative to the signal obtained using the same probe in the presence of the alternative allele containing a single base difference. In Figure 4, melting curves for the Arg-specific probe are shown in the presence of Arg and Gly complements, respectively. The Arg-specific probe is able to generate significant fluorescent signal at 55 °C in the presence of its complement, while there is significantly reduced fluorescence observed for the Arg-specific probe in the presence of the Gly allele containing only a single base difference. Similar results were observed for the converse experiment.

In real time PCR experiments, each Arg/Arg homozygous sample gave strong fluorescence in the presence of the Arg-specific molecular beacon, while essentially no fluorescence was observed in the presence of the Gly-specific molecular beacon or the no-beacon control (Figure 5A). Conversely, each Gly/Gly homozygous sample gave strong fluorescence in the presence of the Gly-specific molecular beacon and essentially no fluorescence in the presence of the Arg-specific molecular beacon or the no-beacon control (Figure 5B). In heterozygous samples (Arg/Gly), a medium-level of fluorescence was observed in reactions containing either molecular beacon probe (Figure 5C). Note that for heterozygous samples, the end-point signals observed were roughly half that of the homozygous samples.

Molecular beacon technology is thus a useful tool for the discrimination of single base differences in human sequences relevant in pharmacogenetic, diagnostic, and disease pre-disposition applications.

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