

## Simultaneous detection and identification of HBV and HTLV-I viruses by Melting curve analysis of multiplex Real-time PCR

Vahid Kia<sup>1</sup>, Mahdi Forouzandeh Moghadam<sup>1\*</sup>, Mahdi Paryan<sup>2</sup>, Abbasali Raz<sup>2</sup>, Siamak Mirab Samiee<sup>3</sup>

1. Department of Medical Biotechnology, Tarbiat Modares University, Tehran, Iran

2. Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

3. Food and Drug Laboratory Research Center, Ministry of Health and Medical Education, Tehran, Iran.

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### Abstract

**Background and Objectives:** HBV and HTLV-I are life threatening infectious agents in patients who receive blood and blood products. Although serological methods have been proved to be useful, detection of these viruses has remained a challenging issue due to the many obstacles. By the advent of Nucleic Acid Testing methods, especially in multiplex format, more precise detection is possible. The objective of this study was to develop a reliable, rapid and cost-effective method to simultaneously detect HBV and HTLV-I.

**Materials and Methods:** We have developed a multiplex Real time-PCR assay for simultaneous detection of HBV and HTLV-I. Primer sets were designed for highly conserved regions of genome of each virus. Using these primers and standard plasmids, we determined the limit of detection, clinical and analytical specificity and sensitivity of the assay. Monoplex and multiplex Real-time PCRs were performed.

**Results:** Analytical sensitivity was considered to be 1000 and 100 copies/ml for HBV and HTLV-I, respectively. High concentration of one virus had no adverse effect on detection of low concentrations of the other one. By analyzing 30 samples, clinical sensitivity of the assay was determined to be 87% and 96% for HBV and HTLV-I, respectively. Using different viral and human genome samples, the specificity of the assay was verified to be 100%.

**Conclusions:** We have developed a reliable, rapid and cost effective method to simultaneously detect HBV and HTLV-I. Our results indicated the high capability of this simple and rapid method for detecting these viruses in clinical samples.

**Keywords:** Multiplex Real-time PCR, HBV, HTLV-I

### Introduction

Donated blood is routinely screened for the presence of viral pathogens (Poiesz, Ruscetti et al. 1980). Hepatitis B virus (HBV) and human T-lymphotropic virus-1 (HTLV-I) are among the life threatening infectious agents transmitted by blood and blood products

(Kalyanaraman, Sarngadharan et al. 1982, Bhagavati, Ehrlich et al. 1988, Pantaleo, Graziosi et al. 1993). Conventional serological techniques used for the detection of the several pathogens are not perfectly satisfactory since they are not capable of detecting early stages of infection when the immune response is developing (Zucker-Franklin, Pancake et al. 1997). Furthermore, antibodies specific to HTLV-I are not always present in infected individuals (Ho, Moudgil et al. 1989).

\*Corresponding author: Dr. Mahdi Forouzandeh Moghadam, School of Medical Sciences, Department of Medical, Biotechnology, Tarbiat Modares University, Jalal Ale Ahmad, Highway, Tehran, Iran; Tel: +98-912-3937320; Fax: +98-21-82883861  
E-mail: foroz@modares.ac.ir

There is a small but highly significant transfusion risk of viral pathogens owing to the inability of existing serological screening methods to either successfully identify recently infected donors who are in the pre-seroconversion window phase of infection or detect various serotypes of these viruses. Recently, application of nucleic acid amplification tests (NATs) have significantly lowered the “window period” (Meng, Wong et al. 2001).

However, NAT technology has a significant drawback of high cost and poor cost-effectiveness (Meng, Wong et al. 2001). In addition, several NAT approaches apply pooling of samples which exposes a relatively high rate of false-positive results, prompts a substantial loss of sensitivity when a low-positive sample is diluted into the pool and creates the need to further identify the infected unit in the pool (Miyachi, Masukawa et al. 2000). To conquer these setbacks, some previous researches developed rapid and sensitive multiplex PCRs (Meng, Wong et al. 2001). Over the last several years, different methods have been developed for sequence-specific probe capture of viral genomes to specific particles like (Busch, Program Abstr. 52nd Annu. Meet. Am. Assoc. Blood Bank): transcription-mediated amplification/nucleic acid sequence based amplification (TMA/NASBA), ligase chain reaction (LCR), and branched DNA signal amplification assay (b-DNA assay) (Meng, Wong et al. 2001).

Although acceptable results have been acquired with PCR assays (Mulder, McKinney et al. 1994), there are some serious disadvantages that have limited their clinical application. The oligonucleotide primers specially designed to hybridize specifically to retroviral target sequences sometimes hybridize to human sequences and produce undesirable amplicons that give impairing signals. Furthermore, sometimes the primers hybridize to each other and generate short “primer-dimer” amplicons (Erlich, Gelfand et al. 1991). Though nested

PCR has been developed to improve primer specificity, it needs post-PCR processing and opening of the tubes which increase the risk of contamination (Mullis and Faloona 1987). Although sterilization strategies and multi-compartment assay chambers have been employed to eliminate contaminating amplicons in untested samples (Longo, Berninger et al. 1990), the exorbitant cost and immense complexity of these schemes have reduced their utility. Practical diagnostic laboratory methods should be simple, fast, inexpensive, and quite sensitive, make use of a high-throughput format that enables the simultaneous monitoring of many samples and preferably allow the detection of different pathogenic agents in the same assay tube. Heretofore, nucleic acid detection methods have been far too complicated to accomplish all of these objectives in a single assay (Vet, Van der Rijt et al. 2002). In this article, we report the development of a deeply sensitive PCR assay that ignores unwanted amplicons, detects only desirable amplicons in real time, is carried out in a hermetically sealed reaction tube, and is able to detect two different pathogenic viruses simultaneously.

### Materials and methods

**Primers:** Several sequences of HTLV-I and also different subtypes of HBV were retrieved from GenBank, NCBI and multiple alignments were performed for each series. The conserved regions of HBV genome (HBsAg) and HTLV-I genome (*tax*) were used to design primers. Compatible primer sets were selected to ensure efficient amplification and detection of most subtypes of each virus. The chosen primer pairs were inspected by BLAST analysis to ensure that they have no homology with irrelevant viral genomes or human sequences. The HTLV-I/HBV primers were chosen to amplify a region of low variability in the *tax* and *HbsAg* genes, respectively. The sequences of the primers were: *HBsAgF*: 5'-TAT GTT GCC CGT TTG TC-3', and *HBsAgR*: 5'-CAC TGA ACA AAT GGC ACT AG-3', and

taxF:5'-GTG TTT GGA GAC TGT GTA CAA G-3', and tax R: 5'-AAG GAG GGT GGA ATG TTG-3'. The oligo nucleotide sequences were analyzed by Oligo 6.0 (Med Probe, Oslo, Norway) and Beacon Designer (Premier Biosoft) for their compatibility in a multiplex PCR assay.

**Clinical samples:** A total of 40 seropositive and 25 seronegative samples from consecutive non-duplicate whole blood were collected from Clinical Laboratory of Day hospital, Tehran, Iran. These samples had been screened by serological experiments for positive result. In addition, they were approved to be positive/negative using Artus HBV RG PCR assay.

**DNA isolation:** To extract HBV genomes from 200 µl of whole blood samples, QiaAmp DNA mini Kit (Qiagen) was used according to the manufacturer's instructions. Purified DNA was eluted in 50 µl of the kit elution buffer and stored at -20°C until use. HTLV-I genome was extracted from peripheral blood mononuclear cells (PBMCs) which had been isolated based on the protocol in Sambrook molecular cloning Laboratory manuals using Ficoll. DNA was extracted from 100 PBMCs/ml counted via hemocytometer.

#### Assessment of HTLV-I and HBV reference whole blood

HTLV-I stock virus contained  $6 \times 10^5$  DNA copies/ml and HBV stock virus contained  $2 \times 10^6$  DNA copies/ml. Prior to DNA purification procedure, the HTLV-I and HBV stocks were diluted in HTLV-I and HBV negative whole blood and mixed to achieve several HTLV-I/HBV scalar dilutions (from  $2 \times 10^3$  to 200 DNA copies/ml).

#### Construction of positive control plasmids

Desired regions of each virus were amplified using patient's samples to prepare standard plasmids. To construct HTLV-I and HBV plasmids, the resulted PCR products were separately ligated into PTZ 57R/T according to the instructions of the 'T/A Cloning Kit K1213' (Fermentas, Germany).

The cloned HTLV-I and HBV fragments that comprise a 260bp region from the *tax* gene and a 240bp region from the *HBsAg* gene were obtained by PCR from HTLV-I and HBV positive samples by PCR.

#### Preparation of standard plasmids

For generating the standards, the respective plasmids were digested by restriction enzymes. The linear plasmids were purified over a 0.8% agarose gel in  $1 \times$  TBE. The band of the correct size was excised and purified from the agarose using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentrations of the final solutions were determined by measuring the OD at 260 nm. For the standard curve generation, a series of 10-fold dilutions of HTLV-I and HBV were prepared, representing  $10^6$ -100 copies of DNA/ml.

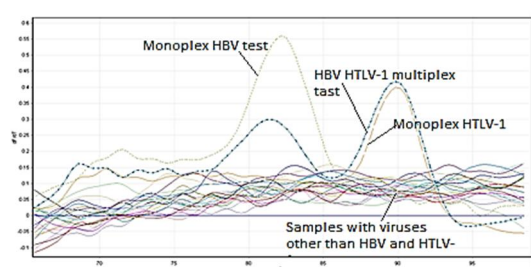
#### Monoplex and Multiplex Real time-PCR for HTLV-I and HBV viruses

The Monoplex Real time-PCR for each virus was carried out in a 25 µl reaction using QuantiFast SyberGreen I PCR kit (Qiagen). The PCR mixture for DNA amplification consisted of 5 µl of DNA extract, forward and reverse primers for HBV (0.9 µM of each), forward and reverse primers for HTLV-I (0.3 µM of each) and  $1 \times$  master mix of QuantiFast SyberGreen I PCR. For both the HTLV-I and HBV DNA amplifications, after one cycle at 95 °C for 5 min, a two-step PCR procedure was used consisting of 15 s at 95 °C and 60s at 60 °C for 45 cycles. In the end of the amplification cycles, melting temperature analysis was performed by a slow increase in temperature (1°C/s) up to 99°C. Amplification, data acquisition, and analysis were performed on a Rotor-Gene 3000 (Corbett Research, Australia). The melting peaks were analyzed to distinguish HTLV-I and/or HBV specific amplicons.

## Results

### Optimization of multiplex realtime-PCR:

Using QuantiFast SYBR Green I PCR master mix the optimal conditions for the Syber Green multiplex real time-PCR were obtained. Most importantly, the primer concentrations were adjusted to 0.3 and 0.9  $\mu$ M for HTLV-I and HBV, respectively, to achieve the maximum sensitivity. Syber Green is a dsDNA binding dye that does not distinguish different dsDNA molecules. Thus, different amplicons should be distinguished by the melting curve analysis. The values of melting temperature ( $T_m$ ) which varied based on difference in length and composition of the amplicons, specifically revealed the presence of HBV and HTLV-I in the samples. The melting curve displayed two separate peaks at 88.8°C and 82.6°C for HTLV-I and HBV, respectively. (Fig. 1)



**Figure 1** Melting curve of multiplex test for HBV and HTLV-I. 260 bp target region of HTLV-1 *tax* gene has peak at 88.8 C and 240 bp region of HBV *HBsAg* gene has a peak at 82.6 C.

### Sensitivity of HTLV-I and HBV real time-PCR

In order to establish the sensitivity of HTLV-I and HBV real-time PCR, a serial dilution ranging from  $10^6$  to  $10^2$  DNA copies/ml was spiked in healthy whole blood samples. HTLV-I and HBV DNA were detected in 100% of the samples at concentrations  $\geq 10^2$  and  $10^3$  copies/ml of whole blood, respectively (Table 1). Since some reports have indicated that multiplex PCR sensitivity might be influenced by the target ratio in the sample, the ability of the assay in detecting low levels of HTLV-I or HBV, close to the sensitivity limit, in the presence of high yields of the other viral nucleic acid when co-amplified, were examined. Samples were prepared by mixing either serial dilutions of HTLV-I (from  $10^5$  to 100 copies/100 PBMCs) with  $10^6$  copies/ml for HBV, or serial dilutions of HBV (from  $10^5$  to 100 copies/ml) with  $10^6$  copies/PBMCs of HTLV-I. HBV and HTLV-I specific melting curves were constantly detected both when 100 copies/100 PBMCs of HTLV-I were amplified with  $10^6$  copies/ml of HBV and  $10^3$  copies/ml of HBV were amplified with  $10^6$  copies/PBMCs of HTLV-I as reported in Table 2.

**Table 1** Multiplex real-time PCR sensitivity.

HBV copies/ml	HTLV-I copies/ml	HBV or HTLV-I positive replicates
$10^6$	0	3/3
$10^5$	0	3/3
$10^4$	0	3/3
$10^3$	0	3/3
$10^2$	0	2/3
0	$10^6$	3/3
0	$10^5$	3/3
0	$10^4$	3/3
0	$10^3$	3/3
0	$10^2$	3/3

**Table 2** Multiplex real-time PCR sensitivity detection in presence of different viral genome concentration.

HBV copies/ml	HTLV-I copies/ml	HBV or HTLV-I positive replicates
10 <sup>6</sup>	10 <sup>5</sup>	3/3
10 <sup>6</sup>	10 <sup>4</sup>	3/3
10 <sup>6</sup>	10 <sup>3</sup>	3/3
10 <sup>6</sup>	10 <sup>2</sup>	1/3
10 <sup>5</sup>	10 <sup>6</sup>	3/3
10 <sup>4</sup>	10 <sup>6</sup>	3/3
10 <sup>3</sup>	10 <sup>6</sup>	3/3
10 <sup>2</sup>	10 <sup>6</sup>	3/3

**Reproducibility**

**Intra-assay:** Five replicates of each standard were tested in a single run. As shown in table 3, the coefficient of variation (CV) was not greater than 4.

**Inter-assay:** standards were tested in five consecutive days to assess the between-run variability (table 4). The CV was not greater than 5%.

**Table 3** Five replicates of each standard were tested in a single run to assess reproducibility (intra-assay) of the assay

No.	Standard concentration (copy/μl)	MeanCt	Standard deviation	CV
1	1E+6	14.76	0.35	2.4
2	1E+5	18.1	0.48	2.7
3	1E+4	21.5	0.66	3.1
4	1E+3	24.6	0.86	3.5
5	1E+2	28.1	1.1	4

**Table 4** Five replicates of each standard were tested in a single run to assess reproducibility(intra-assay) of the assay

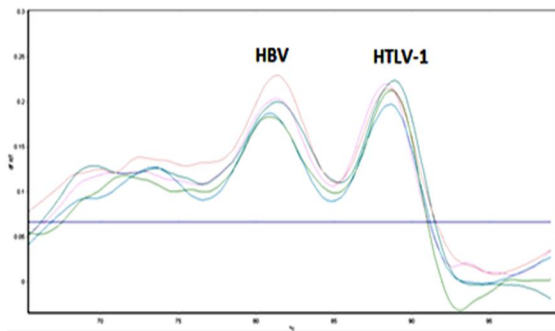
No.	Standard concentration (copy/μl)	MeanCt	Standard deviation	CV
1	1E+6	14.6	0.26	1.8
2	1E+5	18	0.43	2.4
3	1E+4	21.5	0.66	3.1
4	1E+3	24.2	0.87	3.6
5	1E+2	27.5	1.1	4.1

**Specificity of the multiplex real-time PCR assay**

The chosen primer pairs were inspected by BLAST analysis and did not show any

homology with irrelevant viral genomes or human sequences. Sequencing the PCR products from the pHTLV-I and pHBV standard plasmid showed no mutation and confirmed that HBSAg and tax regions were

correctly cloned correctly. All of the HTLV-I and HBV isolates demonstrated a high degree of homology in the primer sequences. The specificity of the assay was tested using DNA extracts from other blood born viruses (HIV, HCV, CMV, HSV-1, HSV2 and EBV) and from blood samples of 20 HTLV-I and HBV seronegative blood donors. The results were negative for the blood born viruses with  $C_t$  values  $>45$  cycles and for the 20 healthy controls (Fig. 2).



**Figure 2:** Multiplex test for HBV and HTLV-1 positive samples and DNA extracts from other blood born viruses. Samples with viruses other than HBV and HTLV-1 showed negative results that confirms the specificity of the assay.

### Analysis of patients

To test the multiplex real time-PCR on whole blood from infected patients, 30 patients infected with HTLV-I and HBV (30 spiked samples with HTLV-I and HBV) and 25 healthy blood donors were selected. Using this technique, HBV genome was detectable in 26 out of 30 (86%) HBV positive samples approved by Artus HBV RG PCR assay. The negative samples had a viral load lower than 984 copies/ml in Artus HBV RG PCR assay. On the other hand, HTLV-I was revealed in 29 out of 30 (96%) analyzed samples. Interestingly, HTLV-I was identified in one patient with in-house real-time PCR HTLV-I viral load  $<100$  copies/100 PBMCs. The analysis of the 25 healthy blood donors did not show any positive results, confirming the specificity of the assay.

### Discussion

Today, molecular diagnosis methods have been highly developed and newer methods are developing rapidly. Among these, NATs have drawn special attention. However, large scale use of them has been limited because these methods are costly and time-consuming (Vargo, Smith et al. 2002). To decrease the cost of NATs, two approaches have been proposed.

First is the use of a pool of donors' plasma which is widely used in the United States and Japan. Clinical tests, however, show that the sensitivity has been reduced because of the dilution of low-titer samples (Vargo, Smith et al. 2002, Stramer, Wend et al. 2011) and a rise in false positive results after using ultra-centrifugation to concentrate the samples (Seifried, Findhammer et al. 2002). In addition, determining positive samples in a pool of plasmas is cumbersome which delays the preparation of blood products. Second is the development of a multiplex method to simultaneously detect more than one virus in a reaction. The advantages of these strategies are less time and money needed for diagnosis. Real-Time PCR is one of the innovations in nucleic acid testing. The advantages of this method are high specificity and sensitivity as a result of using specific primers for target sequences, and the possibility of screening multiple target sequences in a single reaction.

In the present research, we have designed specific primers for highly conserved sequences of each virus' genome which enables us to simultaneously detect both HBV and HTLV-I in plasma samples based on the difference in products' melting curves. By using Real-time PCR, unlike conventional PCR and PCR-ELISA, it is possible to detect low concentration of these viruses with high specificity and sensitivity. Moreover, the risk of cross contamination is eliminated because no post-PCR processing is needed and thus the tubes will not be opened (Mackay, Arden et al. 2002).

To design the primer sets that are capable of detecting all subtypes of each virus, sequences of each virus were retrieved from GenBank,

NCBI and aligned to find the highly conserved regions. In addition by cloning the target region of each virus in a cloning vector, standard plasmids were prepared to examine the sensitivity of the assay. In 100% of the samples the viral DNAs were detected at concentrations more than  $10^2$ copies/100 PBMCs and  $10^3$ copies/ml for HTLV-I and HBV, respectively. Furthermore, the assay is fully optimized that high concentration of one viral genome will not adversely affect the detection of the other one. Thus the assay is perfectly appropriate for the simultaneous detection of HBV and HTLV-I.

To study the specificity of the assay DNA extracts from other blood born viruses and HTLV-I and HBV seronegative blood samples were tested. It showed no positive results which accentuates the specificity of this method. 30 prepared samples positive for HBV and HTLV-I and 25 negative samples were tested to investigate the efficiency of multiplex reaction. HBV and HTLV-I genomes were detected in 87% and 96% of samples, respectively. The four negative results for HBV showed viral load 984 copies/ml as tested by Artus HBV RG PCR assay. No positive result was observed in 25 negative samples emphasizing the specificity of the assay.

The usefulness of detecting HBV and HTLV-I has become an integral part of the clinical diagnosis and management of patients infected with these infectious agents. Moreover, by simultaneous detection, time and cost needed is considerably decreased (Mackay, Arden et al. 2002). Using a duplex Real time-PCR in conjunctions with standard quantitation plasmids provides a highly efficient clinical method that is remarkably accurate with an extremely rapid turnaround time.

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